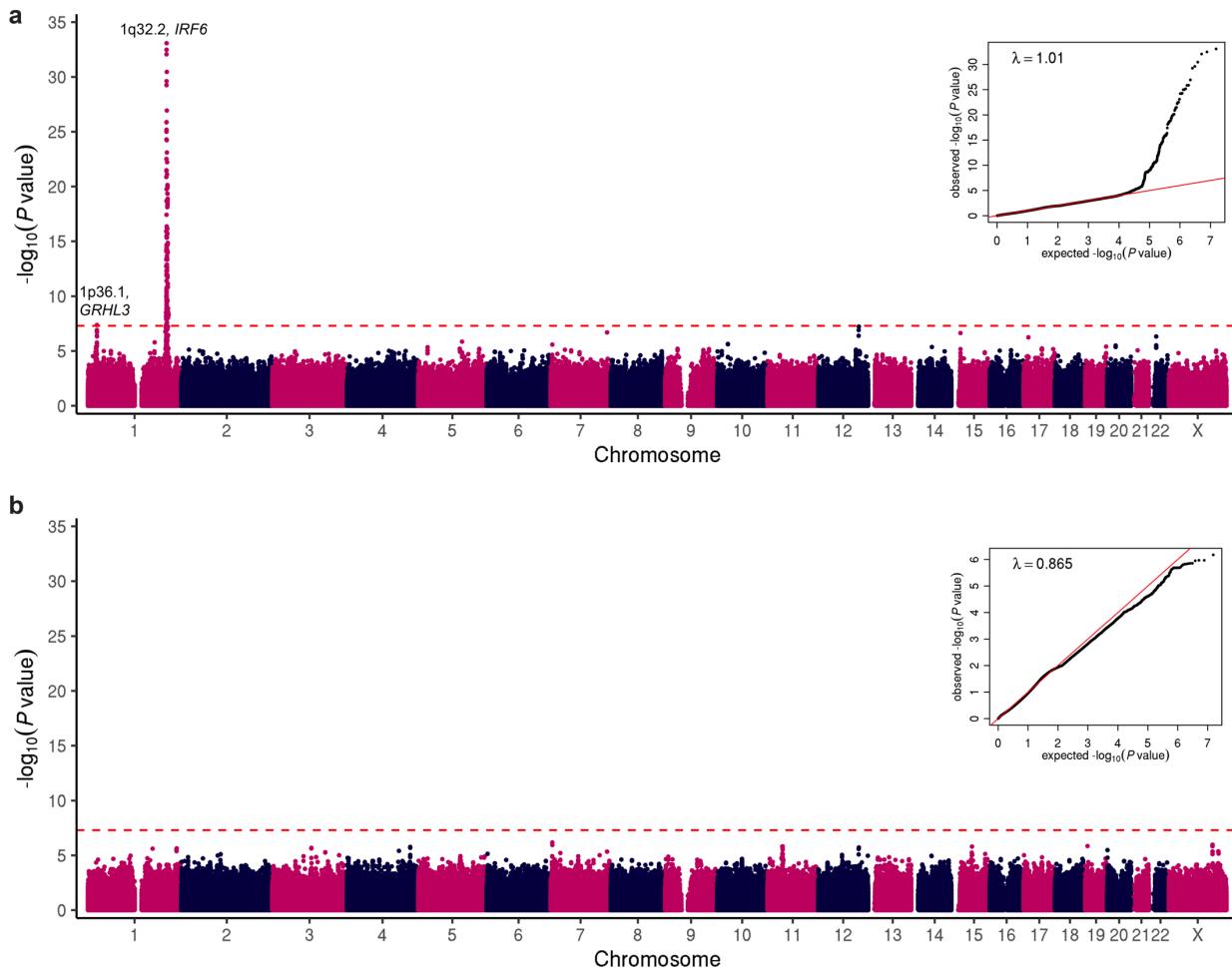


Supplementary Information

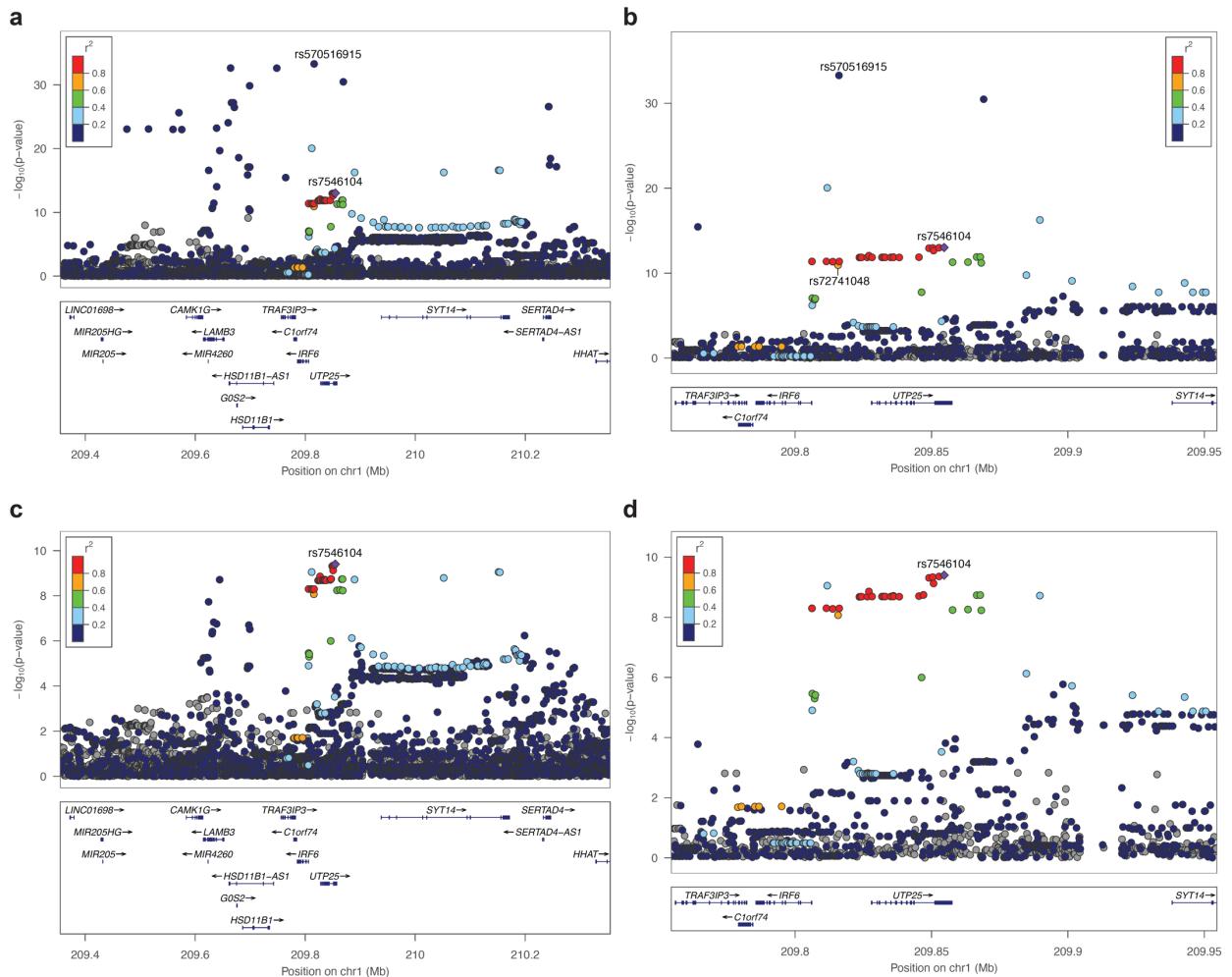
High incidence and geographic distribution of cleft palate in Finland are associated with the *IRF6* gene

Fedik Rahimov, Pekka Nieminen, Priyanka Kumari, Emma Juuri, Tiit Nikopensius, Kitt Paraiso, Jakob German, Antti Karvanen, Mart Kals, Abdelrahman G. Elnahas, Juha Karjalainen, Mitja Kurki, Aarno Palotie, FinnGen, Estonian Biobank Research Team, Arja Heliövaara, Tõnu Esko, Sakari Jukarainen, Priit Palta, Andrea Ganna, Anjali P. Patni, Daniel Mar, Karol Bomsztyk, Julie Mathieu, Hannele Ruohola-Baker, Axel Visel, Walid D. Fakhouri, Brian C. Schutte, Robert A. Cornell, David P. Rice

Supplementary Figures



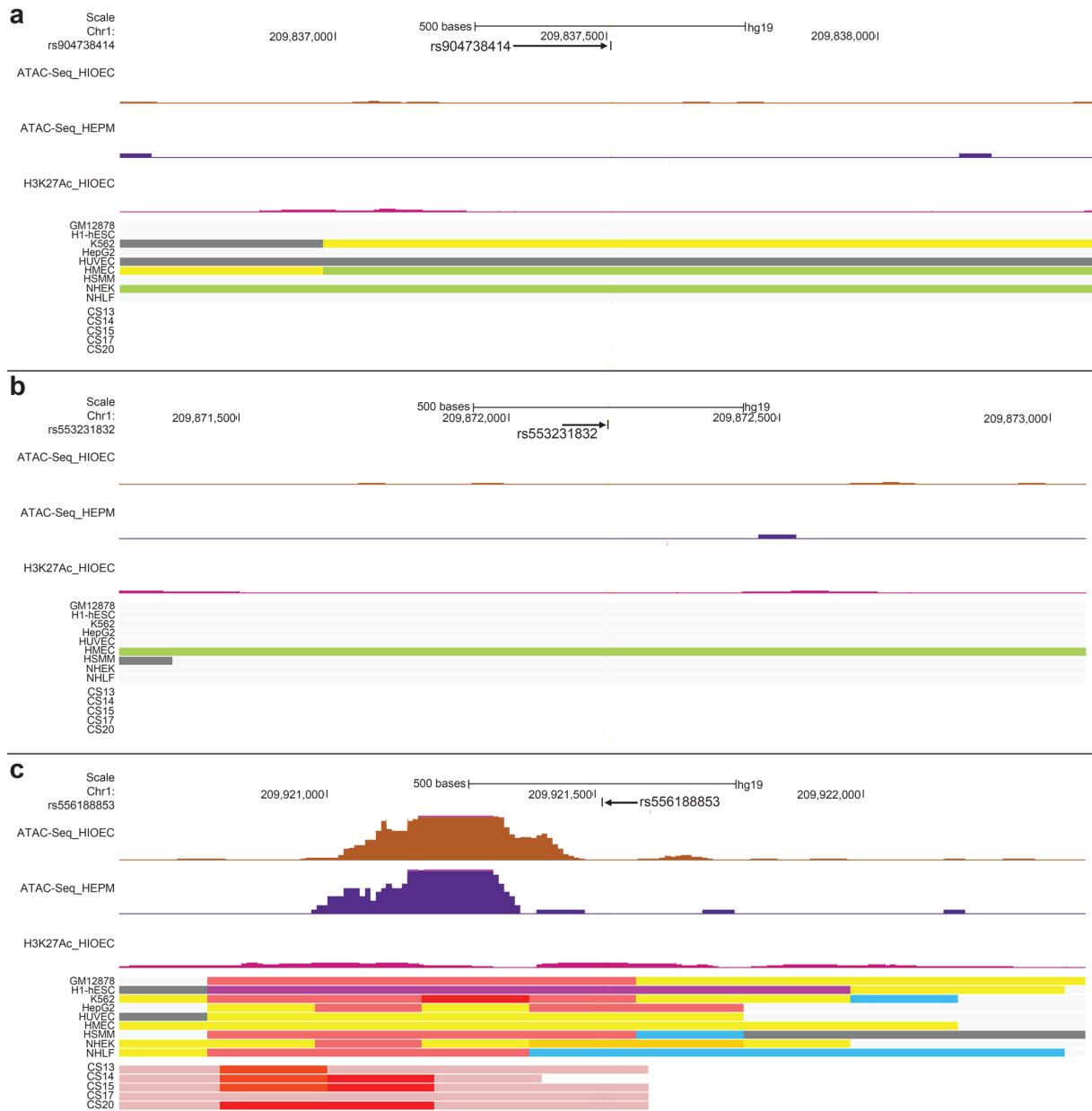
Supplementary Fig. 1: Manhattan plots showing GWAS results of (a) 355 cases with non-syndromic OFC (CL, CLP, and CP combined) and 308,799 population controls, and (b) 151 cases with CL and CLP combined and 308,799 population controls. Negative log₁₀ P values (y axis) are plotted for each tested variant against their chromosomal coordinates (x axis) provided in the human genome build GRCh38/hg38. Two-sided P values are obtained from a likelihood ratio test in regression analysis and are not corrected for multiple comparisons. Red dashed line represents the threshold for genome-wide statistical significance ($P = 5 \times 10^{-8}$ or $\log_{10}(P) = 7.3$) after Bonferroni correction for multiple hypothesis testing. Quantile-quantile plots are shown in inset panels, where the observed (y axis) negative log₁₀ P values are plotted against the expected (x axis) negative log₁₀ P values under null distribution (red line).

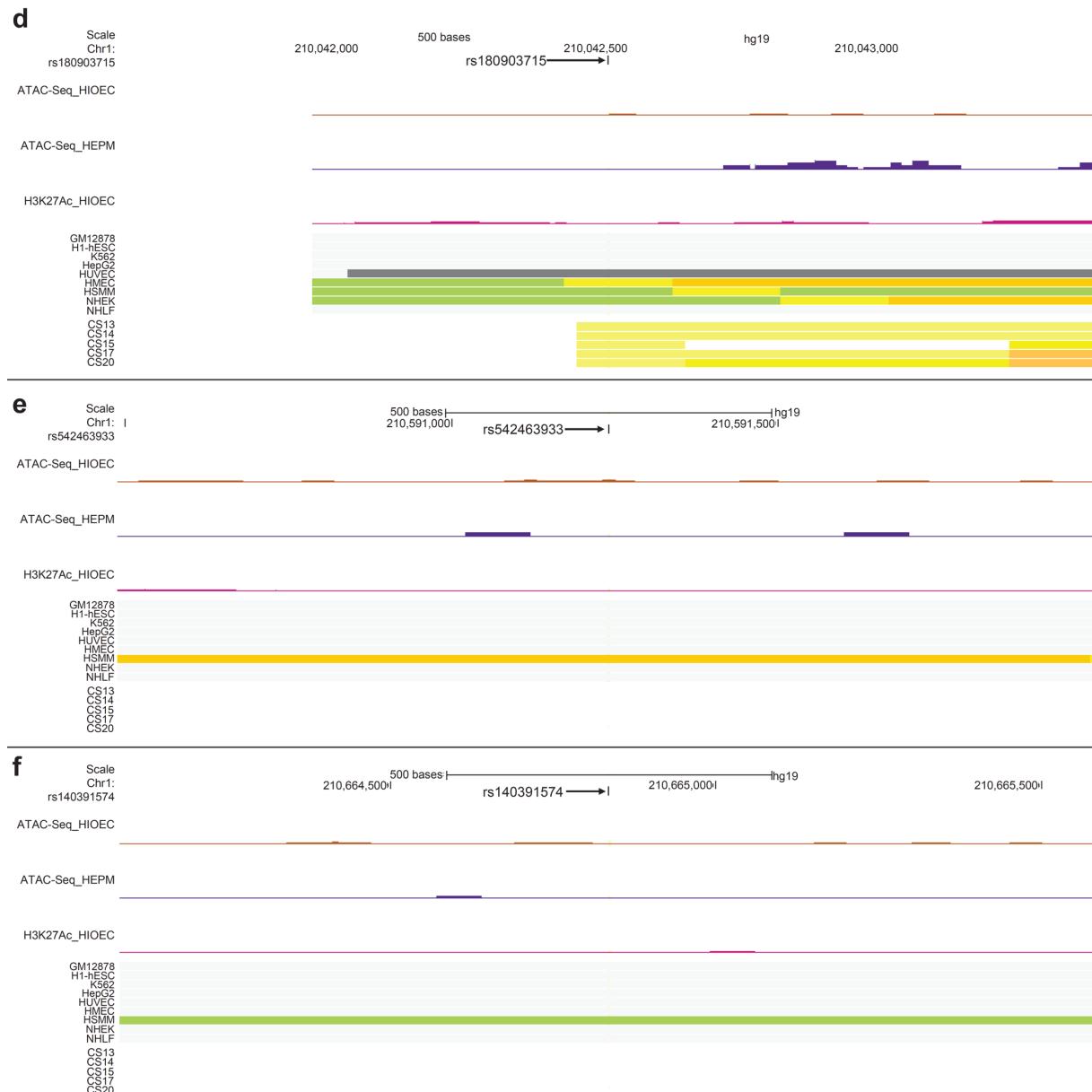


Supplementary Fig. 2: Regional association plots of the chromosome 1q32.2 locus before (a and b) and after (c and d) conditional analysis. For clarity, negative $\log_{10} P$ values (y axis) are shown for variants (x axis) within a 1 Mb (a and c) and a 200 kb (b and d) windows flanking the reference SNP rs7546104, respectively. Two-sided P values are obtained from a likelihood ratio test in regression analysis and are not corrected for multiple comparisons. The reference SNP rs7546104 is labeled with a purple diamond, and pairwise LD (r^2) between the reference SNP and other variants are indicated by color. The r^2 values were estimated from high-coverage whole-genome sequences of 3,775 Finns. Both directly genotyped and imputed SNPs are plotted. Genomic coordinates are shown according to the human genome build GRCh38/hg38. Plots c and d display SNPs in LD with the lead SNP rs7546104, after adjusting for rs570516915 in a conditional analysis. Plots a and c show 1 Mb regions surrounding the index SNP, whereas plots b and d show an expanded view of a 200 kb region focusing on the independent risk haplotype tagged by rs7546104. SNP rs72741048 has been associated with OFC in a large GWAS in the Chinese population¹.

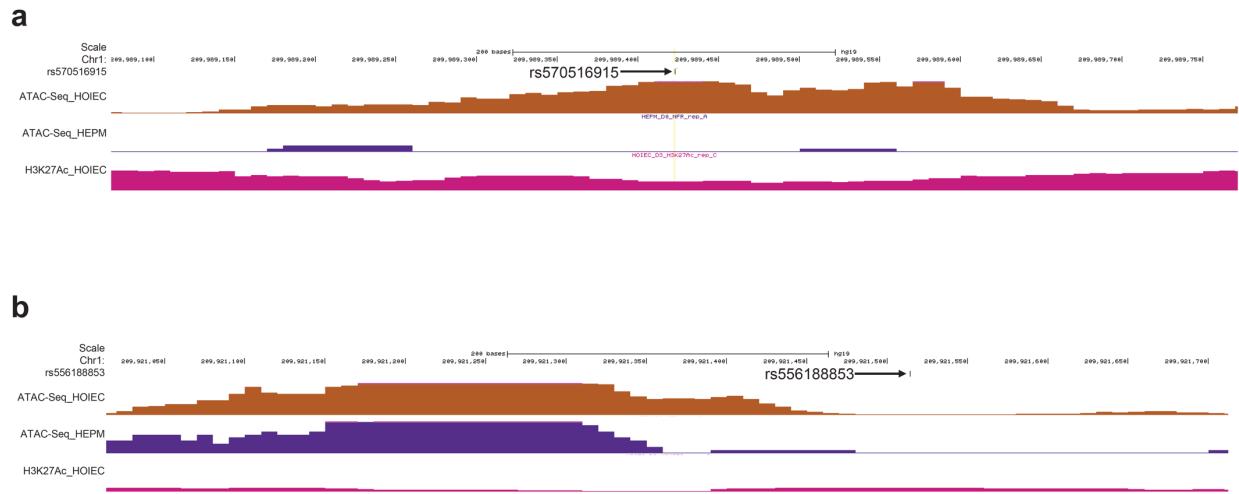
rs570516915, T > G	
Human	CCTGAGAGTTTCGCTCAGGCT
Chimp	CCTGAGAGTTTCGCTCAGGCT
Gorilla	CCCGAGAGTTTCGCTCAGGCT
Orangutan	CCCGAGAGTTTCGCTCAGGCT
Gibbon	CCCGAGAGTTTCGCTCAGGCT
Rhesus	CCTGAGAGTTTCGCTCAGGCT
Crab eating macaque	CCTGAGAGTTTCGCTCAGGCT
Baboon	CCTGAGAGTTTCGCTCAGGCT
Green monkey	CCTGAGAGTTTCGCTCAGGAT
Marmoset	CCTGAGAGTTTCGCTCAGGCT
Squirrel monkey	CCTGAGAGTTTCGCTCAGGCT
Bushbaby	CCTGAGAGTTTCACTCCTGCT
Chinese tree shrew	CCTGAGAGTTTCAGTCGAGCT
Squirrel	CCCAGAGTTTCACTCTGGCT
Lesser Egyptian jerboa	CCC - AAGTTTCGCTCTGGCT
Prairie vole	CCTGGAGTTTCGCTCTGACC
Chinese hamster	CCTGGAGTTTCGCTCTAGCC
Golden hamster	CCTGGAGTTTCGCTCTGGCC
Mouse	CCTGGAGTTTCGCTCTGGCT
Rat	CCTGGAGTTTCGCTCTGGCT
Naked mole rat	CCTCAGAGTTTCACTCTGGCT
Guinea pig	CCTGAGAGTTTCGCTCT - GCT
Chinchilla	CCTGAGAGTTTCGCTCTGGCT
Brush tailed rat	CCCGAGAGTTTCACTCTGGCT
Rabbit	CCCAGGAGTTTCGCTCTGGCT
Pig	CCCAGAGTTTCG - - CTGTTT
Alpaca	CCTGGAGTTTCG - - CTGTTT
Bactrian camel	CCTGGAGTTTC - - CTGTTT
Dolphin	CCCAGAGTTTCG - - CTGTTT
Killer whale	CCCAGAGTTTCG - - CTGTTT
Tibetan antelope	CCCAGAGTTTCG - - GAGTTT
Cow	CCCAGAGTTTCG - - GAGTTT
Sheep	CCCAGAGTTTCG - - GAGTTT
Domestic goat	CCCAGAGTTTCG - - GAGTTT
White rhinoceros	CCCAGAGTTTCGCTCTGGTT
Cat	CCTGAGAGTTTCGCTCTGT
Dog	CCCAGAGTTTCGCTCTGT
Ferret	CCTGAGAGTTTCGCTCTGT
Panda	CCTGAGAGTTTCGCTCTGTCT
Pacific walrus	CCTGAGAGTTTCGCTCTGTGT
Weddell seal	CTTGAGAGTTTCGCTCTGT
Black flying fox	CCCGTGAGTTTCGCCCTGCT
Megabat	CCCGTGAGTTTCGCCCTGCT
David's myotis	CCCGGGAGTTTCGCTCTGGTT
Microbat	CCCGGGAGTTTCGCTCTGGTT
Big brown bat	CCCGGGAGTTTCGCTCTGGTT
Elephant	CCCAGAGTTTCACTCTGGTT
Cape elephant shrew	CCCAGAGTTTCGCCCTGGTT
Manatee	CTCGAGAGTTTCACTCTGGTT
Cape golden mole	CCCGGGAGTTTCACTCTTGTT
Tenrec	CCCAAGGAGTTTCACTCTGGTT
Aardvark	CCCGAGAGTTTCACTCTGGTT
Armadillo	CCTGAGAGTTTC - - CTCTCGTT
Opossum	CCC - AGAGTTTCACTTGGGCT
Tasmanian devil	CCC - AGAGTTTCACTTGGGCT
Wallaby	CCC - AGAGTTTCACTTGGGCT
Platypus	TTCAAGAGTTTC-----

Supplementary Fig. 3: Evolutionary conservation of the rs570516915 variant site in MCS-9.7. Multi-species sequence alignments surrounding the rs570516915 variant site in the MCS-9.7 enhancer for *IRF6* were obtained from the multiz alignment and conservation track of 100 vertebrate species in the UCSC Genome Browser (<https://genome.ucsc.edu>).

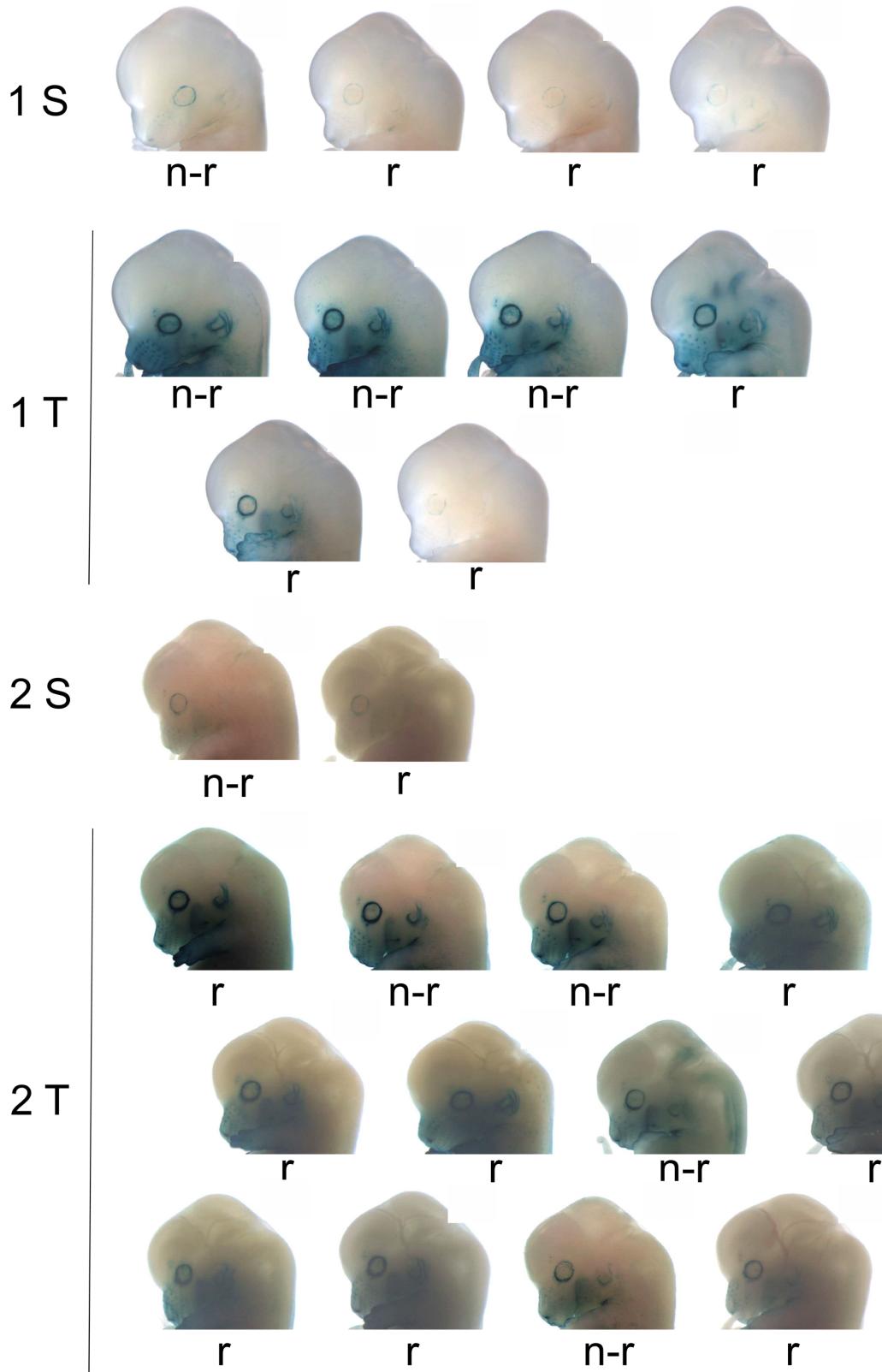


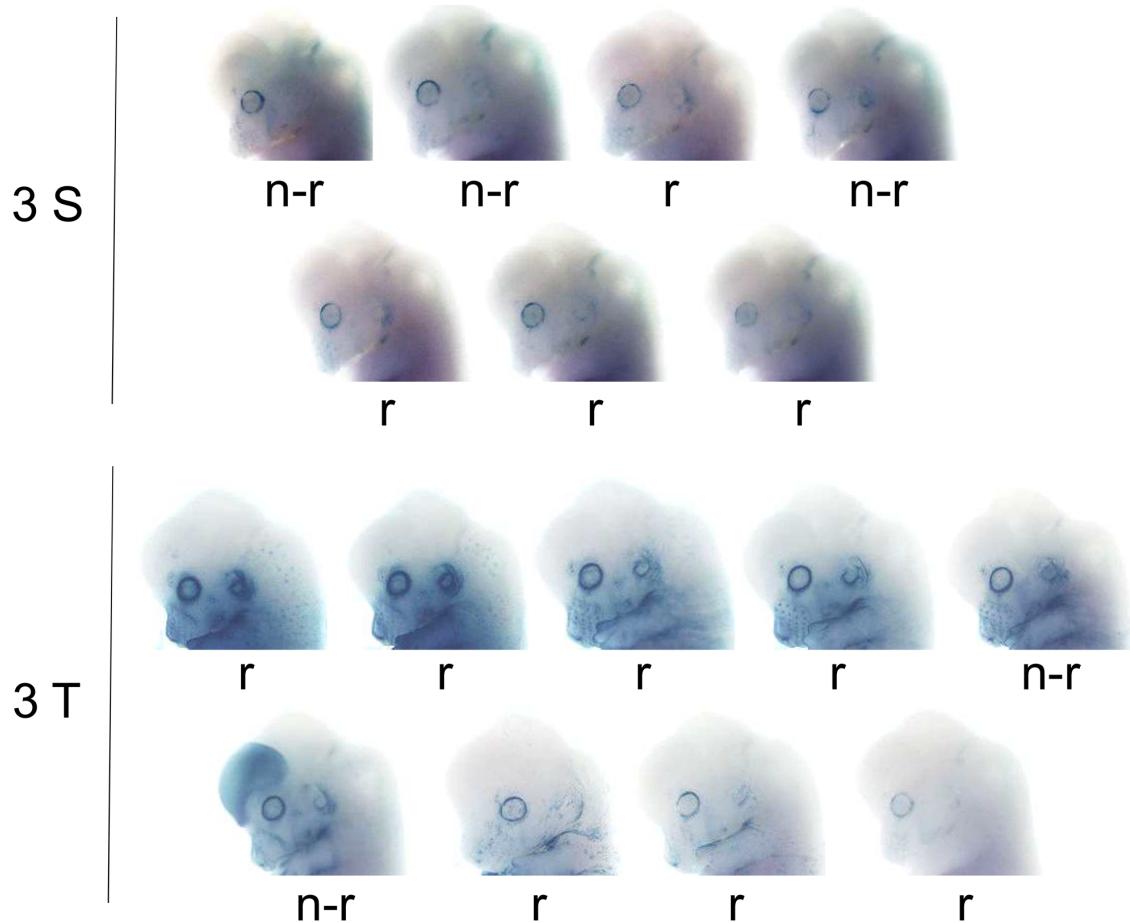


Supplementary Fig. 4: Browser views of the human genome in GRCh37/hg19, illustrating that one (rs556188853) of the 6 SNPs in strong LD with rs570516915 falls into a region with chromatin marks consistent with enhancer activity in epithelial cells (HIOEC and NHEK). First track, SNPs in LD with rs570516915, (a) rs904738414, (b) rs553231832, (c) rs556188853, (d) rs180903715, (e) rs542463933 and (f) rs140391574; second and third tracks, ATAC-Seq peaks from HIOEC and HEPM cells, respectively; fourth track, H3K27Ac from HIOEC cells; fifth track, chromatin status revealed by ChIP-Seq to various chromatin marks from the ENCODE Project cell lines and facial explants from human embryos at Carnegie stage (CS) 13-20, color coded as in Fig. 4a. Additional color codes from ENCODE project cell lines: bright red, active promoter; light red, weak promoter; purple, inactive/poised promoter. Additional color codes from facial explants from human embryos at CS 13-20: red, active TSS; orange red, promoter upstream or downstream TSS; light purple, poised promoter.



Supplementary Fig. 5: Browser views of genomic regions included in the luciferase reporter vectors for the rs570516915 and rs556188853 variants. First track, position of (a) rs570516915 and (b) rs556188853 SNPs; second and third track, ATAC-Seq peaks from the HOIEC and HEPM cells, respectively; fourth track, H3K27Ac peaks from the HOIEC cells.



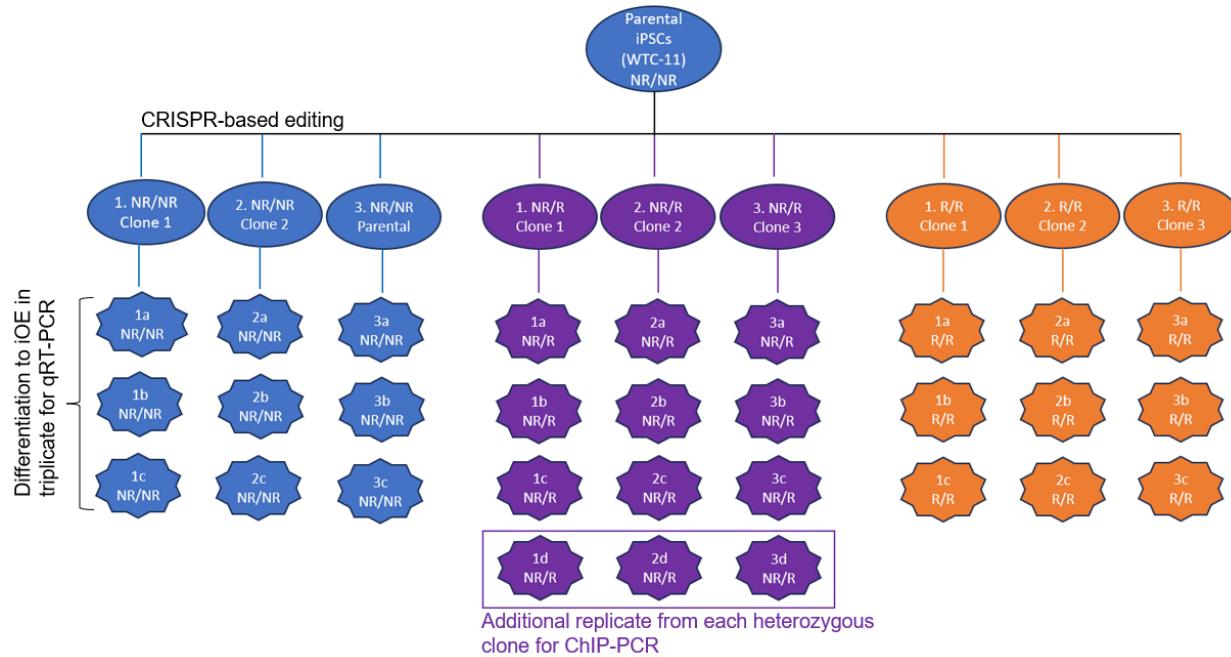


Supplementary Fig. 6: Heads of X-gal-stained transgenic reporter embryos at E13.5 showing effect on staining of inserted non-risk and risk alleles of rs570516915. Embryos were divided into six sets, which consisted of single (S) or tandem (T) insertions from three experimental batches (1, 2 and 3). The heads are ordered according to consensuses from blinded assessments from strongest to weakest expression. Images of heads of sets 3S and 3T were adjusted similarly in each set for brightness and contrast. n-r, non-risk; r, risk.

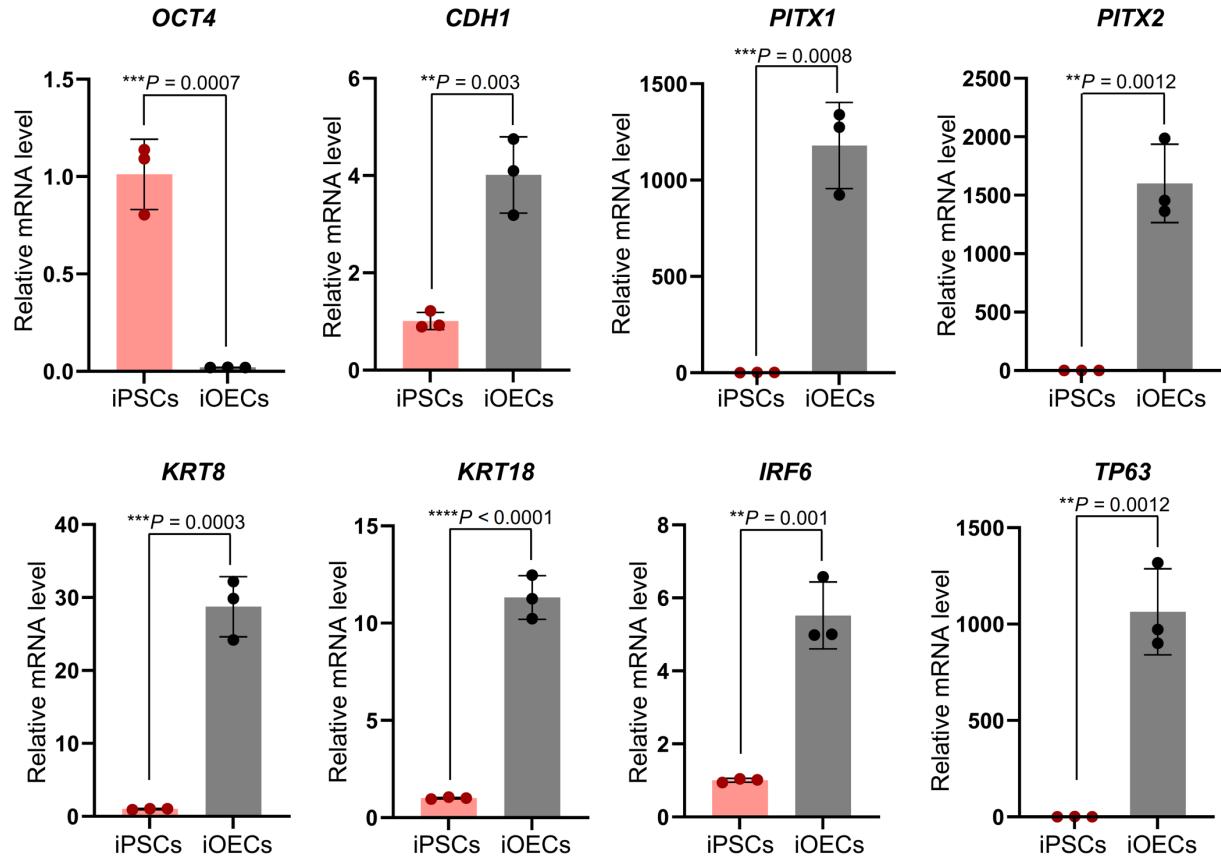
Guide RNA and repair template sequences

Genome with T allele
XXXXXTGGAGCTTGGGGCTGGAACCTCTACCTCGCTCAATGTCGGAGGCCCTGAGAGTTCCGCTCAGGCTCAGAGCAGGCATCGAACCTCCAGTTACTATTCTGTGCTGTGGCAAGXXXXX
HDR template with G allele
TGGAGCTTGGGGCTGGAACCTCTACCTCGCTCAATGTCGGAGGCCCTGAGAGTTCCGCTCAGGCTCAGAGCAGGCATCGAACCTCCAGTTACTATTCTGTGCTGTGGCAAG

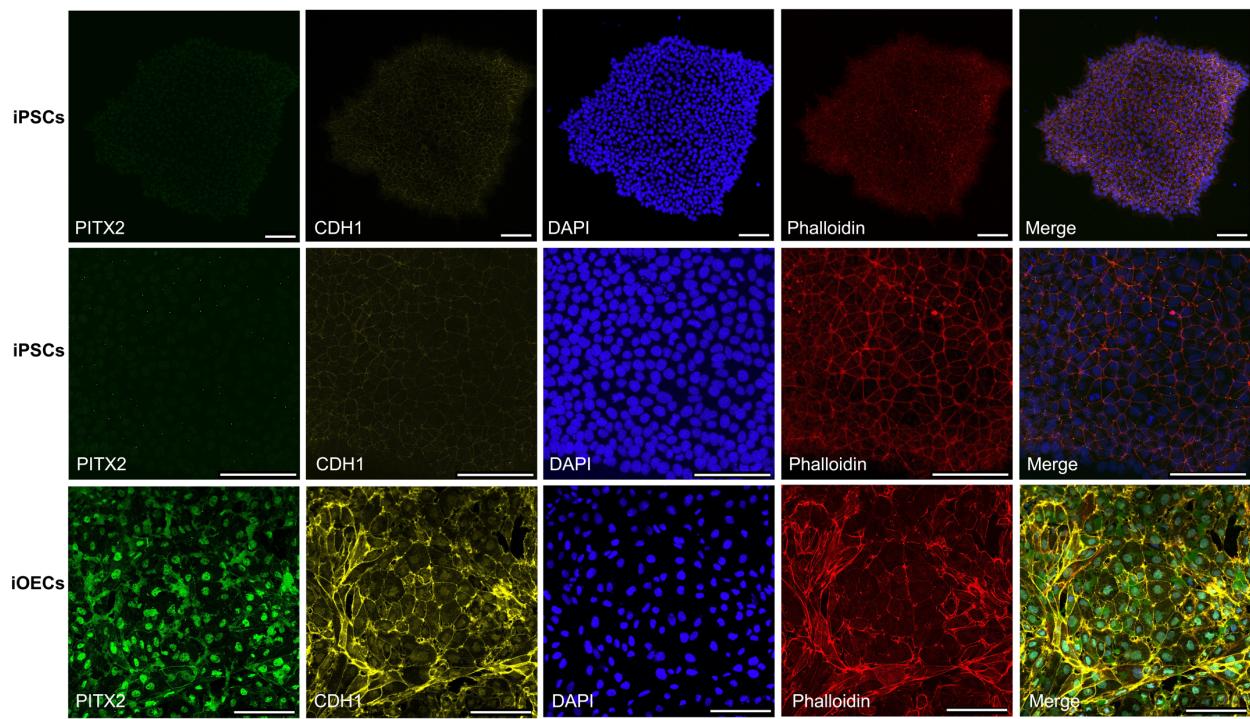
Supplementary Fig. 7: Sequences of the guide RNA (gRNA) and homology-directed repair (HDR) template DNA used for CRISPR-Cas9 editing of the rs570516915 variant sequence. PAM represents the protospacer adjacent motif for the guide used in the study.



Supplementary Fig. 8: Strategy for *in vitro* cell culture experiments: Parental induced pluripotent stem cells (iPSCs) WTC-11, homozygous for non-risk allele of rs570516915 (TT, NR/NR), were edited to be heterozygous (TG, NR/R) or homozygous for the risk allele (GG, R/R) and subjected to a 10-day differentiation protocol to generate induced oral epithelial cells (iOECs). NR, non-risk allele; R, risk allele.



Supplementary Fig. 9: Scattered dot plots illustrating comparison of expression of stem cell marker *OCT4* and different epithelial markers (*CDH1*, *PITX1*, *PITX2*, *KRT8*, *KRT18*, and *TP63*) in iOECs vs iPSCs by qRT-PCR. Expression levels of each gene are normalized to *ACTB* expression. Data are represented as mean values +/- s.d. from three replicates. Statistical significance is determined by Student's *t*-test (two-tailed; *P* values are indicated on the plot).

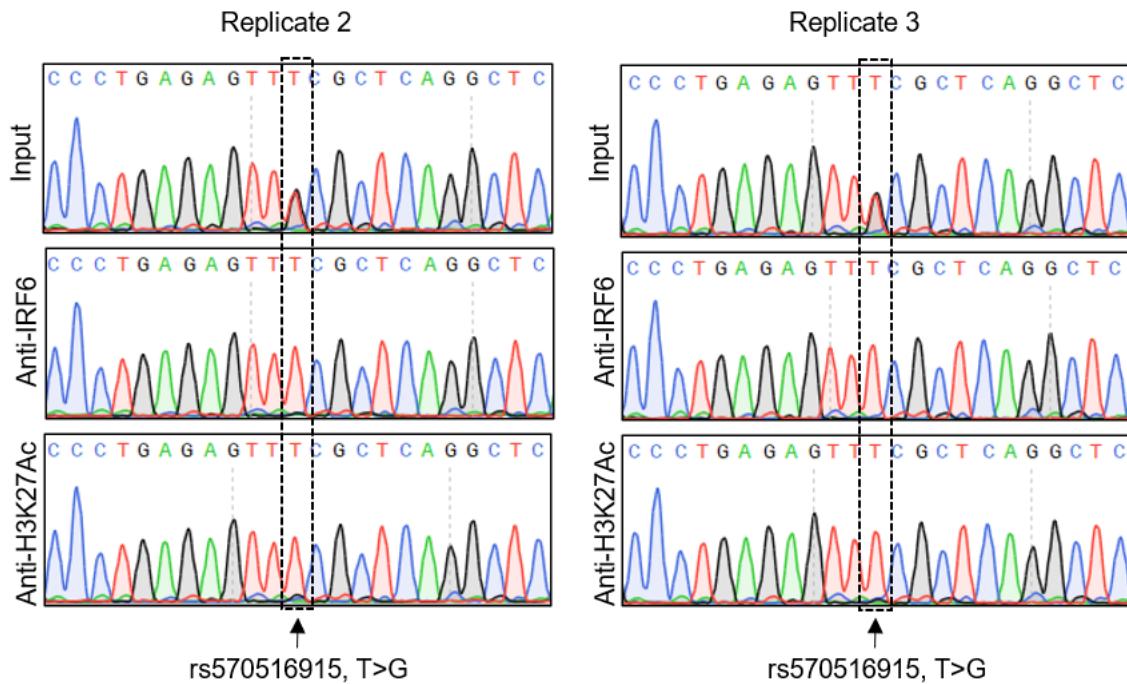


Supplementary Fig. 10: Immunofluorescence staining of human iPSCs and iOECs. Green, PITX2-positive nuclei; yellow, CDH1-positive induced oral epithelial cells (scale bar: 100 μ m).

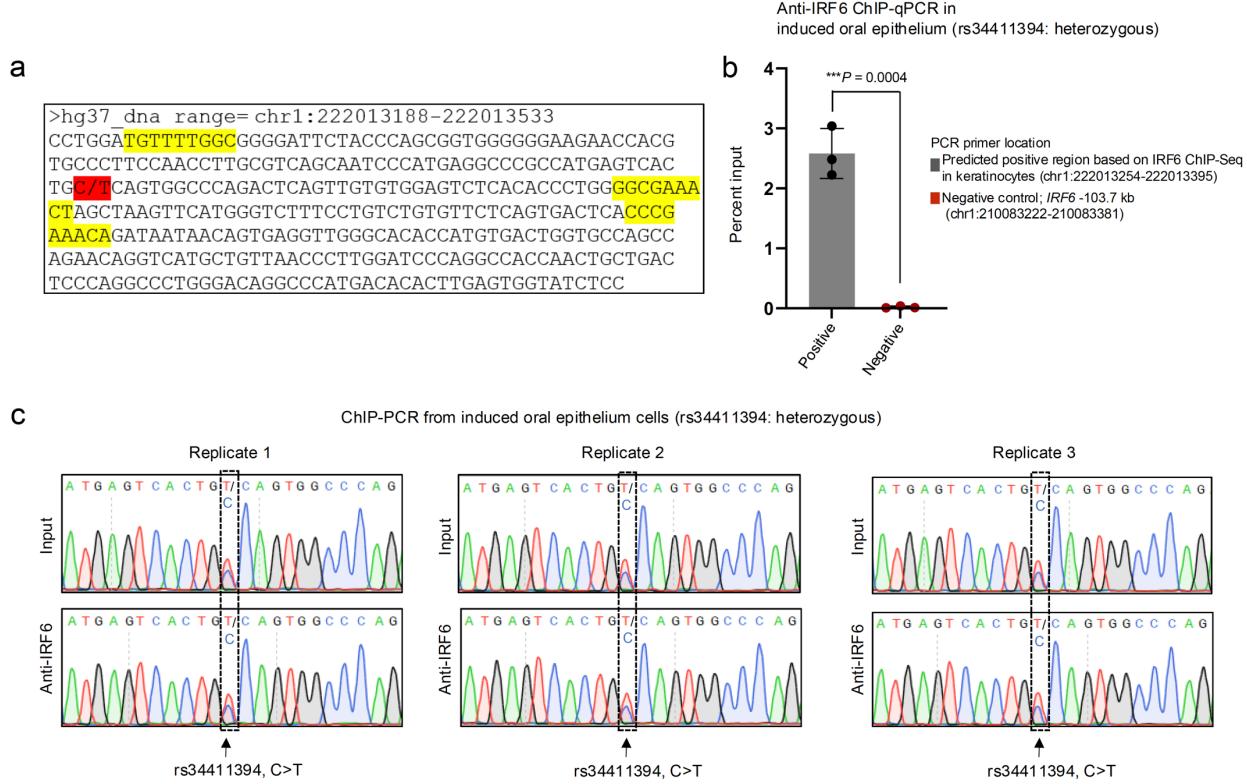
Rank	Difference log(p) for two sequences	rs570516915_A sequence p-value	rs570516915_C sequence p-value	Matrix_ID	Matrix name
1	1.8	0.00382	0.242	PB0036.1	Irf6_1
2	1.49	0.0117	0.36	PB0034.1	Irf4_1
3	1.3	0.0183	0.363	PB0035.1	Irf5_1
4	-1.13	0.354	0.026	PF0167.1	CCTNTMAGA
5	1.02	0.0473	0.498	MA0024.1	E2F1
6	0.906	0.111	0.898	MA0393.1	STE12
7	-0.802	0.533	0.0842	MA0349.1	OPI1
8	0.786	0.0375	0.229	MA0440.1	ZAP1
9	0.742	0.125	0.69	PB0115.1	Ehf_2
10	0.737	0.156	0.852	MA0360.1	RDR1

Supplementary Fig. 11: Prediction of IRF6 binding and the effect of the rs570516915 risk allele on its binding affinity. Sequence shown in Supplementary Fig. 6 was searched in sTRAP² (http://trap.molgen.mpg.de/cgi-bin/trap_two_seq_form.cgi) with the non-risk (rs570516915_A) and risk (rs570516915_C) alleles of rs570516915 (AGCCTGAGCG[A/C]AACTCTCAGG). Complementary nucleotides are shown. *P* values are calculated by comparing the observed transcription factor (TF) affinity to the background, using a generalized extreme value (GEV) parametrization model as described previously³. *P* values are not corrected for multiple testing. As a visual aid, *P* values less than 0.05 are highlighted in green. TF matrix was selected from Jaspar (all_matrices) and human_promoters was selected as a background model.

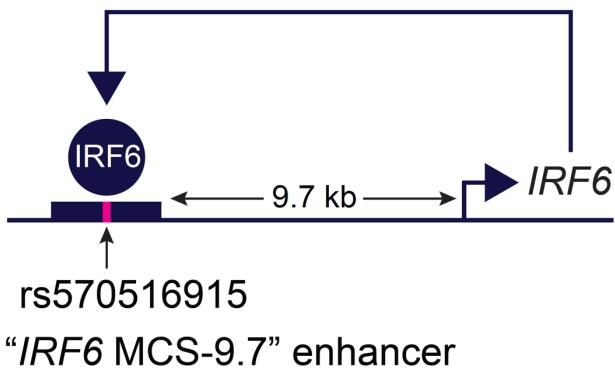
ChIP-PCR from heterozygous induced oral epithelium cells



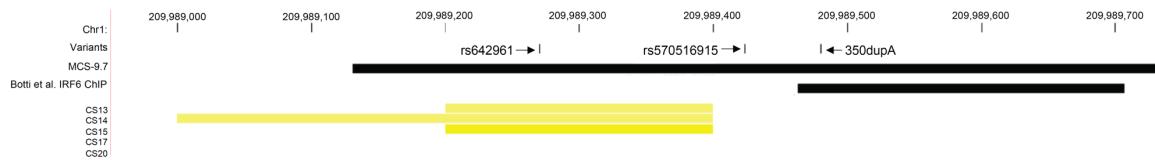
Supplementary Fig. 12: Sanger sequencing chromatograms of anti-IRF6 and anti-H3K27Ac ChIP-PCR products of cells heterozygous for rs570516915 from two ChIP replicates.



Supplementary Fig. 13: No allele bias was detected in chromatin precipitated by anti-IRF6 at a heterozygous SNP that does not lie within a predicted IRF6 binding site. **a** Genomic sequence underlying an anti-IRF6 ChIP-Seq peak in human keratinocytes⁴. IRF6 binding sites predicted by the JASPAR 2024 database⁵ are highlighted in yellow; red, rs34411394 C>T position. **b** Percent of input chromatin precipitated by anti-IRF6 in iOECs heterozygous for rs34411394 using primers within the locus mentioned above or at a predicted negative region. The latter is located 103.7 kb upstream *IRF6* transcription start site. This region does not overlap ATAC-Seq or H3K27Ac ChIP-Seq peaks in HIOEC or NHEK cells and is devoid of predicted IRF6 binding sites. Error bars refer to 3 ChIP replicates and expressed as mean values +/- s.d. Statistical significance is determined by Student's t-test. P value (two-tailed) is indicated on the plot. **c** Sanger sequencing chromatograms of anti-IRF6 ChIP-PCR product of cells heterozygous for rs34411394.



Supplementary Fig. 14: A schematic model depicting autoregulation of *IRF6* through the MCS-9.7 enhancer located 9.7 kb upstream of *IRF6* transcription start site.



Supplementary Fig. 15: Browser view of the human genome in GRCh37/hg19, illustrating that rs570516915 is close to IRF6 ChIP-Seq peak identified by Botti et al. First track, three DNA variants in MCS-9.7, rs642961⁶, rs570516915 and 350dupA⁷; second and third tracks, MCS-9.7⁶ and IRF6 binding peaks identified by Botti et al.⁴; fourth track, chromatin status from facial explants from human embryos at Carnegie stage (CS) 13-20, encompassing the time when palate shelves fuse where yellow bars represent the active enhancer elements.

Supplementary Methods

Sequence of the rs556188853 variant region inserted into the luciferase reporter construct. Variant sequence is highlighted in red.

> hg19_chr1:209921014-209921714

```
CAGGGGCTCCTCACACCGTCCTGAACAGTACCGTGACCACAAAAGAGCAAGGCTGCCTAAGGAAAGACTTGC  
CTCTCGCCGCCCTCCCTTGGGCCGTAGGGTGGCAGCGCCACCCGTGGCTGAATGTGGTGGTACCAACGCTTC  
ACGCAATCTCTGCCAGTGCATCAGTTCTACCCCAGGGGGCACACCTGCACACCAGCGTGCCTACCAAGT  
GAGTCATCGCTAGGGCCCTGAACCTGACCTAGAGCGGGCTGGACTCCGGCTCGGTAACTCCGCCGGCTGCTG  
GGGCCTACGTTGGATTGAGCCGTGGGAACTAGACCTGTTCAATAGCGACAGCTAGTGGTCACCCCTCAAAACAA  
GGTGCCTGAACCTCGCGGGAAAGCGCTGCAAGATATGGGAAATCAGGACAGATGATTGGCCTGAGTGAAGC  
TGGCACATTTCGAAGCCACTTGGTAAATGGTCTG/ATCGAAACCCAAACGCTTAAAGTGTACACGA  
CCTGGCTGCCCTCCAGCCACATCTCGGGCACCTGCCCTGCTTGATTCCCAAGGACAAGAAACTGCCAGTCC  
TCTGAAGCACCCTGATGATCCCAGTGTGTTGCCATGCCATCTCCCTGCCCTGAGCGCTTCACTCCCCAT  
TGCATGGTTA
```

Sequence of the rs570516915 variant region inserted into the luciferase reporter construct. Variant sequence is highlighted in red.

> hg19_chr1:209989073-209989773

```
GTCATCAGGGATTTTAAAAATTAGTTTAAGAGTTTATCCAAGGTCTAAAGCTGGTAATGGTGAGTAG  
GAAGTTGATTCTGCCGATCTGCTGACTCCATGCCCTTCTATTAGGTCACTGAAGGGAACCTGAGGATTGGAGCT  
TTGAATGTTAATCTACCAAAGGCCTGAAGTAATAACCCAGAATGTGAACATGTGACCCTGCCTGCTGG  
GGTGGGAAGAAGGCAGCATGCTCTATCCTGACCTGATTGAGGCCAGGGCTGAATCTGGAGCTTGGGCTGG  
GAACCTCTCACCTCGTCAATGTCTGGAGGCCCTGAGAGTTT/CAGCTCAGGCTCAGAGCAGGCATCGAACCTCC  
CAGTTACTATTCTGTGCTGAGCCAGCTCTGGCAGCTGGATTCCACTGCCTAGGCAGGAAGCTCATCTCAGCCAGTGACCTT  
TAGTCAGGCCAGACCGCTCTGGCAGCTGGATTCCACTGCCTAGGCAGGAAGCTCATCTCAGCCAGTGACCTT  
TTCTCTGTTTGTACAGAGGAATTCCATGCCAGCTATGGGAATGGGGCAATGGGGTGGGTGGCAAAGGTTCC  
CCCTTAAGCCACAAGAGCCATGGAGTGGAGGTAAGCTAACAAACAGAGGAGGAAGGATGGAGGAAGGATCAGGA  
AGATTAGAG
```

Sequence of the rs570516915 variant region on the Finnish haplotype background fused to the LacZ reporter gene for transgenic F0 embryo assay. Variant sequence is highlighted in red.

> hg19_chr1:209988937-209990041

```
CTAAGAGTAATTACCTATGTTAGCTTTCTGGAATTGTTCCAGAATCTTGCCTTGAGGAAAGTTGTGGCTGC  
GTATTCTGCCCTCTGTTGGAAATCCCTGCACAGCTTGCAGCTACTCAGCTGGTCATCAGGGATTTT  
TTAAATTAGTTAAGAGTTATCCAAGGTCTAAAGCTGGTAATGGTGAAGTGGAGCTTGGAAATGTTAATCTTAC  
TCTGTCTGACTCCATGCCCTTCTATTAGGTCACTGAAGGGAACCTGAGGATTGGAGCTTGGAAATGTTAATCTTAC  
CCAAAGGCCTGAAGTAATAACCCAGGATGTGAACATGTGACCCTGCCTGCTGGGGTGGAAAGAAGGCAGC  
ATGCTCTATCCTGACCTGATTGAGCCAGGGCTGAATCTGGAGCTTGGGGCTGGAAACCTCTACCTGCCT  
CAATGTCTGGAGGCCCTGAGAGTTT/CAGCTCAGGCTCAGAGCAGGCATCGAACCTCCAGTTACTATTCTGTGCT  
GTGGCAAGTGCAGCTGTCCTCTTCCCCACCCAGCCGGAAACCAGCAGCATTTCTAGTCAGGCCAGACCC  
GTCCTGGCAGCCTGGATTCCACTGCCTAGGCAGGAAGCTCATCTCAGCCAGTGACCTTCTCTGTTTGT  
ACAGAGGAATTCCATGCCAGCAGTATGGGCAATGGGGTGGGCAAAGGTTCCCCCTTAAGCCACAAGAGC  
CATGGAGTGGAGGTAAGCTAACAGAGGAGGAAGGATGGGAGGAAAGGATCAGGAAGATTAGAGAGTCCATT  
CCTCAGGCTGCTCATCCTCAAATTCAAGTAAATAAGGTGTGGGAAGGATGCACTATATGTCAGCTGCCAG  
CCCTGAAGATCCTCCCCCTAGAGAACTGAGACAGGAGTTCTCACATTCCACATGCAGGAGGGAAAGAGGCTGG  
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CTGTCAGGTGGAAGCTGGCCTCTCATT
```

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3. Manke, T., Roider, H.G. & Vingron, M. Statistical modeling of transcription factor binding affinities predicts regulatory interactions. *PLoS Comput. Biol.* **4**, e1000039 (2008).
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7. Fakhouri, W.D. et al. An etiologic regulatory mutation in IRF6 with loss- and gain-of-function effects. *Hum. Mol. Genet.* **23**, 2711-2720 (2014).

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