**Smad4-Irf6 genetic interaction and TGFβ-mediated IRF6 signaling cascade are crucial for palatal fusion in mice**

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**SUMMARY**

Cleft palate is one of the most common human birth defects and is associated with multiple genetic and environmental risk factors. Although mutations in the genes encoding transforming growth factor beta (TGFβ) signaling molecules and interferon regulatory factor 6 (Irf6) have been identified as genetic risk factors for cleft palate, little is known about the relationship between TGFβ signaling and Irf6 activity during palate formation. Here, we show that TGFβ signaling regulates expression of Irf6 and the fate of the medial edge epithelium (MEE) during palatal fusion in mice. Haploinsufficiency of Irf6 in mice with basal epithelial-specific deletion of the TGFβ signaling mediator Smad4 (Smad4<sup>fl/fl</sup>;K14-Cre;Irf6<sup>+/R84C</sup>) results in compromised p21 expression and MEE persistence, similar to observations in Tgfr2<sup>−/−</sup>;K14-Cre mice, although the secondary palate of Irf6<sup>+/R84C</sup> and Smad4<sup>−/−</sup>;K14-Cre mice form normally. Furthermore, Smad4<sup>−/−</sup>;Irf6<sup>−/−</sup>;Irf6<sup>+/R84C</sup> mice show extra digits that are consistent with abnormal toe and nail phenotypes in individuals with Van der Woude and popliteal pterygium syndromes, suggesting that the TGFβ/SMAD4/IRF6 signaling cascade might be a well-conserved mechanism in regulating multiple organogenesis. Strikingly, overexpression of Irf6 rescued p21 expression and MEE degeneration in Tgfr2<sup>−/−</sup>;K14-Cre mice. Thus, Irf6 and SMAD4 synergistically regulate the fate of the MEE, and TGFβ-mediated Irf6 activity is responsible for MEE degeneration during palatal fusion in mice.

**KEY WORDS:** TGFβ, Irf6, Palatal fusion, Mouse

**INTRODUCTION**

Cleft palate is a serious and common craniofacial birth defect affecting millions of people worldwide (Mossey et al., 2009; Wong and Hagg, 2004). Cleft palate has ethnic and geographic variations in prevalence, and it affects feeding, swallowing, speech, hearing, middle-ear ventilation, respiration and appearance (Iwata et al., 2011). Studies in mouse models and screening in humans have implicated several factors in syndromic cleft palate, such as Irf6 mutation in Van der Woude syndrome (VWS) and popliteal pterygium syndrome (PPS), SMAD4 mutation in juvenile polyposis syndrome, and TGFBR1 or TGFBR2 mutation in Loeys-Dietz syndrome (previously called Marfan syndrome type II). Mutations in TGFβ3, Irf6, CYP (cytochrome P450), MSXI and TBX10 have also been associated with non-syndromic cleft lip with or without cleft palate (NSCL/P) (Iwata et al., 2011). In addition, polymorphic variants associated with NSCL/P within human chromosomes 1q32 (Irf6), 1p22 (ABC48), 8q24.21, 10q25 (VAX1), 17q22 and 20q12 (MAFB) have been identified by genome-wide association studies (Dixon et al., 2011). Thus, mutations in Irf6, SMAD4 and TGFBR2 confer a significant attributable risk for cleft palate.

TGFβ signaling is one of the major signaling cascades crucial for craniofacial development (Iwata et al., 2011). Epithelial-specific deletion of Tgfr2 (Tgfr2<sup>−/−</sup>;K14-Cre) in mice results in persistence of the medial edge epithelium (MEE) and submucous cleft palate (Xu et al., 2006). TGFβ transmits signals through a membrane receptor serine/threonine kinase complex that phosphorylates SMAD2 and SMAD3, followed by the formation of transcriptional complexes with SMAD4 and translocation into the nucleus (Massagué, 2012; Ross and Hill, 2008; Schmierer and Hill, 2007; Shi and Massagué, 2003). TGFβ also activates SMAD-independent signaling cascades, including mitogen-activated protein kinase (MAPK) pathways, such as p38 MAPK (MAPK14 – Mouse Genome Informatics), under certain physiological and pathological conditions (Kang et al., 2009; Xu et al., 2008; Zhang, 2009). Studies using SMAD4-deficient cells, or dominant-negative SMADs, support the possibility that MAPK activation is independent of SMADs (Chen et al., 1998; Giehl et al., 2000; Hocevar et al., 1999; Hu et al., 1999). p38 MAPK activation by TGFβ is accompanied by SMAD-independent, TRAF6 and TAK1 (MAP3K7 – Mouse Genome Informatics), under certain physiological and pathological conditions (Kang et al., 2009; Xu et al., 2008; Zhang, 2009). Studies using SMAD4-deficient cells, or dominant-negative SMADs, support the possibility that MAPK activation is independent of SMADs (Chen et al., 1998; Giehl et al., 2000; Hocevar et al., 1999; Hu et al., 1999). p38 MAPK activation by TGFβ is accompanied by SMAD-independent, TRAF6 and TAK1 (MAP3K7 – Mouse Genome Informatics), phosphorylation (Iwata et al., 2012; Sorrentino et al., 2008; Yamashita et al., 2008). The balance between direct activation of SMADs and MAPK pathways often defines cellular responses to TGFβ.

IRF6 belongs to a family of transcription factors that share a highly conserved, helix-turn-helix, DNA-binding domain and a less conserved, protein-binding domain. Among the genes that have been associated with NSCL/P, Irf6 has been implicated in the largest percentage of cases (Koillinen et al., 2005; Srichomthong et al., 2005). Mutation of Irf6 can lead to the autosomal-dominant conditions VWS and PPS, which are characterized by oral clefting and lower lip pits (Kondo et al., 2002; Moretti et al., 2010). VWS and PPS are allelic variants of the same condition caused by different mutations of the same gene. PPS includes all the features of VWS, plus popliteal pterygia, synonychia, distinct toe and nail abnormality, syndactyly and genito-urinary malformations. An arginine 84 to cysteine (R84C) mutation in Irf6 is the most common mutation found in patients with PPS (Richardson et al., 2006). Although the function of the R84C mutation is still largely...
unknown, recent studies have demonstrated that it results in loss of DNA binding (Kondo et al., 2002; Little et al., 2009). The primary defect in Irf6-deficient mice is in keratinocyte differentiation and proliferation. Homozygous Irf6<sup>−/−</sup> (null) embryos exhibit abnormal skin, limb and craniofacial morphogenesis, including cleft palate (Ingraham et al., 2006). Mice homozygous for Irf6<sup>−/−</sup>, which is an R84C knock-in resulting in expression of mutant Irf6 protein, exhibit a severe intraoral epithelial adhesion caused by a failure of terminal differentiation similar to that in homozygous Irf6<sup>−/−</sup> (null) embryos (Ingraham et al., 2006; Richardson et al., 2006).

Despite the established roles of TGFβ signaling and IRF6 activity during palate formation, the interaction between TGFβ signaling and IRF6 activity is poorly understood. In this study, we investigate the interaction between TGFβ signaling and IRF6 activity. We demonstrate that Irf6 and Smad4 interact genetically, and that TGFβ-mediated Irf6 expression is crucial for p21 (CDKN1A – Mouse Genome Informatics) expression and fate determination of the MEE cells during palatal fusion.

### MATERIALS AND METHODS

#### Animals

To generate Smad<sup>−/−</sup>;K14-Cre;Irf6<sup>−/−</sup> mice, we mated Smad<sup>−/−</sup>;K14-Cre;Irf6<sup>−/−</sup> with Smad<sup>−/−</sup>;K14-Cre mice. To generate Tgfbr2fl/+;K14-Cre mice, we mated Tgfbr2<sup>−/−</sup>;K14-Cre with Tgfbr2<sup>−/−</sup> mice. Genotyping was performed using PCR primers as previously described (Ito et al., 2003; Xu et al., 2008; Richardson et al., 2006). Mouse experiment protocols approved by the Department of Animal Resources and the California Department of Food and Agriculture (K14; KRT14 – Human Gene Nomenclature Database) promoter-driven Irf6-encoding transgene was prepared as follows: an EcoRI-HindIII blunt fragment (7.3 kb) encoding mouse Irf6 was subcloned into the BamHI blunt-ended sites of the pGem 3Z-K14 vector producing pGEM3Z-K14-lrf6, resulting in a construct containing the K14 promoter (2.1 kb) and the β-globin intron (736 bp), the coding sequence for Irf6 (4.1 kb) and the K14 polyadenylation signal (500 bp). The EcoRI-HindIII fragment was isolated free of vector sequence by preparative gel electrophoresis. DNA was further purified using an Elutip column (Schleicher and Schuell, Dessel, Germany) and microinjected in the pronuclei of fertilized oocytes (Jackson/B6DF1 F1) following standard procedures. Transgenic founder mice were identified by PCR analysis. PCR amplification of tail genomic DNA (0.5-1 μl) of each reaction was resolved in 1% agarose gel, and amplified fragments were visualized by ethidium bromide staining. The PCR primers used were 5′-TCAGGAGCGTCGAACAGAGGT-3′ and 5′-ACTCCGATCCCTTTCATTTAAT-3′. To generate Tgfbr2<sup>Δ</sup>;K14-Cre;Irf6<sup>−/−</sup> mice, we mated Tgfbr2<sup>−/−</sup>;K14-Cre;Irf6<sup>−/−</sup> with Tgfbr2<sup>−/−</sup> mice. Genotyping was performed using PCR primers as previously described (Ito et al., 2003; Xu et al., 2008; Xu et al., 2006). All mouse experiments were conducted in accordance with protocols approved by the Department of Animal Resources and the Institutional Animal Care and Use Committee of the University of Southern California.

#### Comparative analysis of transcription factor-binding sites

Genomic sequences of the entire human and murine Irf6 (RefSeq accession NM_006147.3/hg19 and NM_016581.2/mm10), p21 (RefSeq accession NM_000389.4/hg19 and NM_016851.2/mm10) and CDKN1A/p21 (RefSeq accession NM_000389.4/hg19 and NM_000389.4/mm10) were obtained from the University of California, Santa Cruz (UCSC) genome browser, including 2.5 kb upstream and 2.5 kb downstream of the respective transcription start sites (TSSs), based on mouse genome Build 38. These sequences were mapped to seven additional mammalian genomes [chimpanzee (Build 2.1.4), orangutan (Build 2.0.2), rhesus macaque (Build 1.0), human (Build 19), rat (Build 5), dog (Build 3.1) and horse (Build eqCab3)] using the BLAT tool. Multiple alignments for these sequences were obtained using the ClustalW2 tool with default parameters and settings (Larkin et al., 2007). To account for uncertainty in the quality of the horse and dog draft genome sequences, sequence alignments with and without information from these species were performed. Transcription factor-binding motifs relevant to SMAD, p63 (TRP63 – Mouse Genome Informatics) and p38 MAPK pathway elements were searched within and proximal to these genes (i.e. 2.5 kb upstream and 2.5 kb downstream of the mouse TSS for each gene). The recognition sequences searched for SMAD binding were 5′-GTCCT-3′, 5′-AGAC-3′ and 5′-GTCCTAGC-3′ (Denissova et al., 2000; Zawel et al., 1998) and for MEF2 family member binding was 5′-YTAATTTCTTCA-3′ (Black and Olson, 1998). In addition, we evaluated the following potential p63 recognition sites: the canonical p53 family 20-base recognition sequence formed from duplicates of the half-site recognition sequence 5′-RRRWWGYYY-3′ separated by any combination of 0 to 13 bases (Cai et al., 2012; de-Deiry et al., 1992) and the p63-preferential recognition sequence 5′-GTRRWWGYTYYRRCWGYTTY-3′ (Perez et al., 2007). To account for the possibility of degenerate p53 family member-binding sites, we used the p53scan and p63scan algorithms with default options (Kouwenhoven et al., 2010), which allow the consensus half-site to vary slightly in composition. Finally, we also searched for the eight-base recognition sequence 5′-AANNGAAA-3′ for the DNA-binding domains of IRF family members (Fujii et al., 1999) and the more specific ten-base recognition sequence 5′-AAACGAACGAC-3′ for the Irf6 DNA-binding domain (Little et al., 2009).

#### Promoter-proximal search of p53-family responsive elements

The aforementioned consensus recognition sequences of p53 family members (see previous section) were used to search the murine and human p21 genomic sequences for putative p53 and p63 recognition sites proximal to the promoter. Genomic sequences were obtained from the UCSC genome browser, spanning a 5-kb window centered around the transcription start site of each p21 RefSeq transcript variant in the murine genome (build mm10, 2 transcript variants) and human genome (build hg19, 4 transcript variants).

#### Chromatin immunoprecipitation (ChIP) assay

Skin was dissected from embryonic day (E) 13.5 C57Bl/J mouse embryos in PBS. For the separation of skin epithelium from mesenchyme, the explants were incubated in 0.25% dispase (Invitrogen) for 30 minutes at 37°C. The epithelium was further dissociated in a 0.25% trypsin-EDTA solution for 10 minutes at 37°C, fixed with 1% formaldehyde for 15 minutes at room temperature and lysed in cell lysis buffer (Cell Signaling Technology) with a cocktail of proteinase inhibitors (Ultra Complete Mini, Roche). After pre-clearing treatment, cell extracts were incubated with anti-CDK4, anti-MEF2 (Cell Signaling Technology), anti-p63 or IgG control (Santa Cruz Biotechnology) antibodies for two hours according to the manufacturer’s protocol (Cell Signaling Technology). We assayed for the presence of putative target sites in the immune complexes by PCR using primers amplifying the following genomic regions: mouse Irf6 promoter SMAD-binding site (BS) 1, 5′-GCGATCTTCGTCAGTACTGG-3′ and 5′-CTGCTCTTCGTCACCCCTAC-3′; mouse Irf6 promoter SMAD2 BS, 5′-GGACGTATTTCTGGGCTTCC-3′ and 5′-GCGATTATTTGGGTTTCTG-3′; mouse Irf6 promoter SMAD3 BS, 5′-GCTTGCTATTAGCCCCCAACCT-3′ and 5′-GTAGGGGTGTTAGGTTGTT-3′; mouse Irf6 promoter MEF2 BS, 5′-CGATCACACACCATCTTCGC-3′ and 5′-GACAGCGTCTGACTTCTTT-3′; mouse Irf6 promoter SmadBS 2, 5′-TTTGTTTTTCTGTTGGGTT-3′; mouse Irf6 promoter SmadBS 3, 5′-GGTGGGTTTCTGTTGGGTT-3′; mouse Irf6 promoter SmadBS 4, 5′-GGACGTATTTCTGGGCTTCC-3′; mouse Irf6 promoter SmadBS 5, 5′-GTGGGTTTCTGTTGGGTT-3′; mouse Irf6 promoter SmadBS 6, 5′-GGACGTATTTCTGGGCTTCC-3′; mouse Irf6 promoter SmadBS 7, 5′-GGACGTATTTCTGGGCTTCC-3′; and mouse Irf6 promoter SmadBS 8, 5′-GGACGTATTTCTGGGCTTCC-3′. Positions of PCR fragments correspond to National Center for Biotechnology Information (NCBI) mouse genome Build 33.1.

#### Histological examination

Hematoxylin and Eosin (H&E), immunohistochemical and 5-bromo-2-deoxyuridine (BrdU) staining were performed as described previously (Iwata et al., 2012; Iwata et al., 2010). Antibodies used for immunohistochemistry were rabbit polyclonal antibodies against p21 (Santa Cruz Biotechnology), Irf6 (Aviva Systems Biology), phosphorylated histone H3 (Millipore) and Lex/SSEA1 (FUT4 – Mouse Genome Informatics) (Cell Signaling Technology), and mouse monoclonal antibody against p63 (Santa Cruz Biotechnology). Fluorescence images were obtained using a fluorescence microscope (Model IX71, Olympus).
**Immunoblot analysis**

Immunoblots were performed as described previously (Iwata et al., 2006; Iwata et al., 2010). Antibodies used for immunoblotting were rabbit polyclonal antibodies against IRF6 (Aviva Systems Biology) and p21 (Abcam), and mouse monoclonal antibodies against p63 (Santa Cruz Biotechnology) and GAPDH (Chemicon).

**Palatal shelf organ culture**

Timed pregnant mice were sacrificed at E13.5. Genotyping was carried out as described above. The palatal shelves were microdissected and cultured in serum-free chemically defined medium as previously described (Ito et al., 2003; Xu et al., 2008). After 48 or 72 hours in culture, palates were harvested, fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and processed. For p63 experiments, palatal shelves were transfected with small interfering RNA (siRNA) duplexes (500 nM) for p63 or control (Santa Cruz Biotechnology). The 500 nM siRNA solutions were prepared by diluting a siRNA stock (10 μM) in BGJb medium containing Oligofectamine (0.3%) (Invitrogen). The siRNA mixture in transfection medium was incubated with palatal shelves up to 72 hours after siRNA treatment at 37°C in a CO2 incubator. For p21, palatal shelves were transfected with a GFP-tagged p21 overexpression or control vector (Origene). The 2-μg p21 transfection mixture was changed every 24 hours and incubated with palatal shelves up to 72 hours after siRNA treatment at 37°C in a CO2 incubator. For experiments with p21, palatal shelves were transfected with a GFP-tagged p21 overexpression or control vector (Origene). The 2-μg p21 transfection solutions were prepared in BGJb medium containing Lipofectamine LTX and PLUS reagents (Invitrogen) according to the manufacturer’s protocol. The p21 transfection mixture was changed every 24 hours and incubated with palatal shelves up to 72 hours after transfection at 37°C in a CO2 incubator. All experiments were performed with at least five samples.

**Cell culture**

Primary mouse keratinocytes were isolated from newborn mice and cultured in dermal cell basal medium (ATCC) supplemented with the Keratinocyte Cell culture medium was changed every 24 hours and incubated with palatal shelves up to 72 hours after transfection at 37°C in a CO2 incubator. For p63 experiments, palatal shelves were transfected with small interfering RNA (siRNA) duplexes (500 nM) for p63 or control (Santa Cruz Biotechnology). The 500 nM siRNA solutions were prepared by diluting a siRNA stock (10 μM) in BGJb medium containing Oligofectamine (0.3%) (Invitrogen). The siRNA mixture in transfection medium was incubated with palatal shelves up to 72 hours after siRNA treatment at 37°C in a CO2 incubator. For p21, palatal shelves were transfected with a GFP-tagged p21 overexpression or control vector (Origene). The 2-μg p21 transfection mixture was changed every 24 hours and incubated with palatal shelves up to 72 hours after siRNA treatment at 37°C in a CO2 incubator. All experiments were performed with at least five samples.

**Quantitative reverse transcription PCR (RT-PCR)**

Total RNA was isolated from dissected mouse MEE at E14.5 with the QIAshredder and RNeasy Micro Extraction Kit (QIAGEN), as described previously (Iwata et al., 2010). The following PCR primers were used: Irf6, 5′-AGGCGCTGTGATTAAACAAG-3′ and 5′-TGATCCGGGGCTGCAAGTTC-3′; p21 (Cdh1a), 5′-AGGCTGAGACTGTAATGGG-3′ and 5′-AAAGTTCACCGTTCATCGG-3′; Np63, 5′-ACAAAACCTGGAAGCCAGAAA-3′ and 5′-GAGGAGCCGTCTGAATCTG-3′; and Gapdh, 5′-AACATTGGCATTGGGAAG-3′ and 5′-ACACATTGGGGTGAGAACA-3′.

**Scanning electron microscopic (SEM) analysis**

Samples were fixed with a modified Karnovsky fixative solution [2% paraformaldehyde and 2.5% glutaraldehyde in 0.067 M cacodylate buffer (pH 7.4)] for two days. After dehydration through a graded ethanol series, samples were critical-point dried in a Balzer Union apparatus (FL-9496), ion-sputtered with platinum-palladium (10-15 nm), and observed in JEOL JSM-6390 low vacuum scanning electron microscope (JEOL USA, MA, USA) at a low accelerating voltage of 10 kV.

**Whole-mount skeletal staining and micro-CT analysis**

The three-dimensional architecture of the skeleton was examined using a modified whole-mount Alcian Blue-Alizarin Red S staining protocol as previously described (Iwata et al., 2010). Antibodies used for immunoblotting were rabbit polyclonal antibodies against IRF6 (Aviva Systems Biology) and p21 (Abcam), and mouse monoclonal antibodies against p63 (Santa Cruz Biotechnology) and GAPDH (Chemicon). Immunoblots were performed as described previously (Iwata et al., 2006; Iwata et al., 2010). The QIAshredder and RNeasy Micro Extraction Kit (QIAGEN), as described previously (Iwata et al., 2010). The following PCR primers were used: Irf6, 5′-AGGCGCTGTGATTAAACAAG-3′ and 5′-TGATCCGGGGCTGCAAGTTC-3′; p21 (Cdh1a), 5′-AGGCTGAGACTGTAATGGG-3′ and 5′-AAAGTTCACCGTTCATCGG-3′; Np63, 5′-ACAAAACCTGGAAGCCAGAAA-3′ and 5′-GAGGAGCCGTCTGAATCTG-3′; and Gapdh, 5′-AACATTGGCATTGGGAAG-3′ and 5′-ACACATTGGGGTGAGAACA-3′.

**Fig. 1. Decreased IRF6 expression in the MEE of Tgfbr2Δ/Δ,K14-Cre mice.** (A) Quantitative RT-PCR analyses of Irf6 expression in the palates of Tgfbr2Δ/Δ control (n=6) and Tgfbr2Δ/Δ,K14-Cre (n=6) mice at E14.5. (B,C) Immunohistochemical analyses of IRF6 expression (red) in the palates of Tgfbr2Δ/Δ control (B) and Tgfbr2Δ/Δ,K14-Cre (C) mice at E14.5. Nuclei were counterstained with DAPI (blue). (D) Quantitative RT-PCR analyses of Irf6 expression in the palates of Smad4Δ/Δ control (n=6) and Smad4Δ/Δ,K14-Cre (n=6) mice at E14.5. (E) Quantitative RT-PCR analyses of Irf6 expression in palate explants from Smad4Δ/Δ control and Smad4Δ/Δ,K14-Cre mice treated with p38 MAPK inhibitor (+) or vehicle (−) for 48 hours. n=3 per group. (F-I) H&E staining of palate explants from Smad4Δ/Δ,K14-Cre and Smad4Δ/Δ control (n=6) mice treated with p38 MAPK inhibitor (F,H) or vehicle control (G,I) for 72 hours. n=5 per group. (J) Schematic of the upstream region of the mouse Irf6 gene (not to scale), showing locations of putative SMAD-binding (red) or MEF2-binding (green) sites tested in ChIP assays. Putative SMAD- and MEF2-binding sequences are shown below. Arrowheads indicate the position of primers used in ChIP analysis. (K) ChIP analysis of DNA fragments immunoprecipitated with a SMAD4-specific or MEF2-specific antibody or with an isotype-specific control antibody. Immunoprecipitates were PCR amplified with primers flanking the putative SMAD-binding or MEF2-binding region. Input lane shows PCR amplification of the sonicated chromatin before immunoprecipitation. Ab, antibody; BS, binding site. Error bars represent s.d. *p<0.05. Scale bars: 50 μm.
RESULTS

TGFβ signaling in the MEE regulates Irf6 expression during palatal fusion

Loss of TGFβ signaling in the basal epithelium in mice (Tgfbr2-/-;K14-Cre) results in diminished Irf6 expression and failure of apoptosis in MEE cells, followed by MEE persistence, suggesting that TGFβ-mediated Irf6 expression might play a role in degeneration of the MEE (Xu et al., 2006). The MEE is composed of a basal columnar cell layer covered by flat cells that constitute the periderm (Cuervo and Covarrubias, 2004). Irf6 is expressed in the periderm at E13.5, and Irf6 expression shows an obvious and consistent transition from the periderm to regions of Tgfbr2fl/fl;K14-Cre constituting the periderm (Cuervo and Covarrubias, 2004). β-suggesting that TGFβ signaling in the MEE regulates the disappearance of MEE cells during palatal fusion (Ingraham et al., 2006; Richardson et al., 2006). To investigate the fate of the MEE in Irf6 mice, we used the ex vivo organ culture system. E13.5 wild-type control (n=17) and Irf6-/-R84C (n=39) palatal shelves failed to fuse (Fig. 2C) and the MEE persisted with continuous cell proliferation (Fig. 2D-F) and compromised cell death (Fig. 2G-I) following two days of culture with complete phenotype penetrance. We quantified the increased cell proliferation as well as decreased apoptosis in Irf6 mice compared with wild-type littermate control and Irf6-/-R84C MEE cells during palatal fusion (Fig. 2J,K). The MEE in control and Irf6-/-R84C explants disappeared completely following three days in culture; however, the MEE persisted in Irf6-/-R84C explants (supplementary material Fig. S2). These data indicate that IRF6 is crucial for MEE disappearance during palatal fusion.

Southern California Molecular Imaging Center. The data were collected at a resolution of 10 μm. The reconstruction was performed using AVIZO 7.0 (Visualization Sciences Group).

Statistical analysis
A two-tailed Student’s t-test was applied for statistical analysis. For all graphs, data are represented as mean±s.d. A P-value of less than 0.05 was considered statistically significant.
Genetic interaction between Smad4 and Irf6 during palatal fusion

We hypothesized that haploinsufficiency of Irf6 in a Smad4 mutant background would cause an additional reduction of IRF6 activity and result in cleft palate. To investigate a possible genetic interaction between Irf6 and Smad4, we generated Smad4<sup>fl/fl</sup>;K14-Cre;Irf6<sup>+/+<sup> mice. Smad4<sup>fl/fl</sup>;K14-Cre;Irf6<sup>+/R84C</sup> mice exhibited extra digits with complete phenotype.
penetration (n=19/19) (supplementary material Fig. S3). Thus, Smad4<sup>fl/fl</sup>;K14-Cre;Irf6<sup>−/−R84C</sup> mice have a more severe phenotype than do Smad4<sup>fl/fl</sup>;K14-Cre mice, suggesting that Irf6 and SMAD4 function synergistically in regulating embryogenesis. In addition, the extra digits in Smad4<sup>fl/fl</sup>;K14-Cre;Irf6<sup>−/−R84C</sup> mice are consistent with the abnormal toe and nail phenotype in individuals with VWS and PPS. Because some individuals with VWS and PPS have cleft palate, we investigated the palatine in Smad4<sup>fl/fl</sup>;K14-Cre;Irf6<sup>−/−R84C</sup> mice in detail by scanning electron microscopy (SEM), and detected compromised rugae formation (Fig. 3G,H). By histological analysis, we found that all Smad4<sup>fl/fl</sup>;K14-Cre;Irf6<sup>−/−R84C</sup> mice exhibited submucous cleft palate at birth with persistence of the MEE (n=19/19) and in some cases a stretched epithelial bridge (n=6/19) (Fig. 3I-O). Taken together, our findings suggest that a genetic interaction between Smad4 and Irf6 is responsible for MEE disappearance during palatal fusion.

**Rescue of palatal fusion in Tgfbr2<sup>fl/fl</sup>;K14-Cre mice by overexpression of Irf6**

To test whether Irf6 expression level controls MEE cell fate, we generated K14-driven Irf6 transgenic (K14-Irf6<sup>Tg</sup>) mice to overexpress Irf6 in MEE cells (Fig. 4A). Most K14-Irf6<sup>Tg</sup> mice were born healthy and fertile without any noticeable pathological phenotype up to the age of one year (n=114/146) (supplementary material Table S2). We analyzed Irf6 gene expression level by quantitative RT-PCR and found that there is a significant increase in Irf6 expression (2.2-fold) in the MEE of K14-Irf6<sup>Tg</sup> mice compared with control (Fig. 4B). Moreover, we confirmed the overexpression of IRF6 protein in the MEE by immunoblotting (Fig. 4C,D) and immunostaining (Fig. 4E) at E14.5. Approximately 22% of Irf6 transgenic mice (n=32/146) exhibited absence of the calvaria and an open eye phenotype and died within one day of birth (supplementary material Table S2 and Fig. S4). Although these Irf6 transgenic mice exhibited severe calvarial defects and hypoplasia of the palatine bones (Fig. 4I,M), their palatal shelves fused normally without obvious cleft palate or persistence of the MEE (Fig. 4J). Palatal fusion appeared to be unaffected in all K14-Irf6<sup>Tg</sup> mice (n=146) (Fig. 4F-M; supplementary material Table S2).

Next, we generated Tgfbr2<sup>fl/fl</sup>;K14-Cre;Irf6<sup>Tg</sup> mice to restore Irf6 gene expression in Tgfbr2<sup>fl/fl</sup>;K14-Cre mice. By SEM analysis, rugae formation was detectable in control mice, but not in their Tgfbr2<sup>fl/fl</sup>;K14-Cre and Tgfbr2<sup>fl/fl</sup>;K14-Cre;Irf6<sup>Tg</sup> littermates (Fig. 5A-C). We also found that Tgfbr2<sup>fl/fl</sup>;K14-Cre;Irf6<sup>Tg</sup> mice exhibit cleft soft palate, which was partially rescued (in the anterior of the soft palate) in Tgfbr2<sup>fl/fl</sup>;K14-Cre;Irf6<sup>Tg</sup> mice (Fig. 5B,C). In addition, Tgfbr2<sup>fl/fl</sup>;K14-Cre mice have submucous cleft palate throughout the anterior to posterior regions of the hard palate, based on histology (Fig. 5K). At E15.5 and E16.5, the MEE disappeared in control mice, whereas MEE persistence was detectable in Tgfbr2<sup>fl/fl</sup>;K14-Cre mice (Fig. 5D,E,G,H). Tgfbr2<sup>fl/fl</sup>;K14-Cre;Irf6<sup>Tg</sup> mice show a one-day delay in the disappearance of the MEE (Fig. 5F,I). In histological sections, we detected MEE persistence (n=14/14) corresponding with the groove in the midline of the palate in Tgfbr2<sup>fl/fl</sup>;K14-Cre;Irf6<sup>Tg</sup> mice (Fig. 5K). Proper apoptotic degradation of the MEE was restored in Tgfbr2<sup>fl/fl</sup>;K14-Cre;Irf6<sup>Tg</sup> mice (n=7/7), based on histological analysis (Fig. 5L), indicating that TGFβ-mediated Irf6 signaling is functionally important and sufficient for MEE disappearance.

**Irf6 regulates MEE disappearance via p21 expression**

Apoptosis is one of the ultimate fates of the MEE during palatal fusion (Cuervo and Covarrubias, 2004). Loss of Tgfbr2 in the epithelium results in absence of apoptosis and maintenance of proliferation in MEE cells (Xu et al., 2006). We analyzed the cellular defect in persistent MEE cells by assaying both cell proliferation and apoptosis activities. We found that altered cell proliferation in the MEE was restored in Tgfbr2<sup>fl/fl</sup>;K14-Cre;Irf6<sup>Tg</sup> mice to a level comparable to the control (Fig. 6A-D). Similarly, we found that altered apoptotic activity in the MEE was also restored in Tgfbr2<sup>fl/fl</sup>;K14-Cre;Irf6<sup>Tg</sup> mice compared with the control (Fig. 6E-H). Thus, our data demonstrate that loss of Tgfbr2 results in compromised Irf6 expression, continuous cell proliferation, and failure of apoptosis in MEE cells, indicating that TGFβ-mediated Irf6 expression plays a role in the disappearance of the MEE. We previously reported that p21 (also known as Cdkn1a) was decreased in the MEE of Tgfbr2<sup>fl/fl</sup>;K14-Cre mice (Xu et al., 2008). We hypothesized that p21 expression might be regulated by the TGFβ-mediated Irf6 pathway. To investigate p21 expression, we performed immunohistochemical (Fig. 6I-K), immunoblotting
expression was detectable in basal epithelial cells in formation (Fakhouri et al., 2012). Indeed, increased ∆hypothesized that IRF6 might regulate mammals, 5 kb upstream and 5 kb downstream of the TSS), we mice, correlating with that p21 expression was restored in E14.5 (Fig. 6L) and quantitative RT-PCR (Fig. 6M,N) analyses. We found that IRF6 activity during palatogenesis (Laurikkala et al., 2006; Welsh and O’Brien, 2009; Westfall et al., 2003). Because there is no conserved IRF6-binding site in the promoter-proximal genomic region of p21 (in at least six mammals, 5 kb upstream and 5 kb downstream of the TSS), we hypothesized that IRF6 might regulate p21 expression via ∆Np63. ∆Np63 is only expressed in the basal epithelial layer during palate formation (Fakhouri et al., 2012). Indeed, increased ∆Np63 expression was detectable in basal epithelial cells in Tgfr2β 0  ; K14-Cre mice, consistent with a TGFβ-mediated IRF6/∆Np63/p21 signaling cascade at E14.5 and E15.5 (Fig. 7A-F). Moreover, ∆Np63 expression was reversed by overexpression of IRF6 (Fig. 7C,F). We also evaluated ∆Np63 expression levels in control, Tgfr2β 0  ; K14-Cre and Tgfr2β 0  ; K14-Cre; Irf6R84C mice by immunoblotting (Fig. 7G). To confirm that p21 expression is regulated by p63, we analyzed the mouse p21 promoter region (2.5 kb upstream and 2.5 kb downstream of the TSS), and found two potential p63-binding sites in the mouse genomic region of p21 (Fig. 7H). We performed ChIP analysis to test whether p63 could bind to the genomic region of p21, and found that the p21 genomic region immunoprecipitated with the p63 antibody at p63-binding site 1 (+727 bp to +746 bp), but not at p63-binding site 2 (−436 bp to −455 bp) (Fig. 7I). These results indicate that p21 expression is likely to be regulated through the TGFβ/IRF6/ΔNp63 signaling cascade.

Finally, we investigated the functional significance of the IRF6/p21 signaling cascade using an ex vivo organ culture system. We found that either reduction of p63 or overexpression of p21 could restore the degeneration of the MEE (Fig. 8A-F). In addition, primary mouse keratinocytes were treated with Tgfr2β, Irf6 or control siRNA (Fig. 8G). After siRNA knockdown for Tgfr2β, gene expression of Irf6 and p21 was significantly decreased and p63 gene expression was significantly (P<0.05) upregulated. Similarly, Irf6 siRNA knockdown resulted in downregulation of Irf6 and p21 gene expression and upregulation of p63 gene expression. Previous studies indicate that p63 expression is upregulated in E14.5 Irf6R84C/R84C palates (Thomason et al., 2010) and siRNA knockdown for Irf6 results in upregulated p63 expression in human keratinocytes (Moretti et al., 2010). Therefore, we investigated whether p63 expression was altered in Irf6R84C/R84C palates. The expression of p63 was increased in Irf6R84C/R84C palates, consistent with previous findings (Fig. 8H,J). Collectively, these data suggest that p21 expression is regulated through a TGFβ/IRF6/ΔNp63 signaling cascade (Fig. 8J).

DISCUSSION

Submucous cleft palate, which can result from MEE defects during palatal fusion, is one of the most common forms of cleft palate in

**Fig. 6.** p21 expression is restored in Tgfr2β 0  ; K14-Cre; Irf6R84C mice. (A-C) Immunostaining of phosphorylated histone H3 (pH3) in Tgfr2β 0  ; K14-Cre control (A), Tgfr2β 0  ; K14-Cre (B) and Tgfr2β 0  ; K14-Cre; Irf6R84C (C) mice at E14.5. Dashed lines indicate the MEE. Arrows indicate positive signal (green). (D) Percentage of pH3-positive nuclei in the palates of Tgfr2β 0  ; blue bar, n=13), Tgfr2β 0  ; K14-Cre (red bar, n=7) and Tgfr2β 0  ; K14-Cre; Irf6R84C (green bar, n=4) mice at E14.5. (E-G) TUNEL staining of Tgfr2β 0  ; control (E), Tgfr2β 0  ; K14-Cre (F) and Tgfr2β 0  ; K14-Cre; Irf6R84C (G) mice at E14.5. Dashed lines indicate the MEE. Arrows indicate positive signal (green). (H) Percentage of TUNEL-labeled nuclei in the palates of Tgfr2β 0  ; blue bar, n=13), Tgfr2β 0  ; K14-Cre (red bar, n=7) and Tgfr2β 0  ; K14-Cre; Irf6R84C (green bar, n=4) mice at E14.5. (I-K) Immunohistochemical analyses of p21 expression in the palates of Tgfr2β 0  ; control (I), Tgfr2β 0  ; K14-Cre (J) and Tgfr2β 0  ; K14-Cre; Irf6R84C (K) mice at E14.5. Dashed lines indicate the MEE. Arrows indicate positive signal (green). (L) Immunoblotting analysis of Irf6 and p21 in the MEE of Tgfr2β 0  ; control (lane 1), Tgfr2β 0  ; K14-Cre (lane 2) and Tgfr2β 0  ; K14-Cre; Irf6R84C (lane 3) mice. GAPDH was used as a loading control. Bar graphs (below) show the ratio of Irf6 or p21 to GAPDH following quantitative densitometry analysis of immunoblotting data. Tgfr2β 0  expression (blue bar), Tgfr2β 0  ; K14-Cre (red bar), Tgfr2β 0  ; K14-Cre; Irf6R84C (green bar). Three samples were analyzed. (M-N) Quantitative RT-PCR analyses of Irf6 (M) and p21 (N) in the MEE of Tgfr2β 0  ; blue, n=3), Tgfr2β 0  ; K14-Cre (red, n=3) and Tgfr2β 0  ; K14-Cre; Irf6R84C (green, n=3) mice at E14.5. Error bars represent s.d. *P<0.05. Scale bars: 50 μm.
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Humans, but the molecular and developmental mechanism of submucous cleft palate are not well studied, in part because of the paucity of animal models that exhibit this phenotype (Fumano et al., 2012; Pauws et al., 2009). MEE persistence affects palatal bone formation in the hard palate and muscle development in the soft palate; consequently, patients with submucous cleft palate need surgical and other procedures to develop precise physiological functions such as speech and swallowing.

We have generated an animal model in which loss of TGFβ signaling in MEE cells results in submucous cleft palate (Xu et al., 2006). In this study, we show that TGFβ-mediated Irf6 expression is crucial for the fate of MEE cells. MEE degeneration in Tgfb2−/−;K14-Cre mice was restored by overexpression of Irf6, indicating that Irf6 is functionally important for the MEE disappearance mediated by TGFβ signaling. Previous studies indicate that mice with loss of Irf6 function exhibit cleft palate owing to failure of palatal shelf elevation resulting from adhesion between the palatal shelves and the tongue, following a defect in epithelial differentiation (Ingram et al., 2006; Richardson et al., 2006). Because of the failure of palatal elevation in Irf6G84C/G84C mice, we were previously not able to investigate the role and function of Irf6 during MEE disappearance fully. In this study, using an ex vivo organ culture system, we show for the first time that Irf6G84C/G84C mutation results in the absence of apoptosis and the maintenance of proliferation in the MEE, indicating that Irf6 is crucial for MEE cell fate during palatal fusion. Although heterozygous Irf6 mutant mice have normal palate formation, a combination of loss of Smad4 and inhibition of p38 MAPK only led to ~50% reduction of Irf6 expression and persistence of the MEE in ex vivo organ culture. This result suggests that there are other factors or signaling pathways that might regulate Irf6 expression in addition to SMAD4/p38 MAPK signaling pathways (Ferretti et al., 2011; Letra et al., 2012). A recent study has shown that integration of IRF6 and the Notch ligand jagged 2 signaling is essential for controlling palatal adhesion and fusion during palatogenesis (Richardson et al., 2009). Interestingly, overexpression of Irf6 in Tgfb2−/−;K14-Cre mice did not rescue the developmental defects in the palatal mesenchyme (supplementary material Fig. S5), suggesting that the TGFβ-mediated IRF6 signaling cascade plays a cell-autonomous role in regulating the fate of MEE cells during palatal fusion and that TGFβ regulates other downstream target genes that control the development of muscles in the soft palate through tissue-tissue interactions. Furthermore, using mutant mouse models, we found that TGFβ signaling regulates Irf6 and p21 expression and MEE disappearance via both SMAD-dependent and -independent pathways during palatal fusion.

SMAD4 mutations have been found in patients with unselected hereditary hemorrhagic telangiectasia (HHT), which is an autosomal dominant disease of vascular dysplasia (Gallione et al., 2006). The symptoms of HHT include epistaxis, telangiectases, and arteriovenous malformations, which are most often found in the lungs, brain, liver and gastrointestinal tract. In addition, SMAD4 mutations have been identified in families with juvenile polyposis, aortopathay, and mitral valve dysfunction (Andrabi et al., 2011). Approximately 15% of people with juvenile polyposis syndrome have other abnormalities, such as cleft palate, polydactyly, intestinal malrotation, heart or brain abnormalities, and abnormalities of the genitalia or urinary tract. Although most Smad4−/−;K14-Cre mice have normal palates, one copy of the R84C IRF6 mutation in a Smad4 mutant background (Smad4−/−;K14-Cre;Irf6+/R84C mice) resulted in fully penetrant submucous cleft palate and polydactyly. It is important to note that Smad4−/−;K14-Cre;Irf6−/−R84C mice closely phenocopy individuals affected by PPS as described in

Fig. 7. Increased ΔNp63 expression in Tgfb2−/−;K14-Cre mice. (A-F) Immunohistochemical analyses of ΔNp63 expression in the palates of Tgfb2−/− control (A, D), Tgfb2−/−;K14-Cre (B, E) and Tgfb2−/−;K14-Cre;Irf6+/R84C (C, F) mice at E14.5 (A-C) and E15.5 (D-F). Brown, positive signal. Nuclei were counterstained with 0.03% Methylene Blue. Scale bars: 50 μm. (G) Immunoblotting analysis of p63 in the MEE of E14.5 Tgfb2−/− control (lane 1), Tgfb2−/−;K14-Cre (lane 2) and Tgfb2−/−;K14-Cre;Irf6+/R84C (lane 3) mice. GAPDH was used as a loading control. Bar graph (below) shows the ratio of p63 to GAPDH following quantitative densitometry analysis of immunoblotting data. Three samples were analyzed. (H) Schematic of the upstream region of the mouse p21 gene (not to scale), showing locations of putative p63-binding sites tested in ChIP assays. Putative p63-binding sequences are shown below. Arrowheads indicate the position of primers used in ChIP analysis. (I) ChIP analysis of DNA fragments immunoprecipitated with a p63-specific antibody or with an isotype-specific control antibody. Immunoprecipitates were PCR amplified with primers flanking the putative p63-binding region. Input lane shows PCR amplification of the sonicated chromatin before immunoprecipitation. No amplification of target sites was detected when an isotype-specific control antibody was used. Ab, antibody; BS, binding site.
humans with IRF6 mutation, whereas Irf6R84C/R84C mice do not show digit developmental defects. These results suggest that a compromised SMAD-dependent, TGFβ-mediated IRF6 signaling cascade might be responsible for developmental defects associated with PPS. Furthermore, an impaired TGFβ/IRF6 signaling cascade may cause submucosal cleft palate.

The secondary palate is divided into two parts: the anterior bony hard palate, which is about two-thirds of the secondary palate and is composed of bone, and the posterior fleshy soft palate, which is about one-third of the secondary palate and is composed of muscles. Our previous study suggests that loss of Tgfr2 in the epithelium causes cleft soft palate owing to failure of muscle development and misorientation of muscle fibers (Xu et al., 2006). Overexpression of Irf6 in the MEE of Tgfr2ΔNp63−/−K14-Cre mice failed to restore epithelial-mesenchymal interaction and to support soft palate muscle development. We are currently investigating the molecular and cellular mechanisms of soft palate muscle defects in Tgfr2ΔNp63−/−K14-Cre mice.

Cleft soft palate in Tgfr2ΔNp63−/−K14-Cre mice was not rescued in Tgfr2ΔNp63−/−K14-Cre;Irf6R84C/R84C mice. One possible explanation is that there are differential gene expression patterns in the anterior versus the posterior in the MEE. Expression of Irf6, p63 and Tgfb3 is detectable throughout the oral epithelia in wild-type mice with no variation along the antero-posterior axis of the secondary palate (Fakhouri et al., 2012; Richardson et al., 2009; Yang et al., 2008). However, gene expression of Tgfb3 was compromised specifically in the posterior palate in Irf6R84C/R84C mice, indicating that Tgfb3 expression is differentially regulated between the anterior and posterior MEE (Richardson et al., 2009). It also suggests that IRF6 provides a feedback in regulating TGFβ signaling in the posterior region of developing palate.

WNT/β-catenin signaling acts upstream of Irf6 in the lip epithelium during lip fusion (Ferretti et al., 2011). A previous study has shown that loss of WNT/β-catenin signaling results in downregulation of TGFβ, whereas expression of stabilized β-catenin in the palatal epithelium can cause ectopic Tgfb3 expression and fusion of the palatal shelf and mandible (He et al., 2011). This study clearly demonstrates that a precisely controlled TGFβ signaling level is crucial for regulating the fate of MEE cells during palatal fusion. Ablation of p63 results in diminished Irf6 expression in the palate (Moretti et al., 2010; Thomason et al., 2010), and Irf6 can regulate ΔNp63 degradation in human keratinocytes (Moretti et al., 2010). However, we found that upregulated p63 expression in wild-type mice did not affect the expression of Irf6 in MEE cells (data not shown), indicating that there is no apparent feedback loop from upregulated p63 to Irf6 expression.

In summary, our data indicate that a combination of genetic mutations in Irf6 and Smad4 cause submucous cleft palate and that TGFβ-mediated Irf6 activity is crucial for MEE disappearance. This is a significant advancement in our understanding of the mechanism of TGFβ signaling-associated cleft palate. Our findings that TGFβ-mediated Irf6 expression is responsible for MEE disappearance and that overexpression of Irf6 normalizes MEE cell fate determination in Tgfr2ΔNp63−/−K14-Cre mice might provide potential therapeutic approaches for individuals with altered TGFβ signaling and submucous cleft palate. In addition, we propose that combined mutations in Irf6 and Smad4 in humans might be useful diagnostic biomarkers for patients with cleft palate.
Acknowledgements
We are grateful to Dr Julie Mayo for critical reading of the manuscript, and to Dr Harold Slavkin for discussion. We thank Dr Harold Moses for Tgfβ2<sup>−/−</sup> mice. We thank Xuemei Deng, Toshiaki Yokota, Pablo Brinas Jr and the Molecular Imaging Center and Center for Electron Microscopy and Microanalysis of USC for technical assistance.

Funding
This study was supported by grants from the National Institutes of Health National Institute of Dental and Craniofacial Research [DE020065 and DE012711 to Y.C.,] and from the UK Medical Research Council [G0901539 to M.J.D.]. Deposited in PMC for release after 6 months.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.089615/-/DC1

References


Fig. S1. Periderm disappearance is unaffected in Tgfbr2<sup>fl/fl</sup>;K14-Cre mice. (A-H) H&E staining of Tgfbr2<sup>fl/fl</sup> control (A-D) and Tgfbr2<sup>fl/fl</sup>;K14-Cre (E-H) mice at the indicated developmental stages. Boxed areas are shown magnified. Arrows indicate peridermal cells. (I-L) Staining of the periderm with anti-Lex/SSEA1 antibody in Tgfbr2<sup>fl/fl</sup> control (I-K) and Tgfbr2<sup>fl/fl</sup>;K14-Cre (L) mice. Arrows indicate positive signal.
Fig. S2. Loss of IRF6 activity causes MEE persistence. (A-C) H&E staining in palatal explants of control (A), Irf6<sup>+/R84C</sup> (B) and Irf6<sup>R84C/R84C</sup> (C) mice after 3 days culture. (D) Immunostaining of phosphorylated histone H3 (pH3) in Irf6<sup>R84C/R84C</sup> palatal shelf explants after 3 days culture. Dashed lines indicate the MEE. Arrows indicate positive signal (green). Nuclei were counterstained with DAPI (blue). Scale bars: 100 μm.
Fig. S3. Haploinsufficiency of *Irf6* in *Smad4*-*K14-Cre* mice causes syndactyly. (A-C) Limb morphologies of newborn control (A), *Smad4*-*K14-Cre* (B) and *Smad4*-*K14-Cre;Irf6*^+/<R84C^ (C) mice. Open arrow indicates abnormal nails in *Smad4*-*K14-Cre* mice. Arrows indicate extra digits in *Smad4*-*K14-Cre;Irf6*^+/<R84C^ mice. (D-F) Whole-mount Alcian Blue-Alizarin Red skeletal staining of newborn control (D), *Smad4*-*K14-Cre* (E) and *Smad4*-*K14-Cre;Irf6*^+/<R84C^ (F) mice. Open arrow indicates abnormal nails. Arrow indicates extra digit. (G-I) microCT images of the hind-limb in newborn control (G), *Smad4*-*K14-Cre* (H) and *Smad4*-*K14-Cre;Irf6*^+/<R84C^ (I) mice. Arrow indicates extra digit.
Fig. S4. Morphologies of *Irf6* transgenic mice. (A,B) Top panels show side views of wild-type (A) and *Irf6* Tg (B) mice, bottom panels show frontal views. Approximately 22% of *Irf6* transgenic embryos exhibit a defect in calvaria formation and open eye at E18.5. (C) SEM image of the palate of newborn *Irf6* Tg mice. Scale bar: 500 μm. (D-H) H&E staining of the skin (D-F) and oral epithelium (G,H) of wild-type control (D,G) and *Irf6* Tg (E,F,H) mice at E18.5. Boxed area in F is shown magnified in E. Scale bars: 50 μm. (I-N) Whole-mount Alcian Blue-Alizarin Red skeletal staining of wild-type control (I,L) and *Irf6* Tg (J,K,M,N) newborn mice. Arrow indicates a defect in the calvaria. Open arrow indicates a defect in the palatine bone. Eighty percent of *Irf6* Tg mice show normal craniofacial development (middle panels; J,M), but 20% of *Irf6* Tg mice exhibit defects in the calvaria and the palatine bone (right panels; K,N).
Fig. S5. Cleft soft palate in Tgfbr2<sup>fl/fl</sup>;K14-Cre and Tgfbr2<sup>fl/fl</sup>;K14-Cre;Irfl6<sup>Tg</sup> mice. (A-H) H&E staining of Tgfbr2<sup>fl/fl</sup>;K14-Cre (A-D) and Tgfbr2<sup>fl/fl</sup>;K14-Cre;Irfl6<sup>Tg</sup> (E-H) mice at E18.5. Boxed areas are enlarged in panels to the right. Scale bars: 250 μm in left-hand panels; 100 μm in right-hand panels. (I-K) MicroCT analysis of the hard tissues of E18.5 control (I), Tgfbr2<sup>fl/fl</sup>;K14-Cre (J) and Tgfbr2<sup>fl/fl</sup>;K14-Cre;Irfl6<sup>Tg</sup> (K) mice.
### Table S1. Conserved SMAD recognition sequences in the *Irf6* gene

<table>
<thead>
<tr>
<th>Binding site position relative to TSS</th>
<th>Genomic context</th>
<th>Binding site sequence context*</th>
<th>Conservation of 4-bp site</th>
</tr>
</thead>
<tbody>
<tr>
<td>–2375 to –2372</td>
<td>Upstream</td>
<td>TGTGTCTTGA</td>
<td>All but rat</td>
</tr>
<tr>
<td>–1695 to –1692</td>
<td>Upstream</td>
<td>GACGTCTTCT</td>
<td>All but dog/horse</td>
</tr>
<tr>
<td>–274 to –271</td>
<td>Upstream</td>
<td>CCAGTCTCAG</td>
<td>All eight species</td>
</tr>
</tbody>
</table>

*Binding site motif is underlined in all cases. It is noted in red if conserved in all eight species, and in blue if conserved in all but the noted species in the rightmost column. The eight species include: human (Build 19), chimpanzee (Build 2.1.3), orangutan (Build 2.0.2), rhesus macaque (Build 1.0), mouse (Build 38), rat (Build 3.4), dog (Build 2) and horse (Build equCab2).
Table S2. Penetrance of abnormalities in *Irf6* transgenic mice

<table>
<thead>
<tr>
<th>Abnormality</th>
<th>Wild-type mice</th>
<th><em>Irf6</em> transgenic mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skull defect</td>
<td>1.28% (2/156)</td>
<td>21.92% (32/146)</td>
</tr>
<tr>
<td>Open eye</td>
<td>0% (0/156)</td>
<td>21.92% (32/146)</td>
</tr>
<tr>
<td>Cleft lip</td>
<td>0% (0/156)</td>
<td>2.74% (4/146)</td>
</tr>
</tbody>
</table>

Phenotype of wild-type littermate control and *Irf6* transgenic mice was scored at E18.5.