Modulation of noncanonical TGF-β signaling prevents cleft palate in Tgfbr2 mutant mice

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Patients with mutations in either TGF-β receptor type I (TGFBR1) or TGF-β receptor type II (TGFBR2), such as those with Loeys-Dietz syndrome, have craniofacial defects and signs of elevated TGF-β signaling. Similarly, mutations in TGF-β receptor gene family members cause craniofacial deformities, such as cleft palate, in mice. However, it is unknown whether TGF-β ligands are able to elicit signals in Tgfbr2 mutant mice. Here, we show that loss of Tgfbr2 in mouse cranial neural crest cells results in elevated expression of TGF-β2 and TGF-β receptor type III (TβRIII); activation of a TβRI/TβRIII-mediated, SMAD-independent, TRAF6/TAK1/p38 signaling pathway; and defective cell proliferation in the palatal mesenchyme. Strikingly, Tgfbr2, Tgfbr1 (also known as Alk5), or Tak1 haploinsufficiency disrupted TβRI/TβRIII-mediated signaling and rescued craniofacial deformities in Tgfbr2 mutant mice, indicating that activation of this noncanonical TGF-β signaling pathway was responsible for craniofacial malformations in Tgfbr2 mutant mice. Thus, modulation of TGF-β signaling may be beneficial for the prevention of congenital craniofacial birth defects.

Introduction
Craniofacial deformities, such as cleft palate, are among the most common congenital birth defects in humans (1). Altered TGF-β signaling causes syndromic and nonsyndromic cleft palate. For instance, mutations in the genes for TGF-β receptor type I or type II (TGFBR1 [also known as ALK5] or TGFBR2, respectively) are associated with Loeys-Dietz syndrome (previously called Marfan syndrome type II) in humans, which can manifest with craniofacial malformations, including cleft palate, craniosynostosis, hypertelorism, and vascular defects (2, 3). In addition, mutations in FBN1, which encodes an elastic extracellular matrix protein called fibrillin 1, lead to excessive TGF-β signaling and cause Marfan syndrome, which exhibits clinical phenotypes similar to those of Loeys-Dietz syndrome (4–6). Furthermore, individuals with DiGeorge syndrome, which results from a variably sized deletion on chromosome 22 (del22q11), exhibit altered TGF-β signaling (7–9). Thus, TGF-β signaling is crucial in regulating craniofacial development, and altered TGF-β signaling leads to multiple malformations in humans. Interestingly, these studies show that TGF-β signaling is elevated in patients with these syndromes. Nevertheless, we still do not have a clear understanding of the TGF-β signaling mechanism and its functional impact on craniofacial development in patients with mutations in TGFBR genes.

TGF-β signaling mediates a wide range of biological activities in development and disease. TGF-β ligands signal through heterodimeric type I and type II receptors (TGF-β receptor type I [TBR], also known as ALK5 and TGFBR1) and TβRII) that are members of the serine/threonine kinase family. To date, overwhelming evidence supports the notion that both TβRII and TβRII are indispensable in eliciting the biological response of TGF-β (10). The third member of the TGF-β receptor family, TβRIII (β-glycan), has high binding affinity for TGF-β2 and may be critical for keeping TGF-β at the cell surface for the TβRI/TβRII complex (11). TβRII may be required for optimal TGF-β2 function during development. Despite the fact that it is missing an intracellular kinase domain, TβRIII plays a crucial role in regulating embryonic development, because loss of Tgfbr3 results in an early embryonic lethal phenotype in mice (12).

Tgfbr1/Alk5, Tgfbr2, and Tgfbr3 are expressed in the developing craniofacial region, and loss of Tgfbr1/Alk5 or Tgfbr2 results in an array of craniofacial deformities, clearly indicating the functional significance of TGF-β signaling in regulating craniofacial development in mice (13–16). In the canonical TGF-β signaling pathway, the TβRII/TβRI complex activates the receptor-regulated SMADs, which form a transcriptional regulatory complex with SMAD4, move into the nucleus, and control downstream target gene expression (11). In parallel, our recent study showed that ectodermal SMAD4 and p38 are functionally redundant in mediating TGF-β signaling and demonstrated the significance of the SMAD-independent pathway in regulating craniofacial development (17).

In order to test the functional significance of TGF-β signaling in regulating the fate of cranial neural crest (CNC) cells, we generated a mutant animal model, in which loss of Tgfbr2 in CNC cells leads to an array of craniofacial deformities, including cleft palate (13). We have shown that CNC cells have a cell autonomous requirement for TβRII to regulate cell proliferation during palatogenesis. In the present study, we investigated the molecular mechanism of TGF-β signaling-mediated palatogenesis by analyzing TGF-β downstream target genes using Tgfbr2fl/fl,Wnt1-Cre mice. Surprisingly, we detected an elevation of TGF-β2 expression that is responsible for the cell proliferation defect and cleft palate in Tgfbr2 mutant mice. Our study demonstrates that elevated TGF-β2 activates an alternative TGF-β signaling pathway through the TβRI/TβRIII receptor complex and induces a SMAD-independent TNF receptor–associated factor 6/TGF-β–activated kinase 1/p38 signaling pathway.
Differentially expressed Tgfβ and Tgfbr genes based on genome-wide expression profiling analysis of the palates of Tgfb2<sup>fl/fl</sup> and Tgfb2<sup>fl/fl</sup>;Wnt1-Cre mice at E14.5. Data in the Tgfb2<sup>fl/fl</sup>;Wnt1-Cre/Tgfbr2<sup>fl/fl</sup> column are the ratios based on the geometric means of expression scores derived from Tgfb2<sup>fl/fl</sup>;Wnt1-Cre and Tgfb2<sup>fl/fl</sup> control mice. The FDR was calculated based on the genome-wide expression profiling analysis, as described in the Methods section. All relevant transcripts with a more than 1.5-fold change and with less than 5% FDR are shown. AFFY_ID, Affymetrix probe set ID.

Table 1

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(TRAf6/TAK1/p38) signaling cascade in the absence of TβRII. Importantly, genetic manipulation of this alternative TGF-β signaling prevents craniofacial deformities in Tgfbr2 mutant mice and offers what we believe to be a unique opportunity for the prevention of craniofacial malformations.

Results

Identification of TGF-β downstream target genes during palatogenesis. Mice lacking Tgfb2 in CNC cells exhibit an array of craniofacial deformities, including cleft palate (13). We have shown that CNC cells have a cell autonomous requirement for TβRII to regulate cell proliferation during palatogenesis. To investigate the mechanism of altered TGF-β signaling in Tgfbr2 mutant mice, we performed global gene expression analyses of the palatal tissue of Tgfb2<sup>fl/fl</sup>;Wnt1-Cre mice and Tgfb2<sup>fl/fl</sup> control mice at E14.5 (during palatal fusion, n = 5 per genotype) and examined the downstream consequences of dysfunctional TGF-β signaling. In this comparison, we uncovered 293 probe sets representing transcripts that were differentially expressed (≥1.5-fold, <5% false discovery rate [FDR]), 149 probe sets that were more abundant in Tgfb2<sup>fl/fl</sup>;Wnt1-Cre mice (Supplemental Table 2). The genes identified as altered in Tgfb2<sup>fl/fl</sup>;Wnt1-Cre mice were consistent with cell proliferation defects, with significant reductions in the levels of transcripts related to cell cycle control, mitosis, and microtubule-based cell division and with enrichment for transcripts related to the negative regulation of cellular proliferation (Supplemental Table 2).

In a focused analysis of Tgfβ and Tgfbr family gene member expression, we discovered that Tgb2 and Tgb3 transcript levels were more abundant in the palates of the Tgfb2<sup>fl/fl</sup>;Wnt1-Cre mice than in those of the control (Table 1). The microarray data was validated by quantitative RT-PCR analysis of TGF-β ligands and receptors (Figure 1A). As expected, TGF-β2 and TβRII protein levels were also upregulated in the palatal mesenchyme of Tgfb2<sup>fl/fl</sup>;Wnt1-Cre mice at E14.5 (Figure 1B). TGF-β2 has a high affinity for TβRII, a unique feature that distinguishes TGF-β2 from other TGF-β ligands (18, 19). In in vitro studies, TGF-β isoforms differentially bind to TGF-β receptors and play crucial nonoverlapping roles during craniofacial development (20, 21).

To identify proteins relevant to dysfunctional TGF-β signaling after the loss of Tgfβ2, we analyzed protein extracts from cultured mouse embryonic palatal mesenchymal (MEPM) cells of Tgfb2<sup>fl/fl</sup> (control) mice and Tgfb2<sup>fl/fl</sup>;Wnt1-Cre mice (Supplemental Figure 1 and Supplemental Table 3). We found that β-spectrin, an adaptor protein of TβRI, was upregulated in Tgfb2<sup>fl/fl</sup>;Wnt1-Cre MEPM cells (Figure 2A, Supplemental Figure 1C, and Supplemental Table 3). β-Spectrin is associated with TGF-β receptors, and loss of β-spectrin leads to disruption of TGF-β signaling by SMAD proteins in mice, indicating that β-spectrin is involved in SMAD-dependent TGF-β signaling (22). β-Spectrin knockout mice exhibit a phenotype similar to that of Smad2<sup>+/–</sup>;Smad3<sup>+/–</sup> mutant mice, with midgestational death due to gastrointestinal, liver, neural, and heart defects (22). β-Spectrin expression was detectable in primary MEPM cells and palatal mesenchyme of both Tgfb2<sup>fl/fl</sup>;Wnt1-Cre and control mice but appears to be increased in Tgfb2<sup>fl/fl</sup>;Wnt1-Cre mice (Figure 2B, arrows). Consistent with its ability to interact with TGF-β receptors, β-spectrin was mainly localized at the cell membrane in Tgfb2<sup>fl/fl</sup>;Wnt1-Cre mice (Figure 2B, arrows).

Similarly, 14-3-3ζ and phosphorylated 14-3-3ζ were elevated in Tgfb2<sup>fl/fl</sup>;Wnt1-Cre MEPM cells compared with those in control cells (Figure 2A). 14-3-3ζ is a TβRII-interacting protein that is phosphorylated by p38 MAPK α, β, γ, or δ (MAPK14/11/12/13) and plays a key role in protein targeting and protein domain-
specific binding (23). 14-3-3 expression appeared to be increased in 
Tgfbr2fl/fl;Wnt1-Cre MEPM cells, and phosphorylated 14-3-3 was increased in 
Tgfbr2fl/fl;Wnt1-Cre mice (Figure 2D). Notably, we found elevated 14-3-3 expression in the palatal mesenchyme

Figure 2
Identification of molecules with upregulated expression in primary MEPM cells from Tgfbr2fl/fl;Wnt1-Cre mice. (A) Immunoblotting analysis of indicated molecules in primary MEPM cells of Tgfbr2fl/fl and Tgfbr2fl/fl;Wnt1-Cre mice using anti–β-spectrin antibody. Arrows indicate expression of β-spectrin. Original magnification, ×400. (C) Immunohistochemical staining of β-spectrin and DAPI staining in sections of Tgfbr2fl/fl and Tgfbr2fl/fl;Wnt1-Cre mice at E14.0. Scale bar: 50 μm. (D) Immunofluorescence analysis of primary MEPM cells from Tgfbr2fl/fl and Tgfbr2fl/fl;Wnt1-Cre mice using anti–14-3-3ζ/δ (14-3-3ζ/δ) or anti–phosphorylated 14-3-3 (P-14-3-3) antibody. Scale bar: 20 μm. (E) Immunohistochemical staining of 14-3-3ζ/δ and DAPI staining in sections of Tgfbr2fl/fl and Tgfbr2fl/fl;Wnt1-Cre mice at E13.5. 14-3-3ζ/δ expression appears increased in Tgfbr2fl/fl;Wnt1-Cre palate (arrow) compared with that in Tgfbr2fl/fl littermate control. Scale bar: 50 μm. Insets show lower-magnification images (original magnification, ×100). (F) Immunoblotting analysis of indicated molecules in primary MEPM cells from Tgfbr2fl/fl and Tgfbr2fl/fl;Wnt1-Cre mice. P-p38, phosphorylated p38; P-JNK, phosphorylated JNK; P-ERK, phosphorylated ERK. (G) Immunoblotting analyses of indicated molecules in primary MEPM cells of Tgfbr2fl/fl and Tgfbr2fl/fl;Wnt1-Cre mice treated with (+) or without (−) p38 MAPK inhibitor SB203580. P-14-3-3, phosphorylated 14-3-3.
Alternative TGF-β signaling pathway in the absence of TβRII. To analyze the mechanism of signaling initiated in the absence of Tβgr2, we performed communoprecipitation assays using anti-TβRII, TβRIII, and β-spectrin antibodies and extracts of control and Tβgr2/Wnt1-Cre MEMP cells (Figure 3A). β-Spectrin was communoprecipitated with TβRIII and TβRI antibodies in Tβgr2/Wnt1-Cre MEMP cells (Figure 3A). Although the interaction between TβRI and β-spectrin was not altered in Tβgr2/Wnt1-Cre MEMP cells compared with that in control cells, TβRIII was more abundantly communoprecipitated by β-spectrin antibody in Tβgr2/Wnt1-Cre MEMP cells, and vice versa, indicating that β-spectrin stably bound to TβRI and more abundantly bound to TβRIII in the absence of TβRII (Figure 3A). To validate that the interaction between β-spectrin and TβRIII is dependent on TGF-β, we performed communoprecipitations after treatment with TGF-β2. Although the interaction between TβRI and TβRIII was induced by TGF-β2 in both control and Tβgr2/Wnt1-Cre MEMP cells, the interaction between TβRI and TβRIII was more prominent in Tβgr2/Wnt1-Cre MEMP cells, consistent with upregulated expression of TGF-β2 and TβRIII in Tβgr2/Wnt1-Cre MEMP cells (Supplemental Figure 2). Notably, we detected colocalization of TβRI and TβRIII in Tβgr2/Wnt1-Cre MEMP cells, whereas we only detected limited colocalization of TβRI and TβRIII in Tβgr2/Wnt1-Cre control MEMP cells, supporting our finding that TβRI/TβRIII complex is more abundant in the absence of TβRII (Figure 3B). Furthermore, we performed cross-linking analysis to test whether TGF-β2 binds to TβRI and TβRIII in the absence of Tβgr2 using radioactive TGF-β2 ligands. In Tβgr2 control MEMP cells, radioactive TGF-β2 ligands (12.5 kDa) bind to TβRII (53 kDa), TβRIII (70 kDa), and TβRIII (100–200 kDa, highly glycosylated molecule) and form the ligand-receptor complexes of TβRII:TGF-β2 (65.5 kDa), TβRIII::TGF-β2 (82.5 kDa), and TβRIII::TGF-β2 (112.5–212.5 kDa) (Figure 3C). In contrast, radioactive TGF-β2 was more abundantly cross-linked with TβRIII and TβRI/TβRIII in Tβgr2/Wnt1-Cre MEMP cells, and complexes of TβRIII::TβRII::TGF-β2 (165.5–265.5 kDa), TβRIII::TβRII::TGF-β2 (112.5–212.5 kDa), and TβRIII::TGF-β2 (65.5 kDa), but not TβRII::TGF-β2 (82.5 kDa), were detectable (Figure 3C). To confirm our findings further, we performed immunoprecipitation–cross-linking analysis (Figure 3D). We found that TGF-β2 can bind to TβRII and TβRIII, but not TβRI, in Tβgr2/Wnt1-Cre MEMP cells and forms a complex with TβRIII and TβRII/TβRI (Figure 3, D and E). Collectively, our data indicate that a TGF-β2/TβRII/TβRII complex forms in the absence of Tβgr2 and likely transduces TGF-β signaling in palatal mesenchymal cells.

TGF-β signals through a receptor complex of both TβRII and TβRI, causing phosphorylation of SMAD proteins (11, 24). We found that SMAD-dependent TGF-β signaling was compromised in Tβgr2/Wnt1-Cre MEMP cells (Figure 3F). In parallel, patients with TGFBR2 mutations show elevated TGF-β signaling (3, 25). We hypothesized that elevated TGF-β levels in Tβgr2 mutant cells initiate an intracellular signal in the absence of Tβgr2, most likely via a SMAD-independent p38 MAPK pathway, using a hitherto unidentified mechanism.
Figure 3
Altered ligand/receptor assembly and upregulated TAK1 phosphorylation in the absence of Tgfbr2. (A) Immunoblotting analysis of immunoprecipitation products derived from IgG control beads with Tgfbr2<sup>fl/fl</sup> or Tgfbr2<sup>fl/fl;Wnt1-Cre</sup> MEPM cell extracts (lanes 1 and 2, respectively), Tgfbr2<sup>fl/fl</sup> MEPM cell extracts (lane 3), and Tgfbr2<sup>fl/fl;Wnt1-Cre</sup> MEPM cell extracts (lane 4) using the indicated antibodies. The bar graphs show the ratios of TβRIII and β-spectrin after quantitative densitometry analysis of immunoblotting data. Three samples were analyzed for each experiment. Error bars represent SD. (B) Immunoblotting analysis of immunoprecipitation products using anti-His-tag antibody derived from IgG control beads or MEPM cell extracts from Tgfbr2<sup>fl/fl</sup> or Tgfbr2<sup>fl/fl;Wnt1-Cre</sup> mice after overexpression of His-tagged Tgfbr1 and FRP-tagged Tgfbr3, with (+) or without (−) TGF-β2 treatment. (C) Cross-linking analysis after treatment with radioactive iodine-125 (<sup>125</sup>I) TGF-β2 in primary MEPM cells from Tgfbr2<sup>fl/fl</sup> and Tgfbr2<sup>fl/fl;Wnt1-Cre</sup> mice. (D) Immunoprecipitation products derived from IgG control beads (lane 1), Tgfbr2<sup>fl/fl</sup> MEPM cell extracts (lane 2), and Tgfbr2<sup>fl/fl;Wnt1-Cre</sup> MEPM cell extracts (lane 3) after cross-linking with <sup>125</sup>I–TGF-β2. The top arrowhead indicates TβRI::TGF-β2 complex after IP with anti-TβRII antibody, and the bottom arrowhead indicates TβRII::TGF-β2 complex after IP with anti-TβRI antibody. The bracket indicates TβRIII::TβRI::TGF-β2 complex. (E) Immunoblotting analysis of immunoprecipitation products derived from IgG control beads (lane 1), Tgfbr2<sup>fl/fl</sup> MEPM cell extracts (lane 2), and Tgfbr2<sup>fl/fl;Wnt1-Cre</sup> MEPM cell extracts (lane 3) after cross-linking with <sup>125</sup>I–TGF-β2. (F) Immunoblotting analysis of indicated molecules in primary MEPM cells from Tgfbr2<sup>fl/fl</sup> and Tgfbr2<sup>fl/fl;Wnt1-Cre</sup> mice cultured with TGF-β2 for indicated times. The bar graphs show the ratios of phosphorylated SMAD2 relative to SMAD2/3, phosphorylated TAK1 relative to TAK1, or phosphorylated p38 relative to p38 after quantitative densitometry analysis of immunoblotting data.
TGF-β2 triggers a SMAD-independent TGF-β signaling cascade via a TβRII/TβRIII complex in the absence of Tgfbr2 and may adversely affect CNC cell proliferation during palate formation. Therefore, reduction of TGF-β2-induced TAK1/p38 MAPK activation may increase CNC cell proliferation in Tgfbr2<sup>fl/fl</sup>;Wnt1-Cre palates. To test this hypothesis, we took an in vitro approach and treated palatal explants with TGF-β2–neutralizing antibody (TGF-β2 NAb). Inhibition of altered TGF-β signaling with TGF-β2 NAb blocked phosphorylation of both TAK1 and p38 in Tgfbr2<sup>fl/fl</sup>;Wnt1-Cre MEPM cells in a cell-autonomous manner (Figure 5A).

Furthermore, TGF-β2 NAb treatment restored cell proliferation in the palatal mesenchymal cells in Tgfbr2<sup>fl/fl</sup>;Wnt1-Cre palates in the ex vivo organ culture system (Figure 5B). In order to test the hypothesis that activation of alternative TGF-β signaling in Tgfbr2<sup>fl/fl</sup>;Wnt1-Cre mice depends on the noncanonical TGF-β signaling mediated by TβRII/TβRIII, we cultured palates with a NAb for TβRIII (Figure 5, C and D). The cell proliferation defect was rescued by treatment with the NAb for TβRIII in the ex vivo organ culture system, whereas cell proliferation was not affected in control samples treated with a TβRIII NAb (Figure 5C). To examine...
Figure 5
Inhibition of TGF-β2/TβRII–mediated TAK1/p38 MAPK activation rescues a cell proliferation defect in Tgfbr2fl/fl;Wnt1-Cre palates. (A) Immunoblotting analysis of indicated molecules in MEPM cells from Tgfbr2fl/fl and Tgfbr2fl/fl;Wnt1-Cre mice cultured with (+) or without (–) TGF-β2 NAB. The bar graphs show the ratios of indicated molecules after quantitative densitometry analysis of immunoblotting data. *P < 0.05. (B) BrdU incorporation in the palate of Tgfbr2fl/fl;Wnt1-Cre mice after IgG or TGF-β2 NAB treatment. Arrows indicate BrdU-positive cells. Original magnification, ×200. The bar graph shows the percentage of BrdU-labeled nuclei in the indicated genotyping palate treated with TGF-β2 NAB or IgG. *P < 0.05. (C) Quantitation of the percentage of BrdU-labeled nuclei in the indicated genotyping palates treated with TβRII NAB or IgG. *P < 0.05. (D) Immunoblotting analysis of indicated molecules in indicated genotyping MEPM cells cultured with (+) or without (–) TβRII NAB. (E) Quantitation of the percentage of BrdU-labeled nuclei in the indicated genotyping palates treated with TAK1 inhibitor or vehicle. *P < 0.05. (F) Immunoblotting analysis of indicated molecules in indicated genotyping MEPM cells cultured with (+) or without (–) TAK1 inhibitor. (G) Quantitation of the percentage of BrdU-labeled nuclei in the indicated genotyping palates treated with p38 MAPK inhibitor or vehicle. *P < 0.05. (H) Quantitation of the percentage of BrdU-labeled nuclei in primary indicated genotyping MEPM cells cultured with (+) or without (–) TβRI kinase inhibitor or vehicle.
the requirements of TAK1 and p38 MAPK activation for the cell proliferation defect in Tgfb2\(^{-}/-\);Wnt1-Cre palates, we cultured palates with a TAK1 or p38 MAPK inhibitor, (S2)-7-Oxoaenol or SB203580, respectively, and found that the cell proliferation defect was also rescued (Figure 5, E–G).

Previous reports suggest that ubiquitination of TRAF6 by TGF-β is independent of TβRI kinase activity (29). Therefore, we tested whether TβRI kinase activity is necessary for the activation of TAK1/p38 MAPK and inhibition of TβRI kinase activity can rescue a defect in cell proliferation in Tgfb2\(^{-}/-\);Wnt1-Cre palates in the ex vivo organ culture system. An inhibitor of TβRI kinase, A83-01, did not rescue the cell proliferation defect in Tgfb2\(^{-}/-\);Wnt1-Cre palates, consistent with the ectopic noncanonical TGF-β signaling being independent of TβRI kinase activity (Figure 5, H and I, and data not shown). Thus, elevated TGF-β2 triggers a SMAD-independent TAK1/p38 MAPK signaling cascade via a TβRI/TβRIII complex in the absence of TβRII and adversely affects CENC cell proliferation during palate formation.

Increased TGF-β2 seems to be a trigger for alternative TGF-β signaling activation. Therefore, reduction of TGF-β2 expression may lead to a rescue of cleft palate in Tgfb2\(^{-}/-\);Wnt1-Cre mice. To test this hypothesis further, we took an in vivo approach and generated Tgfb2\(^{-}/-\);Wnt1-Cre,Tgfb2\(^{-/-}\) mice in order to reduce TGF-β2 ligand expression level. Indeed, Tgfb2\(^{-}/-\);Wnt1-Cre,Tgfb2\(^{-/-}\) mice formed normal palates, with 82.5% phenotype penetrance (33 out of 40 mice had normal palates), although they still exhibited a calvaria defect and small mandible (Figure 6A and Supplemental Figure 7A). Histological analysis showed that the growth and elevation of the palatal shelf in Tgfb2\(^{-}/-\);Wnt1-Cre,Tgfb2\(^{-/-}\) mice was similar to that in the control at E14.5 (Figure 6B). At E16.5, palatal shelves fused normally in Tgfb2\(^{-}/-\);Wnt1-Cre,Tgfb2\(^{-/-}\) mice (Figure 6B). At birth, the palatal processes of the maxilla and the palatine bones were partially rescued in Tgfb2\(^{-}/-\);Wnt1-Cre,Tgfb2\(^{-/-}\) mice compared with those in the control, whereas there was little palatine bone formation in Tgfb2\(^{-}/-\);Wnt1-Cre mice (Figure 6C).

At the molecular level, the upregulated phosphorylated p38 MAPK and phosphorylated 14-3-3 expression in Tgfb2\(^{-}/-\);Wnt1-Cre palates was restored to control levels in E14.5 Tgfb2\(^{-}/-\);Wnt1-Cre,Tgfb2\(^{-/-}\) palates (Figure 6D). Cell proliferation activity in the CNC-derived palatine mesenchyme was restored to control levels in Tgfb2\(^{-}/-\);Wnt1-Cre,Tgfb2\(^{-/-}\) palates (Figure 6E and F). Thus, reducing the TGF-β2 level reversed the activation of p38 MAPK signaling and restored cell proliferation in the palatine mesenchyme of Tgfb2\(^{-}/-\);Wnt1-Cre mice.

In our model, TGF-β2-mediated TAK1/p38 MAPK activation through TβRI. In order to test that activation of alternative TGF-β signaling in Tgfb2\(^{-}/-\);Wnt1-Cre mice depends on the presence of TβRI, we generated Tgfb2\(^{-}/-\);Wnt1-Cre;Alk5\(^{-/-}\) mice (Figure 7 and Supplemental Figure 7B). In fact, Tgfb1/Alk5 haploinsufficiency resulted in the rescue of palatal fusion in Tgfb2\(^{-}/-\);Wnt1-Cre;Alk5\(^{-/-}\) mice, with 91.7% penetrance (22 out of 24 mice had normal palates), whereas Tgfb2\(^{-}/-\);Wnt1-Cre;Alk5\(^{-/-}\) mice still exhibited cleft palate (Figure 7A). The other craniofacial deformities, such as calvaria defect and small mandible, were mostly rescued in Tgfb2\(^{-}/-\);Wnt1-Cre;Alk5\(^{-/-}\) mice as well (Figure 7A), suggesting that alternative TGF-β signaling activation in the absence of TβRII is a mechanism conserved in other craniofacial regions. Based on histological analysis, the growth and elevation of the palatal shelf in most Tgfb2\(^{-}/-\);Wnt1-Cre;Alk5\(^{-/-}\) mice were similar to those in the control at E14.5 (Figure 7B). At birth, the palatal processes of the maxilla and the palatine bones were mostly rescued in Tgfb2\(^{-}/-\);Wnt1-Cre;Alk5\(^{-/-}\) mice compared with those in the control, whereas there was little palatine bone formation in Tgfb2\(^{-}/-\);Wnt1-Cre,Alk5\(^{-/-}\) and Tgfb2\(^{-}/-\);Wnt1-Cre;Alk5\(^{-/-}\) mice (Figure 7C). Protein expression analysis showed that elevated phosphorylated p38 and phosphorylated 14-3-3 levels detectable in Tgfb2\(^{-}/-\);Wnt1-Cre mice (Figure 7D, lane 1) were restored to the control level (Figure 7D, lane 2) in Tgfb2\(^{-}/-\);Wnt1-Cre;Alk5\(^{-/-}\) mice (Figure 7D, lane 3). Moreover, haploinsufficiency of Alk5 restored cell proliferation activity in Tgfb2\(^{-}/-\);Wnt1-Cre;Alk5\(^{-/-}\) mice (Figure 7, E and F). Finally, haploinsufficiency of Takt1 in Tgfb2 mutant mice (Tgfb2\(^{-}/-\);Wnt1-Cre,Takt1\(^{-/-}\) mice) also resulted in a rescue of cleft palate via a reduction of alternative p38 MAPK activation (Supplemental Figure 8). Thus, our findings indicate that SMAD-independent, TRAF6/TAK1/p38 MAPK activation mediated through TβRI/TβRIII is responsible for the CNC cell proliferation defect and failure of palatal fusion in Tgfb2\(^{-}/-\);Wnt1-Cre mice.

**Discussion**

**TGFBR1 and TGFBR2**

Mutations in human structural genes have been reported in humans with craniofacial malformations and/or vascular abnormalities (2, 3, 31–34). The majority are missense substitutions or nonsense mutations in exon 4 of TGFBR1 and exons 4–7 of TGFBR2 that are predicted to disrupt the kinase domain (34, 35). Our findings may be relevant in human disease, given that altered TGF-β signaling is implicated in multiple congenital malformations and syndromes in humans. Our observation that TGF-β2 and TβRIII expression is elevated in the palatine mesenchyme of Tgfb2\(^{-}/-\);Wnt1-Cre mice led to our discovery of an alternative TGF-β signaling mechanism in the absence of TβRII that is detrimental to craniofacial morphogenesis. Specifically, our study shows that elevated TGF-β2 signals through a TβRI/TβRIII complex to induce a SMAD-independent TRAF6/TAK1/p38 signaling cascade and results in cleft palate in Tgfb2 mutant mice. This is an important advancement in our understanding of the mechanism of TGF-β signaling. Previous studies suggested that TβRIII appears to be dispensable for TGF-β-mediated signal transduction, because it lacks an intracellular domain and most cells that lack functional TβRIII still respond to TGF-β (12, 36). However, these studies did not consider how TGF-β signals in the absence of TβRII. Furthermore, TβRIII is required for murine somatic development, because loss of Tgfb3 results in an early embryonic lethal phenotype (12). In this study, we show that alternative TGF-β signaling can be activated through the TβRI/TβRIII complex, which in turn activates the TRAF6/TAK1/p38 MAPK signaling cascade to inhibit CNC-derived palatal mesenchymal cell proliferation and cause cleft palate.

Although Tgfb2 haploinsufficiency rescued the CNC cell proliferation defect and cleft palate in Tgfb2\(^{-}/-\);Wnt1-Cre mice, calvarial and mandibular defects were not rescued. In contrast, Tgfb1/Alk5 haploinsufficiency completely rescued the CNC cell proliferation defect as well as palatal fusion and mostly rescued calvarial, maxillary, and mandibular defects in Tgfb2\(^{-}/-\);Wnt1-Cre mice. One possible explanation is that TGF-β ligands other than TGF-β2 can signal through the TβRI/TβRIII complex to cause adverse effects on craniofacial development (Supplemental Figure 9). Therefore, a reduction of TGF-β2 signaling alone does not prevent these developmental defects, but a reduction of Tgfb1/Alk5 does. This reasoning is supported by the finding that almost all craniofacial defects, such as cleft palate, microglossia, small mandible, and

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880
Figure 6
Rescue of cleft palate in Tgfb2^{fl/fl};Wnt1-Cre mice via reduction of TGF-β2. (A) Morphologies of newborn Tgfb2^{fl/fl} control, Tgfb2^{fl/fl};Wnt1-Cre, and Tgfb2^{fl/fl};Wnt1-Cre;Tgfb2^{+/–} mice. The bottom row shows the macroscopic appearance of palates at the newborn stage. Arrowheads show calvaria defects. The arrow shows cleft palate, and open arrows show normal palates. Palates were scored as normal or cleft at birth. (B) Hematoxylin and eosin staining of histological sections of Tgfb2^{fl/fl}, Tgfb2^{fl/fl};Wnt1-Cre, and Tgfb2^{fl/fl};Wnt1-Cre;Tgfb2^{+/–} mice at E14.0, E14.5, and E16.5. Arrows indicate palate. Scale bar: 50 μm. (C) Whole-mount Alcian blue–Alizarin red skeletal staining of Tgfb2^{fl/fl}, Tgfb2^{fl/fl};Wnt1-Cre, and Tgfb2^{fl/fl};Wnt1-Cre;Tgfb2^{+/–} newborn mice. Dotted lines indicate the palatal process of maxilla and palatine bones. (D) Immunoblotting analysis of E14.5 Tgfb2^{fl/fl}, Tgfb2^{fl/fl};Wnt1-Cre, and Tgfb2^{fl/fl};Wnt1-Cre;Tgfb2^{+/–} palates. The bar graphs show the ratios of phosphorylated p38 relative to p38, 14-3-3 relative to GAPDH, or TGF-β2 relative to GAPDH after quantitative densitometry analysis of immunoblotting data in Tgfb2^{fl/fl}, Tgfb2^{fl/fl};Wnt1-Cre, and Tgfb2^{fl/fl};Wnt1-Cre;Tgfb2^{+/–} palates. Three samples were analyzed for each experiment. Error bars represent SD. (E) BrdU staining of Tgfb2^{fl/fl}, Tgfb2^{fl/fl};Wnt1-Cre, and Tgfb2^{fl/fl};Wnt1-Cre;Tgfb2^{+/–} mice at E14.0. Scale bar: 50 μm. (F) Quantitation of the number of BrdU-labeled nuclei in the palate of Tgfb2^{fl/fl}, Tgfb2^{fl/fl};Wnt1-Cre, and Tgfb2^{fl/fl};Wnt1-Cre;Tgfb2^{+/–} mice at E14.0. Three samples were analyzed for each experiment. Error bars represent SD. ***P < 0.001.
calvaria defects, are rescued in Tgfr2β/−;Wnt1-Cre;Alk5β/− mice. It is important to note that alternative TGF-β signaling involving TβRI/TβRII is a widely used mechanism and can affect multiple craniofacial organogeneses during development.

The lack of phenotype rescue other than cleft palate in Tgfr2β/−;Wnt1-Cre;Tgfb2−/− mice could also be explained as the result of the CNC-derived palatal mesenchyme being more sensitive than other tissues to the level of noncanonical TGF-β signaling. In support of this theory, progenitor cell differentiation into hepatocytes and biliary cells is dependent on a gradient of TGF-β signaling (37). It is conceivable that TGF-β levels, in conjuction with Tgfr activity, differentially modulate distinct signaling pathways that control unique sets of downstream target genes.

The fact that Alk5β/−;Wnt1-Cre mice exhibit more severe developmental defects than Tgfr2β/−;Wnt1-Cre mice suggests that the TβRI may mediate signals from additional members of the TGF-β family (other than TGF-β1, TGF-β2, and TGF-β3) and regulate downstream target genes during craniofacial development. The gene expression pattern of TGF-β ligands and receptors may also contribute more widespread craniofacial malformations in Tgfr11/−/−;Alk5 mutant mice. We found that there is increased p38 signaling in Tgfr2β/−;Wnt1-Cre mice and are differentially affected downstream signaling mediators in Alk5β/−;Wnt1-Cre mice (J. Iwata et al., unpublished observations), suggesting that noncanonical TGF-β signaling may be controlled via multiple molecular mechanisms during craniofacial development. In parallel, we found that there is no activation of p38 signaling in Alk5β/−;Wnt1-Cre mice. Further studies are necessary to explore the TGF-β signaling mechanism in the absence of Alk5 during embryonic development.

Recent studies revealed that noncanonical TGF-β signaling is also activated in some physiological (17, 38, 39) and pathological conditions, including Marfan and Loeys-Dietz syndromes (25, 40). Previous reports have shown that the p38 MAPK activation mediated by TGF-β is able to signal through a kinase-dead TβRI or constitutively active TβRI in the presence of TβRII in cell lines (29, 30). In contrast, this current study shows that noncanonical TGF-β signaling in the palatal mesenchyme is activated even in the absence of TβRII and results in cleft palate. We found that a low level of activation of noncanonical TGF-β signaling is induced in wild-type MEPM cells, suggesting that some TβRI/TβRII complex may also control p38 MAPK activation in the palatal mesenchyme during normal palate formation.

Previous studies indicate that TGF-β can signal only through TβRI/TβRII complex. In response to TGF-β binding, the cytoplasmic domains of the type I receptors are phosphorylated by the constitutively active type II receptor kinase (41, 42). TβRII kinase activity is crucial for the activation of TβRI. In this study, we have demonstrated that TβRII/TβRIII complex without TβRII mediates elevated TGF-β2 signaling without requiring the kinase activity of TβRII, consistent with the previous study indicating that Tak1/p38 MAPK activation is independent of TβRII kinase activity (29).

A haploinsufficiency of Tak1 in Tgfr2β mutant mice (Tgfr2β/−;Wnt1-Cre;Tak1−/− mice) results in reduced p38 MAPK activation and rescues the craniofacial abnormalities, including cleft palate, consistent with ectopic Tak1/p38 MAPK activation being responsible for these developmental defects in Tgfr2β mutant mice. In addition, siRNA knockdown for Tak1 inhibited p38 MAPK activation. The Tak1 kinase inhibitor (SZ)-7-Oxoozaoenol also blocked p38 MAPK activation and rescued the cell proliferation defect in Tgfr2β/−;Wnt1-Cre palates in the ex vivo organ culture system. Thus, Tak1 is crucial for the activation of alternative TGF-β signaling in the absence of TβRII. Regarding the p38 MAPK activity, SB203580 is a selective inhibitor of p38 MAPK, and it inhibits the activation of downstream targets such as 14-3-3. SB203580 inhibits p38 catalytic activity by binding to the ATP-binding pocket but does not inhibit phosphorylation of p38 by upstream kinases. The upregulated p38 phosphorylation in Tgfb2 mutant cells is partially reversed after treatment with SB203580 (Supplemental Figure 10), suggesting that p38 activation may be attributed in part to p38 autophosphorylation caused by a feedback mechanism or other stimuli (43), but mainly results from the TAK1 cascade. Collectively, our data suggest that the TRAF6/TAK1 pathway contributes to 14-3-3 phosphorylation through the activation of p38 MAPK.

14-3-3 proteins comprise a large family of highly conserved acidic polypeptides (44, 45). Multiple isoforms of 14-3-3 proteins play an essential role in regulating cell proliferation and differentiation and can also interact with a wide variety of proteins related to signal transduction, cell cycle control, vesicular transport, and more (46). 14-3-3 interacts with TβRI and positively regulates TGF-β signaling through TβRI (47). 14-3-3 may thus modulate TGF-β signaling via protein interactions in both SMAD-dependent and -independent pathways and regulate gene expression related to cell proliferation during palateogenesis. These functions will be addressed in future studies.

Our findings that elevated TGF-β2 and ectopic p38 MAPK activation is the molecular mechanism responsible for adversely affecting cell proliferation in the CNC-derived palatal mesenchyme and that reduction of Tgfb2, Tgfr1/−/−Alk5, or Tak1 rescues cleft palate in Tgfr2β/−;Wnt1-Cre mice have the potential for long-term clinical applications. We propose that elevated TGF-β2 and ectopic TAK1/p38 MAPK activation could serve as biomarkers for identifying individuals with an increased risk of having a child with congenital craniofacial malformations. In addition, our molecular mechanisms could be useful for interpreting the results of large-scale population-based genetic studies aimed at identifying inherited risk factors for craniofacial malformations (48, 49). Finally, in the longer term, our results could prove useful in efforts to identify safe and effective maternal nutritional and therapeutic interventions that reduce the risk of offspring developing these congenital malformations.

Methods

Animals. Wnt1-Cre mice were obtained from The Jackson Laboratory and crossed to Tgfr2β/−/− mice (a gift from Harold L. Moses, Vanderbilt University, Nashville, Tennessee, USA). To generate Tgfr2β/−;Wnt1-Cre;Tgfb2−/− mice, we mated Tgfr2β/−;Wnt1-Cre;Tgfb2−/− mice with Tgfr2β/−/− mice. Tgfr2β−/− mice were gifted by Thomas Doetschman (University of Cincinnati, Cincinnati, Ohio, USA). To generate Tgfr2β/−;Wnt1-Cre;Alk5β/−/− mice, we mated Tgfr2β/−;Wnt1-Cre;Alk5β/−/− mice with Tgfr2β/−/−;Alk5β/−/− mice. Alk5β/−/− mice were provided by Michael D. Schneider (Baylor College of Medicine, Houston, Texas, USA). Genotyping was performed using PCR primers, as described previously (13, 50).

Histological examination. Hematoxylin and eosin staining and BrdU staining were performed as described previously (13, 52). Immunohistochemical staining was performed as described previously (53). Antibodies used for immunohistochemistry were anti-β-spectrin rabbit polyclonal (Abcam) and anti-14-3-3ζ rabbit polyclonal antibodies (Abcam). Fluorescence images were obtained using a fluorescence microscope (model IX71, Olympus).
Figure 7
Rescue of cleft palate in Tgfbr2<sup>−/−</sup>;Wnt1-Cre mice via reduction of TGF-β receptor type I. (A) Morphologies of newborn Tgfbr2<sup>−/−</sup>;Alk5<sup>−/−</sup> (control), Tgfbr2<sup>−/−</sup>;Wnt1-Cre;Alk5<sup>−/−</sup>, Tgfbr2<sup>−/−</sup>;Wnt1-Cre;Alk5<sup>+/−</sup>, and Tgfbr2<sup>−/−</sup>;Wnt1-Cre;Alk5<sup>−/−</sup> mice. The bottom row shows the macroscopic appearance of palates at the newborn stage. Arrowheads show calvaria defects. Arrows show cleft palate, and open arrows show normal palates. Palates were scored as normal or cleft at birth. (B) Hematoxylin and eosin staining of sections of Tgfbr2<sup>−/−</sup>;Alk5<sup>−/−</sup> (control), Tgfbr2<sup>−/−</sup>;Wnt1-Cre;Alk5<sup>−/−</sup>, Tgfbr2<sup>−/−</sup>;Wnt1-Cre;Alk5<sup>−/−</sup>, and Tgfbr2<sup>−/−</sup>;Wnt1-Cre;Alk5<sup>−/−</sup> palates at E14.0 and E14.5. Arrows indicate palate. Scale bar: 50 μm. (C) Whole-mount Alcian blue–Alizarin red skeletal staining of Tgfbr2<sup>−/−</sup>;Alk5<sup>−/−</sup> (control), Tgfbr2<sup>−/−</sup>;Wnt1-Cre;Alk5<sup>−/−</sup>, Tgfbr2<sup>−/−</sup>;Wnt1-Cre;Alk5<sup>−/−</sup>, and Tgfbr2<sup>−/−</sup>;Wnt1-Cre;Alk5<sup>−/−</sup> newborn mice. Dotted lines indicate the palatal process of maxilla and palatine bones. (D) Immunoblotting analysis of E14.5 Tgfbr2<sup>−/−</sup>;Wnt1-Cre (lane 1), Tgfbr2<sup>−/−</sup> control (lane 2), and Tgfbr2<sup>−/−</sup>;Wnt1-Cre;Alk5<sup>−/−</sup> (lane 3) palates. (E) BrdU staining of Tgfbr2<sup>−/−</sup> control, Tgfbr2<sup>−/−</sup>;Wnt1-Cre, and Tgfbr2<sup>−/−</sup>;Wnt1-Cre;Alk5<sup>−/−</sup> mice at E14.0. Scale bar: 50 μm. (F) Quantification of the number of BrdU-labeled nuclei in the palate of Tgfbr2<sup>−/−</sup> control, Tgfbr2<sup>−/−</sup>;Wnt1-Cre, and Tgfbr2<sup>−/−</sup>;Wnt1-Cre;Alk5<sup>−/−</sup> mice at E14.0. Three samples were analyzed for each experiment. Error bars represent SD. ***P < 0.001.
Primary MEPM cells and transfection. Primary MEPM cells were obtained from E13.5 embryos. Briefly, palatal shelves were dissected at E13.5 and trypsinized for 30 minutes at 37°C in a CO₂ incubator. After pipetting thoroughly, cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum supplemented with penicillin, streptomycin, l-glutamate, sodium pyruvate, and nonessential amino acids. MEPM cells were treated with or without p38 MAPK inhibitor SB203580 (1 μM), TAK1 inhibitor (5Z)-7-Oxozeaenol (80 nM), or TβR1 kinase inhibitor A83-01 (120 nM) for 24 hours. MEPM cells were treated with TGF-β2 (10 ng/ml) for the indicated time. MEPM cells were cultured with NAb for TGF-β2 (2 μg/ml) or TβRIII (2 μg/ml) for 24 hours. pCMV5B-Tgfb2, His-Tgfb1, or mFRP-Tgfb3 (addgene) was transiently introduced into MEPM cells using Lipofectamine LTX Plus (Invitrogen) according to standard procedures. The cells were lysed 48 hours later, and then the lysates were analyzed by immunoblotting, as described previously (53). For 14-3-3ζ, phosphorylated 14-3-3, and β-spectrin staining, MEPM cells were fixed and stained with anti-14-3-3ζ/b (Abcam), phosphorylated 14-3-3 (Affinity BioReagents), and β-spectrin (Abcam) antibodies as described previously (53). All fluorescence images were obtained using a fluorescence microscope (model IX71, Olympus). Pictures were taken using MicroSuite Analytical Suite software (Olympus).

Immunoblotting analysis. Immunoblots were performed as described previously (54, 55). Antibodies used for immunoblotting were as follows: rabbit polyclonal antibodies against TβRII, p38, JNK, phosphorylated JNK, phosphorylated SMAD1/5/8, phosphorylated SMAD1/5, TAK1, phosphorylated TAK1 (Ser412), and TAB1 (all from Cell Signaling Technology); SMAD1/5/8, TRAF6, 14-3-3ζ, TβRII, and FRP-tag (all from Abcam); phosphorylated 14-3-3ζ (Affinity BioReagents); Lys63-specific ubiquitin (Millipore); BMPRII and 14-3-3ζ (both from Cell Signaling Technology); and mouse monoclonal antibodies against phosphorylated p38 and phosphorylated SMAD2/3 (BD Biosciences), mouse monoclonal antibodies against SMAD4 and TβRIII (both from Santa Cruz Biotechnology Inc.); mouse monoclonal antibodies against β-spectrin and TGF-β2 (both from Abcam), mouse monoclonal antibody against His-tag (Invitrogen), and mouse monoclonal antibody against GAPDH (Chemicon).

Immunoprecipitation. Extracts in cell lysis buffer containing 10 mM Tris (pH 7.5), 100 mM NaCl, 1% Triton X-100, and protease inhibitor cocktail (Complete, Invitrogen) were lysed by sonication. Lysates containing an equal amount of protein (total volume 1 ml) were centrifuged at 1,000 g for 5 minutes at 4°C to remove debris. The supernatant was precleared with 10 μl pure goat anti-rabbit or anti-mouse IgG gel and specific immobilized antibodies (EY Laboratories Inc.). 10 μl of beads and 2 μg of anti-TβRI, TRAF6 (both from Abcam), TβRIII, β-spectrin (both from Santa Cruz Biotechnology Inc.), TAK1, or His-tag (both from Cell Signaling Technology) antibody were then added to the lysate, and the mixture was rotated for 12 hours at 4°C. The immunoprecipitate/gel complex was washed 5 times with ice-cold cell lysis buffer. The complex was boiled for 5 minutes in SDS sample buffer in the presence of β-mercaptoethanol to elute proteins and centrifuged at 1,000 g for 5 minutes at 4°C. For the ubiquitin assay, cell lysates were heated in the presence of 1% SDS and β-mercaptoethanol to disrupt noncovalent protein-protein interaction and then diluted with lysis buffer (1:10). The supernatant was subjected to SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and analyzed by immunoblotting with indicated antibodies (see the legend for Figure 3).

Affinity cross-linking. Cells were incubated with 125I-labeled TGF-β2 (1 ng/ml) in a binding buffer (128 mM NaCl, 5 mM KCl, 1.2 mM CaCl₂, 5 mM MgSO₄, 50 mM HEPES buffer [pH 7.4]). After incubation for 30 minutes on ice, cross-linker solution (200 μM disuccinimidyl suberate in binding buffer) was added for 30 minutes on ice. The reaction was stopped with a detachment buffer (0.25 M sucrose, 1 mM EDTA, 10 mM Tris buffer [pH 7.4]). The precipitate was dissolved in SDS-PAGE sample buffer, boiled for 5 minutes, and subject to SDS-PAGE analysis.

Quantitative RT-PCR. Total RNA was isolated from mouse embryonic palates dissected at each developmental stage with the QiAshredder and RNasy Mini extraction kit (QIAGEN), as described previously (54). PCR primers were purchased from Santa Cruz Biotechnology Inc. Two-tailed Student’s t test was applied for statistical analysis of qPCR data. A P value of less than or equal to 0.05 was considered statistically significant. For all graphs, data are represented as mean ± SD.

siRNA transfection. MEPM cells (2 × 10⁶ cells) were plated in a 6-well cell culture plate until the cells reached 60%-80% confluence. siRNA duplex and reagents were purchased from Invitrogen and Santa Cruz Biotechnology Inc., respectively. siRNA mixture in transfection medium was incubated with cells for 7 hours at 37°C in a CO₂ incubator, as described previously (54). A total of 5 × 10⁶ cells was cultured for 30 minutes with or without TGF-β2 (10 ng/ml).

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 (13). After 24 hours in culture treated with TAK1 inhibitor (SZ)-7-Oxozeaenol (80 nM), TβRII kinase inhibitor A83-01 (120 nM) or SB431542 (1 μM), p38 MAPK inhibitor SB203580 (10 μM), or NAB for TGF-β2 (20 μg/ml) or TβRIII (20 μg/ml), palates were harvested, fixed in 4% paraformaldehyde/0.1 M phosphate buffer (pH 7.4), and processed. At least 3 samples were analyzed for each experiment. Error bars represent SD.

Whole-mount skeletal staining. The 3-dimensional architecture of the skeleton was examined using a modified whole-mount Alcian blue–Alizarin red S staining protocol, as previously described (13, 54).

Statistics. Two-tailed Student’s t test was applied for statistical analysis. For all graphs, data represent mean ± SD. A P value of less than 0.05 was considered statistically significant.

Study approval. All mouse experiments were conducted in accordance with a protocol approved by the Department of Animal Resources and the Institutional Animal Care and Use Committee of the University of Southern California.

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