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Cooperation between the transcription factors p63 and IRF6 is essential to prevent cleft palate in mice

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Cleft palate is a common congenital disorder that affects up to 1 in 2,500 live human births and results in considerable morbidity to affected individuals and their families. The etiology of cleft palate is complex, with both genetic and environmental factors implicated. Mutations in the transcription factor–encoding genes p63 and interferon regulatory factor 6 (IRF6) have individually been identified as causes of cleft palate; however, a relationship between the key transcription factors p63 and IRF6 has not been determined. Here, we used both mouse models and human primary keratinocytes from patients with cleft palate to demonstrate that IRF6 and p63 interact epistatically during development of the secondary palate. Mice simultaneously carrying a heterozygous deletion of p63 and the Irf6 knockin mutation R84C, which causes cleft palate in humans, displayed ectodermal abnormalities that led to cleft palate. Furthermore, we showed that p63 transactivated IRF6 by binding to an upstream enhancer element; genetic variation within this enhancer element is associated with increased susceptibility to cleft lip. Our findings therefore identify p63 as a key regulatory molecule during palate development and provide a mechanism for the cooperative role of p63 and IRF6 in orofacial development in mice and humans.

Introduction
Development of the secondary palate involves a complex series of integrated events that are frequently disturbed, resulting in the congenital malformation cleft palate. With an estimated incidence of 1 in 2,500 live births, depending on geographic origin, racial, and ethnic variation, and socioeconomic status (1, 2), cleft palate results in considerable morbidity to affected families, as individuals who exhibit this condition may experience problems with eating, speaking, and hearing that can be corrected to varying degrees by surgery, dental treatment, speech therapy, and psychosocial intervention (3, 4). The frequent occurrence and significant healthcare burden imposed by cleft palate emphasize the need to identify the molecular and cellular interactions that lead to facial clefeting, with the ultimate aim of improving diagnosis, treatment, counseling, and care for affected individuals and their relatives.

In approximately 50% of cases, cleft palate occurs as an isolated entity; the remainder arise as part of a syndrome in which structures other than the palate are affected (5). The genetic basis of nonsyndromic clefting is complex, as variations in numerous genes, together with environmental factors, are known to play a role in its etiology (3–5). Recent advances in delineating the molecular mechanisms underlying cleft palate have therefore resulted largely from analysis of syndromic forms of cleft palate; for example, mutations in the p53 family member p63 and in interferon regulatory factor 6 (IRF6) underlie congenital malformation syn-

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major isoform expressed in basal epithelial cells and is essential for epidermal and palatal development (17, 21, 22). Heterozygous mutations in *p63* underlie 7 autosomal-dominant developmental disorders that are characterized by varying combinations of cleft lip, cleft palate, ectodermal dysplasia, and limb abnormalities (7). To date, 2 mouse models of *p63*, consisting of severe limb abnormalities, a thin and undifferentiated epidermis of *E18.5* embryos exhibited signs of differentiation with distinct nasal and oral characteristics. *ps*, palatal shelf; *t*, tongue; *ns*, nasal septum; *n*, nasal epithelium; *o*, oral epithelium. Scale bars: 500 μm (A–D); 200 μm (E–H); 400 μm (I and J); 50 μm (K and L).

**Results**

**Epistatic interaction between Irf6 and p63 in palatal development.** In light of the striking phenotypic overlap exhibited by syndromes resulting from mutations in *IRF6* and *p63*, we investigated the potential interaction between *p63* and *Ir6f* using an epistatic approach in which we intercrossed *p63*+/– and *Ir6f*+/R84C mice. We examined 17 litters of *p63*+/–*Ir6f*+/R84C mice between the ages of E14.5 and P0 (*N* = 145). Whereas mice heterozygous for the mutant *p63* allele (*n* = 27) or a mutant *Ir6f* allele alone (*n* = 38) appeared grossly normal and comparable to their wild-type littermates (*n* = 45), approximately 89% of compound heterozygous *p63*+/–*Ir6f*+/R84C embryos (*n* = 35) exhibited a cleft of the secondary palate. Scanning electron microscopy and histological analysis of a developmental series of embryos indicated that the initial stages of palatal development appeared normal. The palatal shelves of *p63*+/–*Ir6f*+/R84C embryos grew vertically down the sides of the tongue and elevated at the expected developmental stage (Figure 1, A–C and E–G); however, in 31 of 35 *p63*+/–*Ir6f*+/R84C embryos, the palatal shelves failed to fuse (Figure 1, D and H). Examination of 5 litters at E14.5 (yielding 15 *p63*+/–*Ir6f*+/R84C embryos) revealed that the palatal shelves achieved intimate contact (Figure 1H); nevertheless, with continued growth of the craniofacial complex, the *p63*+/–*Ir6f*+/R84C palatal shelves separated such that by E18.5, a large oronasal space was apparent (Figure 1J). Despite this observation, histological analysis indicated that the oral and nasal palatal epithelia of *p63*+/–*Ir6f*+/R84C embryos differentiated normally (Figure 1, K and L). Newborn *p63*+/–*Ir6f*+/R84C mice exhibited gasping behavior and died shortly after birth. Histological and immunofluorescence analyses indicated that the epidermis of E18.5 *p63*+/–*Ir6f*+/R84C embryos differentiated normally (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI40266DS1).
To gain greater insight into the pathogenesis of the cleft palate observed in p63<sup>+/–</sup>Irf6<sup>+/R84C</sup> mice, we examined the morphology of the medial edge epithelium (MEE) from E13.5 to E15.5. As immunostaining for desmoplakin, α- and β-catenin, and E-cadherin indicated no gross differences in the distribution of these adhesion molecules between wild-type and p63<sup>+/–</sup>Irf6<sup>+/R84C</sup> embryos (Supplemental Figure 2 and data not shown), we examined the palatal epithelia in greater detail using transmission electron microscopy. At E14.5, the MEE of wild-type mice consisted of a 2- to 3-cell-thick layer of basal and intermediate cells containing well-rounded nuclei, covered by a surface layer of flattened periderm cells (Figure 2A). In contrast, the equivalent region in p63<sup>+/–</sup>Irf6<sup>+/R84C</sup> embryos contained basal and intermediate cells with irregular nuclei covered by morphologically abnormal periderm cells (Figure 2B). We therefore examined the periderm in greater detail using immunostaining for keratin 17 (K17). Deconvolution images from E13.5 p63<sup>+/–</sup>Irf6<sup>+/R84C</sup> embryos indicated that K17 immunostaining appeared filamentous and incorrectly localized (Figure 2, C and D). At E14.5, K17 expression was not confined to the most superficial cells; rather, K17 appeared to be expressed throughout the epithelium (Figure 2, E and F). This observation was confirmed by deconvolution analysis of K17/K14 dual-stained sections. In wild-type mice, the basal cells were positive for K14 only, with a superficial layer of periderm cells positive for K14 and K17; in contrast, in p63<sup>+/–</sup>Irf6<sup>+/R84C</sup> embryos, the entire MEE stained positively for both K14 and K17. (K–M) At E15.5, whereas the MEE of wild-type mice degenerated, K17-positive cells persisted over the MEE of p63<sup>+/–</sup>Irf6<sup>+/R84C</sup> embryos. (M) Higher-magnification view of the boxed region in L. (N–Q) In vitro culture indicated that after 72 hours of forced contact, the palatal shelves of p63<sup>+/–</sup>Irf6<sup>+/R84C</sup> mice fused. K14 expression in both wild-type and p63<sup>+/–</sup>Irf6<sup>+/R84C</sup> palates was evident in remnants of the nasal and oral epithelia only (P and Q, asterisks). bm, basement membrane; p, periderm cell; b, basal cell; ne, nasal epithelium. Scale bars: 5 μm (A and B); 10 μm (C–F, I, J, and M); 50 μm (G and H); 100 μm (K, L, and N–Q).

Figure 2
Cleft palate observed in p63<sup>+/–</sup>Irf6<sup>+/R84C</sup> mice. (A and B) TEM analysis of the MEE at E14.5 revealed highly disordered basal and periderm cells in p63<sup>+/–</sup>Irf6<sup>+/R84C</sup> mice compared with their wild-type littermates. (C–J) Deconvolution analysis of the palatal shelves. (C and D) At E13.5, K17 appeared filamentous and incorrectly localized in the MEE (D, arrows). (E and F) At E14.5, whereas K17 expression was confined to the periderm of wild-type mice, K17 was expressed throughout the MEE in p63<sup>+/–</sup>Irf6<sup>+/R84C</sup> embryos. (G–J) K14 and K17 dual staining. (G and I) In wild-type mice, K14-positive basal cells were covered by K17/K14-positive periderm cells. (H and J) In p63<sup>+/–</sup>Irf6<sup>+/R84C</sup> embryos, the entire MEE stained positively for both K14 and K17. (K–M) At E15.5, whereas the MEE of wild-type mice degenerated, K17-positive cells persisted over the MEE of p63<sup>+/–</sup>Irf6<sup>+/R84C</sup> embryos. (M) Higher-magnification view of the boxed region in L. (N–Q) In vitro culture indicated that after 72 hours of forced contact, the palatal shelves of p63<sup>+/–</sup>Irf6<sup>+/R84C</sup> mice fused. K14 expression in both wild-type and p63<sup>+/–</sup>Irf6<sup>+/R84C</sup> palates was evident in remnants of the nasal and oral epithelia only (P and Q, asterisks). bm, basement membrane; p, periderm cell; b, basal cell; ne, nasal epithelium. Scale bars: 5 μm (A and B); 10 μm (C–F, I, J, and M); 50 μm (G and H); 100 μm (K, L, and N–Q).
To assay whether the palatal shelves of p63<sup>+/–</sup>/Irf6<sup>+/R84C</sup> embryos were competent to fuse, we used in vitro organ culture. Palatal shelves dissected from E13.5 wild-type and p63<sup>+/–</sup>/Irf6<sup>+/R84C</sup> mice were placed in contact and cultured for 24, 48, or 72 hours. Histological analysis confirmed that after 72 hours, palatal fusion was statistically significant compared with wild-type littermates (P = 0.01 for both comparisons, Mann-Whitney U test; Figure 4A). As isolated palatal epithelial cells have not been cultured successfully to our knowledge, we investigated the relationship between p63<sup>+/–</sup>/Irf6<sup>+/R84C</sup> mice, p63 expression in wild-type littermates (Figure 3, A and B), and p63 expression in wild-type mouse primary keratinocytes. Using siRNA, we knocked down p63 (Figure 4B) and found a statistically significant decrease in the level of Irf6 transcript (P = 0.05, Mann-Whitney U test; Figure 4C). Western blot analysis also indicated decreased protein levels (Figure 4D). These data suggested that p63 may regulate Irf6 expression.

To extend the observation obtained in mice, we assessed whether Irf6 expression is affected in a human disease model. We established human primary keratinocyte cell lines from 3 patients with ectrodactyly-ectodermal dysplasia-clefting (EEC) syndrome, who exhibit missense mutations in the DNA-binding domain of p63 (7). These patients have phenotypes typical of EEC syndrome, including cleft lip and palate. Real-time qPCR analysis of the R204W, R279H, and R304W mutant cell lines after 48 hours of differentiation indicated that p63 transcript levels were similar to
those detected in cell lines established from control individuals, and the presence of the early differentiation markers K1 and K10 suggested that differentiation had occurred normally at this stage (data not shown). Interestingly, all 3 cell lines exhibited reduced levels of IRF6 transcript compared with cells established from control individuals, and the decrease was statistically significant in cell lines R279H and R304W (P = 0.001 and 0.05, respectively; Kruskal-Wallis 1-way ANOVA followed by post-hoc Dunn’s test; Figure 4E). These data indicate that the correct function of p63 is important for appropriate expression of IRF6.

IRF6 is activated by p63 through an enhancer element. To understand how IRF6 is regulated, we tested whether IRF6 is a direct transcriptional target of p63. ChIP in combination with deep sequencing analysis (ChIP-seq analysis) using chromatin isolated from a normal human primary keratinocyte cell line and a pan-p63 antibody identified a single p63 binding site within a 100-kb genomic region encompassing the IRF6 locus. This site was located approximately 10 kb upstream of the transcription start site of IRF6 and was a double peak with strong p63 binding affinity, as indicated by the number of sequenced tags (peak heights 229 and 58; Figure 5A). Peak detection using the model-based analysis of ChIP-seq (MACS) data program (28) revealed that the binding site from the double peak covered approximately 900 bp. Interestingly, peak 229 of this p63 binding site overlapped an enhancer element of IRF6 recently identified by searching for multispecies conserved sequences (Supplemental Figure 3), and a SNP in this enhancer has previously been associated with higher risk of cleft lip (13). This binding site was located within a broader site identified previously in a genome-wide study of p63 using ChIP-on-chip analysis of a carcinoma cell line (19), in which a second binding site was detected in intron 1 of IRF6 that was not detected in our ChIP-seq analysis. The binding profile of p63 in close proximity to IRF6 was confirmed by independent ChIP-seq analysis using a second primary keratinocyte cell line established from a different normal individual (data not shown). The algorithm p53scan, which is suitable for binding motif searches for p53 family members (29), identified 2 consensus binding motifs in peak 229 and 1 additional motif in peak 58 (Figure 5B and Supplemental Figure 3).

To confirm the p63 binding site identified in ChIP-seq analysis, we performed independent ChIP-qPCR experiments using 2 p63 antibodies that recognize different epitopes in the p63 protein. Compared with 2 known targets of p63, the p21 and BPAG genes (30, 31), ChIP efficiencies expressed as a percentage of input chromatin at peak 229 and peak 58 regions were higher than, or at least similar to, those of the known binding sites (Figure 5C). Use of 2 different p63 antibodies confirmed that the observed binding was specific for p63 (Figure 5C). Of note, the independent ChIP-qPCR analysis confirmed the lack of p63 binding to the previously reported site in the first intron of IRF6 (ref. 19 and Supplemental Figure 4A). As enhancer elements have been shown to exhibit tissue- and species-specific activities (32), we performed ChIP-qPCR in palatal shelves dissected from wild-type E13.5 mice. Consistent with our data from human primary keratinocytes, we observed p63 binding to the upstream enhancer region of Irf6, but not to the intron 1 region in Irf6 (Supplemental Figure 4, B and C). We next assayed whether p63 binding is affected in patient keratinocyte cell lines with heterozygous p63 mutations. For both peak 229 and peak 58 regions, we observed a marked reduction of p63 binding in keratinocytes with the EEC syndrome mutations R204W, R279H, and R304W (Figure 5D). However, as both wild-type and mutant p63 proteins were present in the patient keratinocytes, it is probable that the detected residual DNA binding is mediated by the wild-type protein. To test this hypothesis further, we generated stable SAOS2-derived cell lines that express wild-type or ΔNp63α containing the mutation R304W in the absence of endogenous p63. ChIP experiments performed with these cell lines showed strong binding of wild-type p63 at the identified peak 58 binding site, whereas binding was abolished in the ΔNp63α R304W cell line (Supplemental Figure 4D).

p63 can act as either an activator or a repressor, depending on the target gene. To investigate how the binding site regulates IRF6 transcription, we cloned the region surrounding peak 229 (Figure 5B) in a firefly luciferase reporter construct. In a transient transfection

Figure 4

Ir6 in p63-deficient cells. (A) qPCR analysis of palatal shelves dissected from E13.5 wild-type, p63ΔN/–, and p63ΔC/ΔC embryos indicated that Ir6 transcripts were reduced to approximately 90% and 63%, respectively, of normal levels. **P = 0.01 versus p63ΔC/ΔC and wild-type (Mann-Whitney U test). (B) siRNA knockdown of p63 in mouse primary keratinocytes reduced p63 levels 5-fold. *P = 0.05 versus control scrambled siRNA (Mann-Whitney U test). (C) Ir6 transcript levels after p63 siRNA knockdown were reduced more than 60%. *P = 0.05 versus control scrambled siRNA (Mann-Whitney U test). (D) Western analysis reveals reduced Ir6 protein levels in mouse primary keratinocytes after p63 siRNA knockdown. Quantitation, shown above the Western blot, shows Ir6 levels relative to tubulin. Samples were run on the same gel but were noncontiguous (white line). (E) qPCR analysis of IRF6 in human primary keratinocytes derived from normal control individuals and patients with EEC syndrome (R204W, R279H, and R304W) showed reduced IRF6 levels when the DNA-binding function of p63 was impaired. *P = 0.05, ***P = 0.001 versus control (Kruskal-Wallis 1-way ANOVA followed by post-hoc Dunn’s test). Data represent mean ± SEM.
The p63 binding site obtained from ChIP-seq is shown as a black bar below the double peak (peak 229 and peak 58), and p63 binding sites reported previously by ChIP-on-chip (19) are shown as gray bars. (B) The 2 p63 binding motifs identified within peak 229 are highlighted in yellow, with the most conserved cytosine and guanine bases shown in red. (C) ChIP-qPCR analysis of p63 binding using p63 antibodies 4A4 and H129. Specific binding of p63 to the positive controls p21 and BPAG as well as to peak 229 (p229) and peak 58 (p58) regions, but not to negative controls myoglobin exon 2 (myo) and a no-gene region (chr11), was observed. (D) ChIP-qPCR analysis of R204W and R304W cell lines indicated reduced p63 binding. (E) Transient transfection assays showed that wild-type p63 strongly activated transcription through peak 229. In contrast, activation by the p63 mutants R204W, R279H, and R304W was greatly reduced, with the highest level (approximately 2-fold) in R304W. (F) Site-directed mutagenesis of the conserved cytosine and guanine bases showed that both motifs in peak 229 were responsive to p63 and that mutation abolished transcription. WT, reporter of wild-type peak 229; M1, mutation of motif 1; M2, mutation of motif 2; M1+M2, mutation of both motifs.

Discussion

Recent research has been highly successful in identifying the genetic mutations underlying syndromic forms of cleft lip and palate; for example, mutations in TBX22 cause X-linked cleft palate with ankyloglossia (33); in FOXE1, Bamforth-Lazarus syndrome (34, 35); in PVR1L, cleft lip and palate-ectodermal dysplasia syndrome (36); in MSX1, cleft lip and palate with tooth agenesis (37); in FLNA, otopalatodigital syndromes types 1 and 2 (38); in FGFR1, autosomal-dominant Kallmann syndrome (39); and in TGFPA, branchio-oculo-facial syndrome (40). Moreover, variation within IRF6, TBX22, PVR1L, and MSX1 is a contributing factor to nonsyndromic forms of cleft lip and palate (41–46). Despite these successes, the molecular pathways in which the proteins encoded by these genes function during development of the lip and palate remain poorly characterized, as such studies are not feasible in human embryos. The identification and use of mouse models for orofacial clefting allows such networks to be characterized and the morphogenetic, cellular, and molecular changes involved in clefting to be dissected (10, 11, 47).

In the current study, we used 2 different mutant mouse models to identify and characterize what we believe to be a novel interaction: mice heterozygous for p63 and the Irf6 knockin mutation R84C exhibited cleft palate. Although Irf6<sup>−/−</sup> mice occasionally exhibited mild intraoral adhesions between the mandible and the maxilla that caused restricted opening of the mouth, failure to suckle, and neonatal lethality (14), neither of these mouse models displayed cleft palate in the heterozygous state, presumably because a critical threshold level for each of these genes during palate development in mice is reached. Importantly, we further demonstrated that IRF6 expression was dependent on functional ΔNp63α protein, which activated IRF6 transcription through an enhancer element located approximately 10 kb upstream of the transcription start site of IRF6. These results provide a molecular basis for the epistatic interaction between IRF6 and p63 that is required for appropriate palatal development and thereby prevents cleft palate. Previous research has shown that p63 plays a central role in maintaining cellular prolifera-
Together, observations from our previous studies and data from human and mouse models suggest that downregulation of p63 and IRF6 is essential for the appropriate specification of MEE and the differentiation of mammary epithelial cells by promoting cell cycle arrest and preventing cell migration, resulting in failure of formation of strong adhesion complexes between adjacent palatal shelves. In contrast, R84C/R84C IRF6 knockout mice exhibit a hyperproliferative phenotype (13). In the current study, we clearly show that p63 also strongly transactivates this element, raising the question of whether p63 and AP-2α cooperate to regulate IRF6.

As cleft lip and cleft palate are not fully penetrant in patients with p63 mutations, genetic modifiers have been suggested to play a role in the disease pathway (7). Previous findings associating genetic variants in IRF6 with different cleft phenotypes (13), together with data obtained in the current study, lead us to speculate that IRF6 may act as a modifier in p63 disease phenotypes. Similarly, disease-causing IRF6 mutations have only been detected in approximately 70% of VWS families (60). Given that IRF6 can be regulated by the upstream p63 binding site, genetic variants in this site may disrupt normal transcriptional regulation of IRF6. Therefore, we believe the p63 binding site identified in this study offers a new opportunity to search for the genetic causes of unresolved cases of nonsyndromic cleft lip and cleft palate as well as VWS and PPS that map genetically to the IRF6 locus, but in which the causative mutation has not been identified.

Our discovery of an interaction between 2 key transcription factors that contribute to cleft lip and cleft palate provides opportunities for further dissection of the pathways involved in the complex process of mammalian palate development. Further elucidation of how these genes are regulated and the identification of both shared and individual targets for p63 and IRF6 in future studies may unearth new candidates as contributing factors for the etiology of facial clefting.

**Methods**

Mouse breeding, genotyping, and phenotype analysis. Generation and genotyping of p63+/– and IRF6Δ/ΔΔ mice have been described previously (14, 22). p63 knockout mice were provided by F. McKeon (Harvard Medical School, Boston, Massachusetts, USA), and IRF6Δ/ΔΔ mice were provided by R. Richardson (University of Manchester, Manchester, United Kingdom). Mice were housed in accredited animal facilities at the University of Manchester. All procedures were approved by the University of Manchester Ethical Review Committee and are licensed under the Animal (Scientific Procedures) Act 1986, issued by the Home Office. Compound heterozygous embryos were obtained by intercrossing p63+/– and IRF6Δ/ΔΔ mice, the morning of the vaginal plug being considered E0.5. Tissues dissected from E14.5 to P0 embryos were fixed in either 4% paraformaldehyde or Bouins reagent and processed for histological examination, scanning electron microscopy, and in situ hybridization, cell proliferation assays, and transmission electron microscopy have been described previously (14, 22). For palate culture, P0 embryos were fixed in either 4% parafomaldehyde or Bouins reagent and processed for histological examination, scanning electron microscopy, or immunohistochemistry using standard protocols (22). Antibodies were obtained from P. Coulombe (K17; Johns Hopkins University, Baltimore, Maryland, USA); F. McKeon (p63, 4A4); D. Garrod (desmoplakin, 115F; University of Manchester, Manchester, United Kingdom); Covance Research Products (loricrin and K10); BD Biosciences (E-cadherin); Abcam (α- and β-catenin), and R&D Systems (activated caspase 3). The section was in situ hybridization, cell proliferation assays, and transmission electron microscopy have been described previously (14, 22). For palate culture, E13.5 palatal shelves were dissected from p63+/– IRF6Δ/ΔΔ embryos and wild-type littermates and cultured as described previously (61).

Cell culture. Patients with EEC syndrome caused by mutations in the p63 gene were requested to donate a skin biopsy for research purposes. All procedures were approved by the ethical committee of the Radboud University Nijmegen Medical Centre (‘Commissie Mensgebonden Onderzoek Arnhem-Nijmegen’). After informed consent was obtained, 3 punch biopsies (3 mm) were taken from the lower back of each patient. Skin biopsy procedures, establishment of human primary keratinocytes from skin biopsies, and...
maintenance of primary keratinocyte cell lines were carried out as described previously (17). The Tet-on inducible expression system established in SAOS2 cells and described previously (29) was used to generate myc-tagged mouse ANP63α wild-type and R304W stable cell lines. Constructs for generation of SAOS2 stable cell lines were provided by M. Lohrum (Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen, Nijmegen, The Netherlands). Doxycyclin (Sigma-Aldrich) was used to induce expression of myc-ANP63α wild-type and myc-ANP63α R304W proteins at similar levels.

** Constructs and transfection assays. ** The genomic regions of p63 binding site peak 229 was amplified by PCR using gateway cloning primers (Supplemental Table 1) and cloned into a modified Smal site in pGL3-Enhancer Vector, which contains a firefly luciferase reporter gene followed by a SV40 enhancer. To mutate the p63 binding motifs, the most conserved cytosine and guanine bases (Figure 5, red text) were mutated to adenosine. The ANP63α wild-type expression plasmid has been described previously (17). Point mutations were introduced into this plasmid to generate R204W, R297H, and R304W mutations. Transfection and luciferase assays were described previously (17).

**RNA extraction and cDNA synthesis.** RNA isolation was performed from RNA extraction and cDNA synthesis. Total RNA was transcribed into cDNA according to the manufacturer’s protocol using reverse transcriptase PCR iScript cDNA Synthesis kit (BioRad) and random primers in combination with MMLV reverse transcriptase (Invitrogen). The cDNA produced was purified by NucleoSpin Extract II kit (Macherey-Nagel, Bioco) according to the manufacturer’s protocol. ChIP and ChIP-seq. Primary keratinocytes and SAOS-2 cultures were cross-linked with 1% formaldehyde for 10 minutes, and chromatin was collected.

**qPCR.** qPCR primers were designed using Primer 3 (http://frodo.wi.mit.edu), and qPCR reactions were performed in a 7500 Fast Real Time PCR System apparatus (Applied Biosystems) using iTaq SYBR Green Supermix (BioRad) according to the manufacturer’s protocol. For qPCR of cDNA analysis, 3 exon-spanning primer sets for IRF6 were used (Supplemental Table 2). Human acidic ribosomal protein was used as a housekeeping gene to normalize the amount of cDNA expression between wild-type and mutant samples were calculated by the 2^(-ΔΔCt) method (65, 66). The relative expression was averaged using 3 primer sets. For qPCR of ChIP analysis, 1 primer set was used for each tested binding region (Supplemental Table 3), and ChIP efficiency of certain binding sites was calculated using percentage of ChIPped DNA against input chromatin. Occupancy used in ChIP experiment with SAOS2 cells was calculated using ChIP efficiency of IRF6 P58 region standardized by that of myoglobin exon 2 region. For qPCR of palatal cDNA, mouse Gapdh (4352932E) and mouse Irf6 (Mm00516797_m1) TaqMan probes (Applied Biosystems) were used. 20-μl reactions were established in 1× PCR buffer (Invitrogen) containing 5 U Taq polymerase (Invitrogen), 100 mM MgCl₂, 4 mM dNTP, and 4 μl cDNA. Reactions were performed in triplicate on an OpticonII thermal cycler (MJ Research). Relative levels of expression were calculated from a standard curve and normalized to Gapdh.

**Bioimaging.** Immunofluorescence and histology images were acquired on a Leica DMRB and DMLB, respectively, using ×2.5 to ×63 Plan Fluotar lenses and a SPOT RT camera (Diagnostic Instruments). For deconvolution, images were acquired on a Delta Vision RT (Applied Precision) restoration microscope using ×40/1.3 Uplan FLN and ×60/1.42 Plan Apo objectives and the Sedat filter set (Chroma 89000). The images were collected using a Coolsnap HQ (Photometrics) camera with a Z. optical spacing of 0.2 μm. Raw images were deconvolved using Softworx software, and single optical sections of a deconvolved stack are shown.

**Statistics.** Mann-Whitney U statistical tests were performed for Figure 4, A–C, using SPSS software. Kruskal-Wallis 1-way ANOVA followed by post-hoc Dunn’s test was performed for Figure 4E, using Prism software. A P value less than 0.05 was considered significant.

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5. Jones MC. Etiology of facial clefts: prospective evalua-

11. Gritli-Linde A. The etiopathogenesis of cleft

16. Little HJ, et al. Missense mutations that cause Van

14. Richardson RJ, et al. IRF6 is a key determinant

Mantovani R, Guerrini L. Complex transcriptional


30. Westfall MD, Mays DJ, Sniezek JC, Pietropaolo NJ. The Delta6 p3α alphaphosphopeptides binds the p21 and 14–3–3 σ sigma promoters in vivo and has transcriptional repressor activity that is reduced by fusion to nontissue-specific CE. Mol Cell Biol. 2003;23(7):2246–2274.


