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# Tak1, Smad4 and Trim33 redundantly mediate TGF-β3 signaling during palate development

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ABSTRACT

Transforming growth factor-beta3 (TGF-β3) plays a critical role in palatal epithelial cells by inducing palatal epithelial fusion, failure of which results in cleft palate, one of the most common birth defects in humans. Recent studies have shown that Smad-dependent and Smad-independent pathways work redundantly to transduce TGF-β3 signaling in palatal epithelial cells. However, detailed mechanisms by which this signaling is mediated still remain to be elucidated. Here we show that TGF-β activated kinase-1 (Tak1) and Smad4 interact genetically in palatal epithelial fusion. While simultaneous abrogation of both *Tak1* and *Smad4* in palatal epithelial cells resulted in characteristic defects in the anterior and posterior secondary palate, these phenotypes were less severe than those seen in the corresponding *Tgfb3* mutants. Moreover, our results demonstrate that Trim33, a novel chromatin reader and regulator of TGF-β signaling, cooperates with Smad4 during palatogenesis. Unlike the epithelium-specific *Smad4* mutants, epithelium-specific *Tak1:Smad4*- and *Trim33:Smad4*-double mutants display reduced expression of *Mmp13* in palatal medial edge epithelial cells, suggesting that both of these redundant mechanisms are required for appropriate TGF-β signal transduction. Moreover, we show that inactivation of Tak1 in *Trim33:Smad4* double conditional knockouts leads to the palatal phenotypes which are identical to those seen in epithelium-specific *Tgfb3* mutants. To conclude, our data reveal added complexity in TGF-β signaling during palatogenesis and demonstrate that functionally redundant pathways involving Smad4, Tak1 and Trim33 regulate palatal epithelial fusion.



## Introduction

Cleft palate, one of the most common birth defects in humans, is caused by a failure in palatogenesis (Chai and Maxson, 2006). During mammalian development, the secondary palate, which separates the oral cavity from the nasal cavity, develops as bilateral outgrowths (palatal shelves) of the maxillary processes of the first pharyngeal arch (Bush and Jiang, 2012). Palatal shelves first grow vertically down along the sides of the tongue; then they rapidly elevate and fuse in the midline (Ferguson, 1987). Failure in any of these three processes can result in cleft palate. The palatal shelf growth and patterning are governed by complex interactions between the ectoderm-derived epithelium, and the underlying mesenchyme derived from the cranial neural crest. One of the later events in palatogenesis, albeit a critical one, is epithelial fusion. During this process, the medial edge epithelium (MEE) in tips of the opposing palatal shelves first forms the midline seam, which subsequently disappears. Many studies, both in humans and mice, have shown that signaling initiated by TGF-β3 is required for successful epithelial fusion (Dudas et al., 2007).

TGF-β3 binds and activates a heterotetrameric receptor complex composed of two type II and two type I receptors. Ligand-receptor interactions result in phosphorylation of TGF-β R-Smads 2 and 3 and subsequent complex formation with a common Smad (Co-Smad or Smad4). R-Smad/Co-Smad complexes then accumulate in the nucleus, where they function as transcriptional co-regulators (Shi and Massague, 2003). This so called canonical (or

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Smad-dependent) signaling is regulated by many proteins interacting either with receptor complexes or Smads, e.g., SARA, Axin, inhibitory Smads (I-Smads) and Trim33 (Derynck and Zhang, 2003; Lamouille et al., 2014; Massague and Xi, 2012). Previous studies have shown that Trim33 (Tif1γ, ectodermin) may regulate TGF-β superfamily signaling in different ways depending on the biological context. Dupont et al. suggested that Trim33 is a negative regulator of TGF-β signaling by functioning as a mono-ubiquitin ligase capable of disrupting activated R-Smad/Co-Smad complexes, while He et al. showed that Trim33 can bind to activated R-Smads in competition with Smad4 and mediate distinct TGF-β signaling processes (He et al., 2006). It also has been suggested that Trim33/R-Smad complexes can function as chromatin readers by making target genes accessible to R-Smad/Co-Smad complexes or by controlling the time the Smad complexes are bound to promoter sequences. In addition to the canonical (Smad-dependent) pathway, TGF-βs can also trigger non-canonical (Smad-independent) signaling processes leading to activation of various downstream mediators, such as small rho-related GTPases, TGF-β-activated kinase-1 and downstream map kinase cascades including p38 Mapk, and Ikk-α (Derynck and Zhang, 2003).

*Tgfb3* is strongly and specifically expressed in the MEE (Fitzpatrick et al., 1990; Pelton et al., 1990), and mouse embryos deficient in *Tgfb3,* as well as epithelium-specific *Tgfbr1* or *Tgfbr2* mutants suffer from defective palatal epithelial fusion (Dudas et al., 2006; Kaartinen et al., 1995; Proetzel et al., 1995; Xu et al., 2006). Xu et al. previously showed that, at least in palatal explant cultures in vitro, Smad-dependent (Smad4-mediated) and Smad-independent (p38Mapk-mediated) pathways act redundantly during palatal epithelial fusion (Xu et al., 2008). Here, we show that cooperation between different TGF-β downstream mediators is even more extensive. By using tissue-specific mouse mutants in conjunction with a whole-head roller culture assay, we demonstrate that both *Tak1:Smad4*- and *Trim33:Smad4*-double conditional mutants display specific palatal fusion defects, and that simultaneous deletion/inactivation of all three proteins in palatal epithelial cells results in palatal phenotypes typically seen in epithelium-specific *Tgfb3* mutants.

## Experimental procedures

### *Mice*

*Tgfb3*$^{F}$, *Tak1*$^{F}$ *Ikka*$^{F}$ and *Trim33*$^{F}$ mice have been described earlier (Doetschman et al., 2012; Kim and Kaartinen, 2008; Liu et al., 2008; Yumoto et al., 2013). *K14-Cre* and *Smad4*$^{F}$ mice were obtained from S. Millar (Andl et al., 2004) and C. Deng (Yang et al., 2002), respectively. To generate mutant embryos, Cre-positive male mice heterozygous for floxed (F) gene(s) were crossed with female mice carrying corresponding homozygous floxed allele(s) (see Table 1). For timed matings, the presence of a vaginal plug was designated as embryonic day 0 (E0). DNA for genotyping was prepared from tail tissues using DirectPCR lysis reagents (Viagen Biotech). Mouse lines were maintained in mixed genetic backgrounds. All experiments involving the use of animals were approved by the Institutional Animal Use and Care Committee at the University of Michigan-Ann Arbor (Protocol #00004320).

**Table 1**
Crosses used to generate mutant embryos

| Male | Female |
|---|---|
| *Tgfb3*$^{FWT}$:*K14Cre*$^{+}$ | *Tgfb3*$^{FF}$ |
| *Smad4*$^{WT}$:*K14Cre*$^{+}$ | *Smad4*$^{FF}$ |
| *Tak1*$^{FWT}$*Smad4*$^{FWT}$:*K14Cre*$^{+}$ | *Tak1*$^{FF}$*Smad4*$^{FF}$ |
| *Ikka*$^{FWT}$*Smad4*$^{FWT}$:*K14Cre*$^{+}$ | *Ikka*$^{FF}$*Smad4*$^{FF}$ |
| *Trim33*$^{FWT}$*Smad4*$^{FWT}$:*K14Cre*$^{+}$ | *Trim33*$^{FF}$*Smad4*$^{FF}$ |

### *Histology, immunohistochemistry and cell death assays*

Embryos were collected into sterile DPBS and fixed at 4 °C overnight in freshly prepared 4% paraformaldehyde in PBS. Samples used for wax embedding were washed, dehydrated through a graded ethanol series (20, 50, 70, 95 and 100%) and an overnight step in 50% Ethanol/50% Toluene, one hour step in 100% toluene, one hour step in 50% toluene/50% fresh Blue Ribbon Tissue Embedding/Infiltration Medium (Leica Surgipath) before being oriented and embedded in fresh Blue Ribbon Tissue Embedding/Infiltration Medium (Leica Surgipath) after three changes. Seven μm sections (histology and immunohistochemistry) were cut, mounted on Superfrost plus slides (Fisher) and stained with hematoxylin and eosin according to standard protocols. Fusion% was calculated as described by (Sun et al., 1998). Briefly, the length of confluence was divided by total length of adherence and multiplied by 100. For immunohistochemistry, sections were rehydrated, and after antigen retrieval (5–20 min at 95–100 °C in 10 mM citrate buffer, pH6.0) proliferating cells were detected using Ki67 antibody (#M7249; Dako). Cells positive for phosphorylated p38Mapk were detected using p-p38 Mapk antibody (#4511; Cell Signaling). Antibody binding was visualized with Alexa Fluor 594 secondary antibody (Life Technologies). Apoptotic cells were detected using a TUNEL assay (Dead End, Promega) following manufacturer's instructions. Fluorescent images were viewed on an inverted fluorescent Leica DMI3000B microscope and documented using an Olympus DP72 camera.

### *In situ hybridization*

Embryos were processed and embedded in paraffin as described above. 10μm sections were cut and mounted on glass slides. RNA probes were labeled using a DIG-labeled NTP mix (Roche Applied Science) according to manufacturer's instructions, stored at −80 °C and diluted in hybridization buffer. Section ISH was performed as described (Moorman et al., 2001). After staining, sections were fixed and mounted in Immu-Mount (Thermo Scientific). Probe templates for *Tgfb3* and *Mmp13* were prepared as described (Blavier et al., 2001; Dudas et al., 2004).

### *Real-time quantitative PCR*

Tissues were harvested from tips of palatal shelves from E14 and E15 mouse embryos, placed into 200 μl of RLT (RNeasy mini kit, Qiagen), and RNAs were isolated by using RNeasy columns (Qiagen). cDNAs were synthesized by using Omniscript reverse transcriptase (Qiagen) according to the manufacturer's protocols. Real time quantitative PCR experiments were done either by using Universal Probe library-based assays (Roche Applied Science) or by using TaqMan assay reagents (Applied Biosystems) (see Table 2). 30 μl assays were quantified using Applied Biosystems ABI7300 PCR and ViiA7 detection systems and software. Data were normalized to β-actin mRNA levels using the $2^{-\Delta\Delta Ct}$ method.

### *Whole-head roller culture assays*

Roller culture assays were modified from (Goudy et al., 2010). Heads from E15 embryos were collected in DPBS and mandibles, tongues, and brains were removed. The resulting mid-face samples were cultured for 24 h at 37 °C in roller bottles (60 ml serum bottles) (60 rotations/minute) in serum-free BGJb medium without penicillin and streptomycin. The bottles were gassed at the

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