

**MOLECULAR AND CELLULAR MECHANISMS UNDERLYING THE CLEFT
PALATE PHENOTYPE OF *TRPS1* MUTANT MICE**

by

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MOLECULAR AND CELLULAR MECHANISMS UNDERLYING THE CLEFT PALATE PHENOTYPE OF *TRPS1* MUTANT MICE

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University of Pittsburgh School of Dental Medicine, 2018

Trichorhinophalangeal syndrome (TRPS) is an autosomal dominantly inherited condition caused by heterozygous mutations of the *TRPS1* gene. This gene codes for the GATA transcriptional factor TRPS1. Patients with TRPS exhibit multiple skeletal, hair, dental and craniofacial defects, including cleft palate. Using mouse models, one of the goals of this study is to characterize the skeletal abnormalities of *Trps1*-deficient mice. Skeletal staining using Alcian blue/Alizarin red revealed apparent underdevelopment of the zygomatic arch, sternum, vertebrae and anterior cranial base in *Trps1*^{-/-} mice. We also found that the nose and mandible of *Trps1*^{-/-} mice were significantly shorter. Additionally, cleft palate was detected in *Trps1*^{-/-} mice. In order to understand the role of *Trps1* in palatogenesis, immunohistochemistry was used to delineate the expression pattern of *Trps1* protein in wildtype (WT) mouse tissues. We demonstrated that *Trps1* was expressed in palatal shelf mesenchyme and epithelium, specifically at the medial edge epithelium. Along the anterior-posterior axis, epithelial *Trps1* signal appeared to be increased in the posterior region of the palate. Lack of fusion observed in *Trps1*^{-/-} mouse palatal shelves led us to examine proteins involved in the fusion process. Thus, immunohistochemistry was used to compare the expression of *Tgfβ3*, *Twist1*, and β -catenin in WT and *Trps1*^{-/-} mice. *Tgfβ3*, β -catenin and *Twist1* were all expressed in WT palatal epithelium as well but were downregulated in *Trps1*^{-/-} palatal epithelium. In summary, *Trps1* plays a vital role in proper skeletal and craniofacial development, including palatal shelf fusion. *Trps1* is also involved in the regulation of other proteins required for palatal fusion.

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PREFACE

I would like to thank Daisy Monier, Dr. Yong Wan and Brandi Lantz (School of Dental Medicine University of Pittsburgh) for technical assistance and Elda Munivez (Baylor College of Medicine) for assistance with processing tissue samples used in this study. Funding was provided by National Institute of Dental and Craniofacial Research of the National Institutes of Health (award number R01DE023083 to D.N.) and by the Center for Craniofacial Regeneration and Department of Orthodontics, School of Dental Medicine University of Pittsburgh.

1.0 INTRODUCTION

1.1 CLEFT LIP AND/OR PALATE

Cleft lip and/or palate (CL/P) is one of the most common birth defects in North America and it occurs in approximately 1 out of every 700 live births (Dixon et al. 2011). According to the Centers for Disease Control and Prevention (CDC), there are approximately 4440 new cases of CL/P per year in the United States (Parker et al. 2010). CL/P may occur bilaterally or unilaterally with a predilection for the left side (Mastroiacovo et al. 2011). It may also present as an isolated defect or as part of another disorder, thus referred to as non-syndromic or syndromic CL/P, respectively.

The emotional stress endured by CL/P patients and their families can be dramatic. This issue was highlighted in a study showing that 30% of mothers of affected children have contemplated suicide (Natsume et al. 2013). The treatment of CL/P involves multiple surgeries to establish proper function and esthetics, which can further add onto the financial and psychological burden of the patients and their families. Furthermore, the costs inflicted on the health care industry are substantial as the CDC estimated that the lifetime expense of treating CL/P patients per year is \$679 million (National Institute of Dental and Craniofacial Research 2018). Therefore, it is imperative that research in this field focuses on optimizing the management of patients with orofacial clefts in order to minimize these stresses.

The care of patients with CL/P is complex and involves numerous sessions with a variety of specialists, including but not limited to oral surgeons, orthodontists, pediatrics, prosthodontists, psychologists, plastic surgeons, and speech therapists. These patients often must undergo multiple surgical procedures starting from infancy to adulthood. The typical treatment protocol includes early lip repair within the first postnatal year and palatal reconstruction before speech develops at approximately 12 to 18 months. Placement of bone graft at the cleft site should be delayed until the adjacent teeth are ready to erupt, typically at 7 to 9 years old. Orthodontic treatment is often required to correct any malocclusion and prosthetic replacement of missing teeth may be necessary. Severe skeletal discrepancies are corrected with orthognathic surgery near the end of the patient's growth at around 15 to 18 years old and final soft tissue revisions, including rhinoplasty, is done at the final stage after the completion of nasal growth (American Cleft Palate-Craniofacial Association 1993).

Currently, the gold standard for CL/P treatment remains surgical repair. However, the procedure can be physically and emotionally demanding as we have previously discussed. There are also co-morbidities associated with autogenous bone grafts in surgical cleft repair, including pain, infection, bleeding, nerve damage, and donor bone fracture (Tavakolinejad et al. 2014). Other therapeutic options that have been explored include tissue engineering using stem cells from bone marrow or adipose tissue, which has significantly less morbidities than traditional grafting techniques (Gimbel et al. 2007; Panetta et al. 2008; Pourebrahim et al. 2013). More novel approaches involve *in utero* molecular therapy to deliver signaling molecules to mouse embryos for prenatal cleft palate correction (Wu et al. 2013; Jia et al. 2017). The innovation of molecular therapies aimed at preventing cleft palates before birth can significantly reduce the

financial and social burdens discussed and has great potential in advancing the craniofacial regeneration field.

1.2 PALATOGENESIS

In order to gain insight on these molecular therapies, one must first understand the process of normal palatal development, also known as palatogenesis. Palatogenesis has been studied extensively using mouse models because mouse and human palatal development are highly comparable. During normal development, the primary and secondary palates arise from the medial nasal and maxillary process, respectively. These early stage palatal shelves grow vertically downwards on either side of the tongue (Figure 1.1A). As development progresses, the palatal outgrowths elevate and reorient themselves horizontally above the tongue (Figure 1.1B). Following palatal shelf elevation, cell proliferation allows the medial edge epithelium (MEE) of the two palatal shelves to approximate each other at the midline. Once the palatal shelves make contact, cell adhesion occurs and a resultant midline epithelial seam (MES) is formed (Figure 1.1C). The MES subsequently disintegrates to allow for mesenchymal confluency in a process termed palatal fusion. There are three primary cellular mechanisms thought to be responsible for palatal shelf fusion: epithelial cell apoptosis, migration and transition to the mesenchymal state via the epithelial-to-mesenchyme transition (EMT) process (Bush and Jiang 2012). A disruption at any stage of this complicated process can result in a cleft palate.

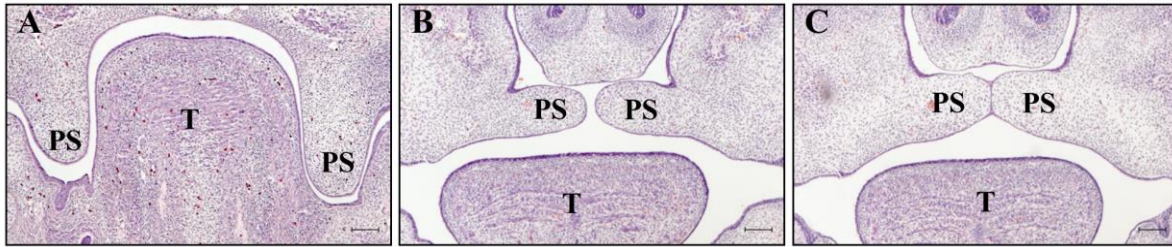


Figure 1.1. Stages of palatogenesis in mouse embryo. A) Vertical palatal shelf growth. B) Palatal shelf elevation. C) Formation of midline epithelial seam. *Scale bar 100 μ m. PS- palatal shelf; T- tongue.* (unpublished)

1.3 GENETIC REGULATION OF PALATOGENESIS

Palatogenesis may be disrupted by a multitude of genetic and environmental factors. This complex process is tightly controlled by numerous interactions between epithelial and mesenchymal cells within the palatal shelves. Several signaling pathways are involved at each stage of development, including sonic hedgehog (Shh), fibroblast growth factor (Fgf), bone morphogenetic protein (Bmp), transforming growth factor beta (Tgf β), Twist1, and Wnt/ β -catenin pathway. Shh is expressed specifically in oral epithelium and promotes cell proliferation and palatal outgrowth via its receptor Patched 1 (Ptc) (Rice et al. 2006; Lan and Jiang 2009). Fgf10 and Bmp2 both regulate cell proliferation within the palatal mesenchyme and also maintain a positive feedback loop with Shh to stimulate palatal outgrowth (Zhang et al. 2002; Rice et al. 2004; Lan and Jiang 2009; Baek et al. 2011). Tgf β and Twist1 are key players in palatal fusion by regulating MES apoptosis and EMT (Martinez-Alvarez et al. 2000; Xu et al. 2006; Yu et al. 2008). Finally, the Wnt/ β -catenin pathway has been implicated in palatal shelf elevation and palatal fusion through its regulation of other signaling molecules (Yu et al. 2010; He et al. 2011). These examples serve to illustrate the intricate level of cross-communication

between multiple signaling pathways and between epithelial and mesenchymal cells to ensure proper palatal development. Amongst all the cellular mechanisms at play, an interruption at any level can lead to a cleft palate. By identifying the defective pathways, it is possible to work towards an intervention targeting specific pathways to recover lost cellular function.

1.3.1 Epithelial-to-mesenchyme transition (EMT) regulation

Epithelial-to-mesenchymal transition (EMT) is a cellular process that occurs during both normal development and pathological conditions where an epithelial cell transforms into a mesenchymal cell. However, this process is reversible through mesenchymal-to-epithelial transition (MET). Epithelial cells are tightly connected to each other through gap junctions while mesenchymal cells are loosely organized. Therefore, the EMT process requires epithelial cells to lose their adhesive characteristics and acquire the ability to migrate (Thiery et al. 2009). This is important for palatogenesis because EMT disrupts the integrity of the MES, thus resulting in degradation of the MES for successful palatal fusion.

The suppression of E-cadherin, one of the most common adhesion molecules, disrupts epithelial integrity and is thus the hallmark of EMT (Thiery et al. 2009). E-cadherin is mainly expressed in epithelial cells, including the palatal shelf MEE during development (Montenegro et al. 2000). Tgfb β is known to promote EMT by regulating proteins that inhibit E-cadherin expression, including Snail, Twist1, and Irf6 (Yu et al. 2008; Jalali et al. 2012; Ke et al. 2015). Studies carried out in mouse palates have shown that these proteins are closely related to each other. For example, Snail and Twist expressions are positively correlated with each other (Pungchanchaikul et al. 2005). In addition, Irf6 promotes the expression of Snail and it also affects epithelial adhesion stability during palatogenesis by forming a complex with E-cadherin

endocytosis regulators called Non-Metastatic Expressed (NME) proteins (Ke et al. 2015; Parada-Sanchez et al. 2017). In summary, several molecular pathways work together to tightly control the EMT process during palatal fusion.

1.3.2 TGF β 3

Tgf β 3 is part of the Tgf β superfamily of growth factors and it plays a key role in palatal development. The association between *TGF β 3* mutation and cleft palate in both humans and animal studies further highlights the importance of this signaling molecule in palatogenesis (Proetzel et al. 1995; Carinci et al. 2007; Rienhoff et al. 2013). This is supported by the fact that mice with a *Tgfb3* knockout mutation presented with complete cleft palates but the phenotype could be rescued with *in utero* injections of viral vectors containing the *Tgfb3* gene into the amniotic sac (Wu et al. 2013). *Tgfb3* is specifically expressed at the MEE during palatal formation (Pelton et al. 1990). Its role during palatal fusion remains unclear but it has been implicated in multiple cellular processes including palatal adhesion, EMT, cell migration, and apoptosis (Proetzel et al. 1995; Martinez-Alvarez et al. 2000; Jalali et al. 2012).

Prior to fusion, the opposing palatal shelves must adhere to each other after making contact to form the MES. Tgf β 3 promotes palatal shelf adhesion via filopodia and the regulation of cell-adhesion molecules such as chondroitin sulfate proteoglycan (CSPG) (Taya et al. 1999; Gato et al. 2002). This is supported by the observation that Tgf β 3-deficient mouse palatal shelves tended to slide off one another instead of adhering properly (Proetzel et al. 1995). On the other hand, some studies have suggested that Tgf β 3 promotes fusion through EMT instead of cell adhesion (Kartinen et al. 1997; Jalali et al. 2012; Ke et al. 2015). Jalali et al. (2012) demonstrated that Tgf β 3 significantly downregulated the expression of *E-cadherin* during

palatogenesis and also increased cell migration of MES cells. The role Tgf β 3 plays during EMT has been highlighted in the previous section. Furthermore, there is evidence to suggest that Tgf β 3 induces apoptosis during palatal fusion. Studies have shown that the absence of Tgf β 3 in mouse palates significantly reduced cell death in the MEE (Martinez-Alvarez et al. 2000; Dudas et al. 2004). Some proposed that Irf6 acts as the mediator in the Tgf β 3 pathway to promote p21-mediated cell death of the MEE (Iwata et al. 2013; Li et al. 2017). In conclusion, the importance of Tgf β 3 for palatal fusion is well-established and it may play multiple roles in this process.

1.3.3 Twist1

Twist1 protein is known to be a key regulator of EMT and is expressed within the MEE during palatogenesis (Yu et al. 2008; Kitase et al. 2011). It functions downstream of Tgf β 3 to promote *Snail1* expression via the PI-3K signaling pathway within the palatal shelf epithelium (Yu et al. 2008; Yu et al. 2013). The proposed mechanism for this is Twist1 recruits E-proteins to stimulate *Snail1* promoter activity, which in turn inhibits *E-cadherin* expression during palatal fusion (Yu et al. 2013). Interestingly, a mutation of *TWIST1* in humans results in Saethre-Chotzen syndrome, a disorder that features cleft palate as well (Gallagher et al. 1993). This further illustrates the significance of Twist1 during palatal development.

1.3.4 β -catenin

β -catenin protein is part of the canonical Wnt signaling pathway, whose role in palatal development has been heavily debated. There is evidence supporting the role of Wnt signaling in palatal shelf elevation, adhesion and fusion. Some studies have shown that the loss of Wnt

receptors Frizzled1 and Frizzled2 resulted in a failure of palatal shelf elevation (Yu et al. 2010). Others argue that since β -catenin is primarily detected in palatal epithelium, it likely functions as a cell adhesion molecule instead (Cobourne 2012). Conversely, there is also support for its role as a signaling mediator because both *Tgfb3* expression and apoptosis were found to be downregulated in the MEE of β -catenin-deficient mice (He et al. 2011). These mutant mice ultimately developed a cleft palate similar to *Tgfb3* knockout mice (He et al. 2011). These findings indicate that β -catenin is responsible for the regulation of *Tgfb3* in palatal shelf epithelium during embryogenesis. There is also a link between β -catenin and Twist, where β -catenin promoted *Twist* expression while Twist downregulated *β -catenin* expression via a negative feedback loop (Yu et al. 2008).

1.4 SYNDROMIC CLEFT LIP AND/OR PALATE

Syndromic CL/P refers to the occurrence of CL/P when it is associated with other disorders such as Pierre Robin, Treacher Collins and Saethre-Chotzen syndrome (Gallagher et al. 1993; Venkatesh 2009). Other common features of these syndromes are hypoplastic midface, mandible and temporomandibular joint (TMJ) (Chang and Steinbacher 2012; Gangopadhyay et al. 2012). The skeletal abnormalities observed in these disorders indicate that the cellular mechanisms involved in skeletal development may involve similar molecular networks as syndromic CL/P pathogenesis. For example, the aforementioned Saethre-Chotzen syndrome is a result of a *TWIST1* mutation, a gene that has been found to be essential for palatal fusion (Yu et al. 2008). In addition to cleft palate, patients with this syndrome also present with skeletal deformities such as craniosynostosis, facial asymmetry, short stature and brachydactyly (Gallagher et al. 1993).

Therefore, it is possible that the proteins that play a vital role in palatal development, including Tgf β 3, β -catenin and Twist1, may also be important in the regulation of skeletal development.

1.5 TRICHORHINOPHALANGEAL SYNDROME (TRPS)

Cleft palate has been reported in rare cases of trichorhinophalangeal syndrome (TRPS) (Morioka et al. 1999; Solc et al. 2017). TRPS is an autosomal dominantly inherited disorder caused by *TRPS1* gene mutations that result in haploinsufficiency of TRPS1, a zinc finger transcription factor that binds to DNA at GATA sequences (Momeni et al. 2000). Momeni et al. (2000) was the first to map the *TRPS1* gene on chromosome 8q24. TRPS is categorized into 3 subtypes: TRPS I, TRPS II (also known as Langer-Giedion syndrome), and TRPS III. TRPS I is a consequence of a nonsense mutation within the *TRPS1* gene that produces a truncated protein with a complete loss of function (Momeni et al. 2000). TRPS II is caused by a deletion of both the *TRPS1* and *EXT1* gene, which is located distal to the *TRPS1* gene on chromosome 8q24 (Hall et al. 1974; Ludecke et al. 1995). Consequently, patients with TRPS II present with a combination of features from TRPS I and multiple exostoses as a result of the additional *EXT1* deficiency (Hall et al. 1974). Finally, TRPS III is considered to be a more severe version of TRPS I that is caused by missense mutations of the *TRPS1* gene (Ludecke et al. 2001).

Phenotypic evaluation of patients and their families revealed that parents of sporadic patients and other healthy relatives do not possess these mutations, which suggests a dominant nature of *TRPS1* mutations (Ludecke et al. 2001). The characteristic clinical presentation of TRPS patients include sparse hair, bulbous nose, short stature, micrognathia, cone-shaped epiphyses of phalanges and dental abnormalities (Giedion 1966; Bennett et al. 1981; Ludecke et

al. 2001; Kantaputra et al. 2008). Some common oral features seen include microdontia, delayed tooth eruption, malocclusion, supernumerary teeth and high arched palate (Bennett et al. 1981; Kantaputra et al. 2008). While these 3 subtypes of TRPS have common clinical features, there is significant clinical variability between them. For example, most patients with TRPS I present with normal intelligence while TRPS II is usually associated with impaired mental development due to a greater mutation size (Brandt et al. 1997; Nardmann et al. 1997). However, mental retardation can also be seen in patients with TRPS I when there is a large chromosomal deletion (Hamers et al. 1990). It is likely that a minimum size of chromosomal deletion is required before mental retardation is observed (Bowen et al. 1985). The skeletal phenotypes are also highly variable between these categories. Compared to type I, patients with TRPS III have more severe brachydactyly and shorter stature (Ludecke et al. 2001). Patients with TPRS II are even shorter than those with type III because of the compounding effects of a deleted *EXT1* gene, which also plays a role in skeletal development (Ludecke et al. 2001). Despite these differences however, the facial phenotype of patients in all subtypes of TRPS remain quite similar (Ludecke et al. 2001).

1.5.1 Role of *Trps1* in embryogenesis

The abnormalities observed in patients with TRPS suggest that the *TRPS1* gene plays a major role during embryonic development. Studies using mouse embryos have shown that *Trps1* is expressed in vital organs such as the brain, kidney and lungs (Kunath et al. 2002). Demonstration of pulmonary and renal defects in *Trps1*-deficient mice along with supporting case reports of TRPS patients experiencing respiratory and renal failure illustrate the importance of *TRPS1* in organogenesis (Lu et al. 1997; Malik et al. 2002; Gai et al. 2009; Tasic et al. 2014). *Trps1* is also

necessary for proper development of hair follicles, tooth bud, cartilage, skeletal elements of long bones, facial bones, ribs and vertebrae (Kunath et al. 2002; Malik et al. 2002; Kantaputra et al. 2008; Napierala et al. 2008). Skeletal staining experiments have supported the presence of thoracic defects in mice with a homozygous mutation of *Trps1* (*Trps1*^{-/-}) by showing that the mutant mice had delayed vertebral ossification, scoliosis and reduced thoracic volume (Malik et al. 2002).

Within the craniofacial region, *Trps1* is highly expressed in the first and second branchial arches during early embryogenesis (E11.5), which ultimately give rise to multiple craniofacial structures (Kunath et al. 2002; Kantaputra et al. 2008). Preliminary data from our lab also showed that in later stages of development (E12.5 - E13.5), *Trps1* expression becomes localized to the subcutaneous nasal region, maxilla, mandible, and tongue (Figure 1.2A-B). When we examined *Trps1* specifically in the secondary palate at E15.5, its expression was found to be confined to the lateral boundaries of the palatal shelves (Figure 1.2D). In summary, *Trps1* is expressed in several regions during embryogenesis, including the oral cavity, and is essential for the development of various organs and skeletal elements. However, data regarding the specific role of *Trps1* in craniofacial development remains limited.

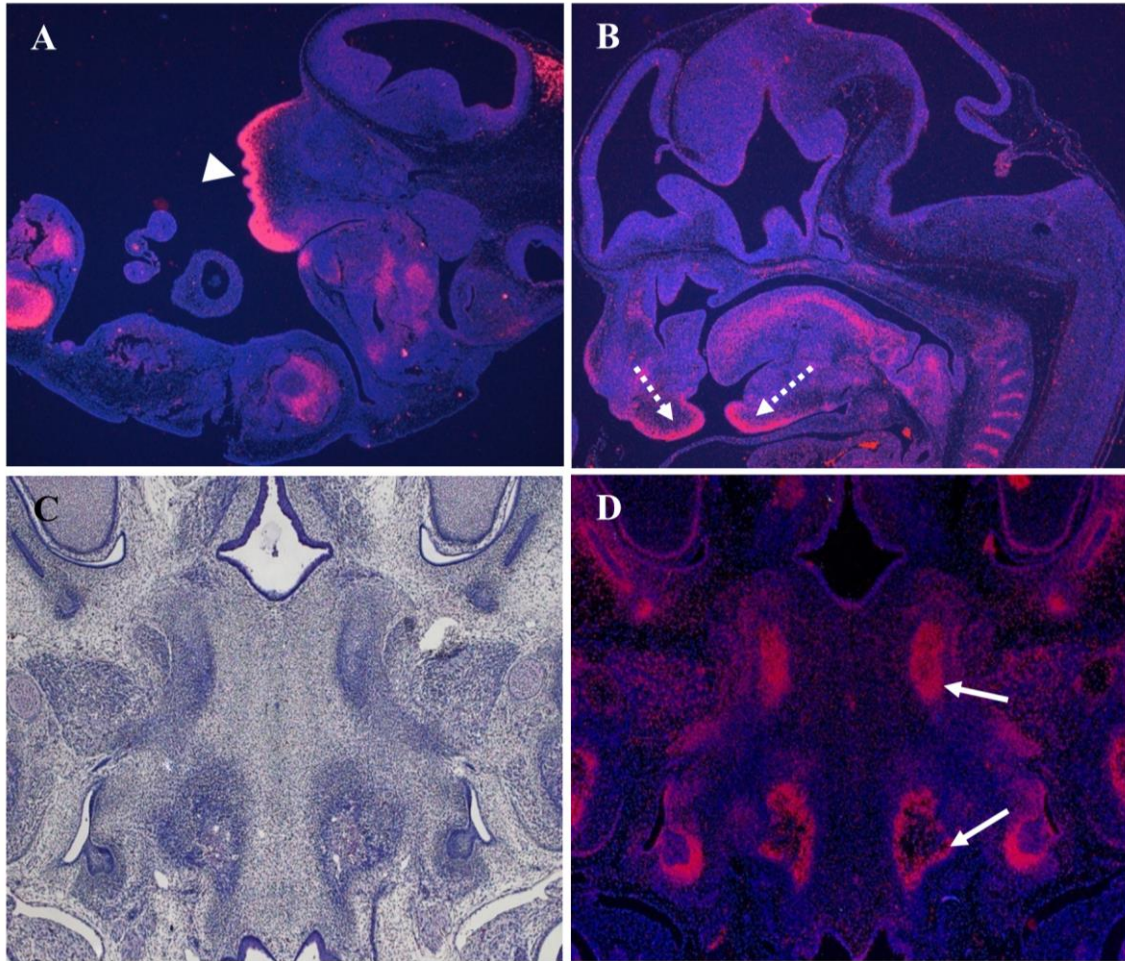


Figure 1.2. *Trps1* expression within craniofacial regions during embryogenesis. A,B) Sagittal view of a developing mouse embryo RNA *in situ* hybridization at E12.5 (A) illustrating *Trps1* expression in subcutaneous snout region (arrowhead) and at E13.5 (B) showing *Trps1* in the maxilla & mandible (dotted arrows), tongue and intervertebral disks. C,D) H&E staining (C) and RNA *in situ* hybridization (D) of E15.5 secondary palate in the transverse view demonstrating *Trps1* expression within the lateral boundaries of the palate (solid arrows). (unpublished)

1.5.2 Cleft palate in *Trps1* mutant mice

TRPS has been studied extensively using mouse models because *Trps1*-deficient mice have craniofacial features that strongly resemble the phenotype of TRPS patients. Mice with a heterozygous *Trps1* mutation (*Trps1*^{+/-}) displayed subtle craniofacial defects such as abnormal palatal arch, shortened mandible and abnormal zygomatic arch (Malik et al. 2002). However, mice with a homozygous *Trps1* mutation (*Trps1*^{-/-}) had more severe malformations including cleft palate (Kantaputra et al. 2008) (Figure 1.3). This suggests that *Trps1*-deficiency has a dose-dependent effect on the extent of the resultant craniofacial deformities.

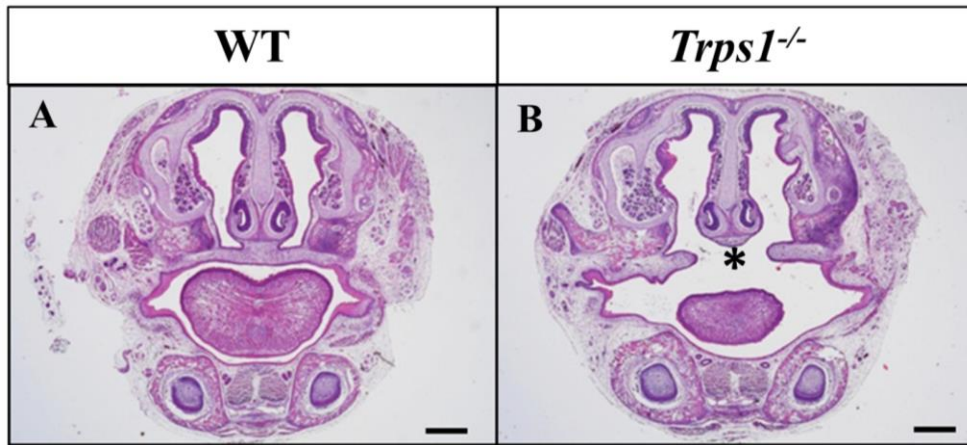


Figure 1.3. Cleft palate phenotype in *Trps1*^{-/-} mice. H&E staining of coronal head sections of E18.5 mice showing A) wildtype (WT) mice underwent normal palatal development while B) *Trps1*^{-/-} mice had cleft palates (*) Scale bar 500 μ m. (unpublished)

A deficiency in *Trps1* has been shown to disturb palatal shelf fusion in previous experiments performed by our laboratory using cultured palatal shelves from *Trps1*^{-/-} mice. After 48 hours of *ex vivo* culture, fusion of all WT palatal shelves occurred (Figure 1.4A) but none of

the *Trps1*^{-/-} palatal shelves initiated the fusion process (Figure 1.4B). Additionally, it appears that palatal adhesion may also be affected in *Trps1*^{-/-} mice because expression of the cell adhesion mediator CSPG was lost on the epithelial surface of mutant palatal shelves and nasal septum (Figure 1.4C-F). Given that palatal shelf adhesion is a prerequisite for palatal fusion, this suggests that *Trps1* may be involved in cellular processes even before to the fusion stage. We noticed that mesenchymal expression of CSPG was not affected in *Trps1*^{-/-} mice, which indicates that *Trps1*-deficiency results in a cell type-specific loss of CSPG in the epithelium. Therefore, this specific loss of CSPG on palatal shelf epithelium surface is likely the reason that *Trps1*^{-/-} mouse palates were unable to initiate fusion.

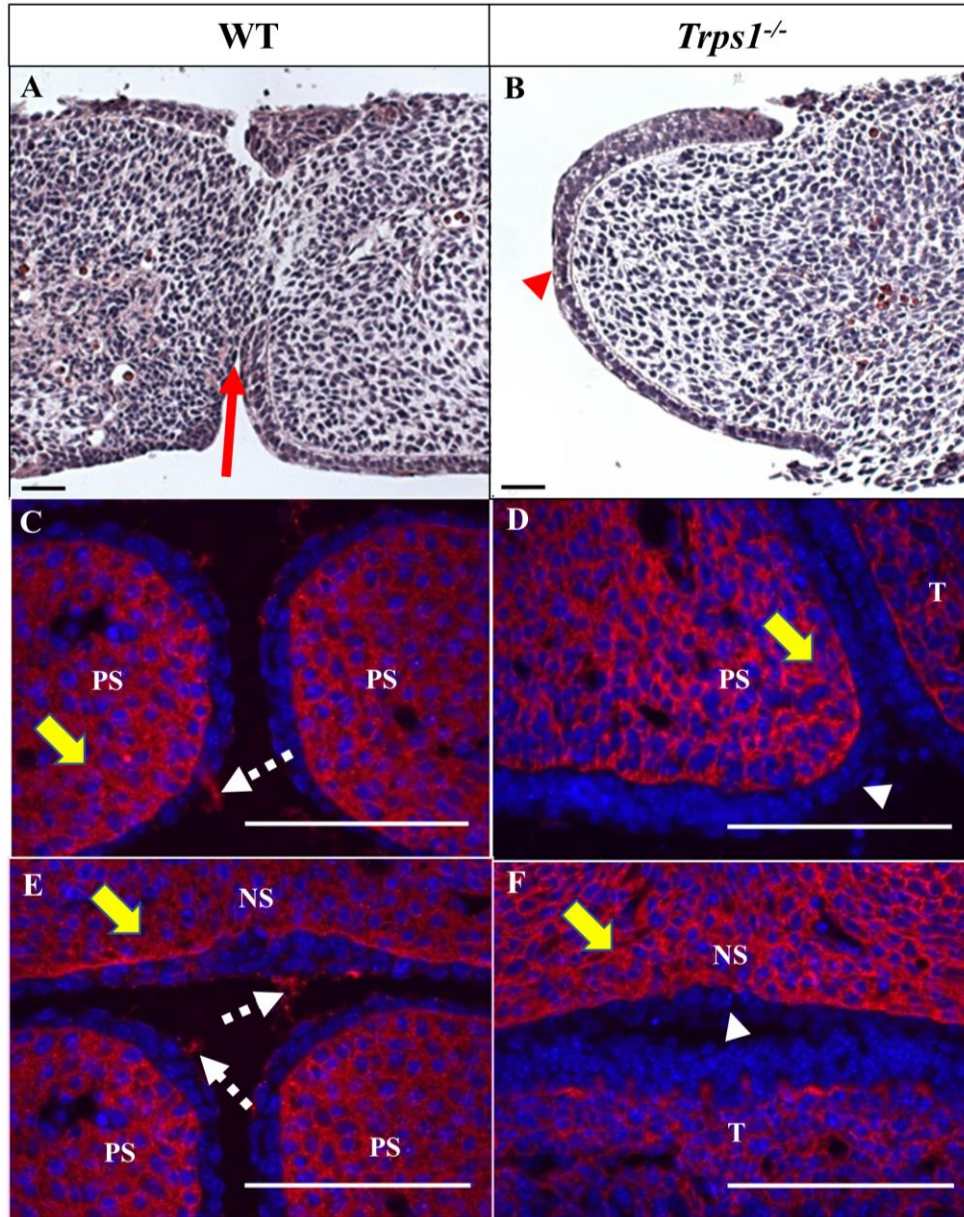


Figure 1.4. *Trps1* is required for the initiation of the palatal shelves fusion and expression of CSPG on palatal and nasal septum surfaces. A,B) H&E staining of cultured palatal shelves from E13.5 WT (A) and *Trps1*^{-/-} (B) mouse embryos. Fusion of palatal shelves was initiated in all WT palatal shelves (arrow, *n*=11) while *Trps1*^{-/-} palatal shelves were unable to initiate fusion (arrowhead, *n*=11). C-F) Immunofluorescent staining for CSPG (red) on E14.5 WT (C,E) and *Trps1*^{-/-} (D,F) mouse palatal shelves. CSPG typically present on the epithelial surface of WT palatal shelves and nasal septum (dotted arrows) were lost in *Trps1*^{-/-} mice (arrowheads), while mesenchymal expression remained unaffected (block arrows). Scale bar 50 μ m. NS- nasal septum, PS- palatal shelf, T- tongue. (unpublished)

2.0 OBJECTIVE OF STUDY

Although the phenotype of TRPS patients clearly indicates that *TRPS1* is involved in skeletal and craniofacial development, the specific role of *TRPS1* in these processes is not well understood. The objectives of this study are: 1) to characterize the craniofacial and skeletal phenotype of *Trps1*^{-/-} mice; 2) to define the expression pattern of *Trps1* during palatogenesis; and 3) to identify dysregulated protein expressions during palatal fusion caused by a *Trps1*-deficiency. Our hypothesis is that a deficiency in *Trps1* will result in craniofacial skeletal defects, including cleft palate, and downregulate the expression of proteins essential for palatal fusion.

3.0 MATERIAL AND METHODS

3.1 MOUSE MODEL

Trps1^{-/-} mice were generated previously by excision of the exon coding for the DNA binding domain of the TRPS1 protein (Malik et al. 2002). These mice were maintained on 129svev and C57BL/6J backgrounds. All animal work performed were approved by IACUC.

3.2 WHOLE MOUNT SKELETAL STAINING

For timed matings, the day the plug was observed was designated E0.5. At day E18.5, pregnant mice were sacrificed in an isoflurane chamber following proper euthanasia guidelines. E18.5 C57BL/6J wildtype (WT) ($n=5$) and *Trps1*^{-/-} ($n=4$) mice pups were collected. Following complete removal of skin and excess soft tissues, the embryos were fixed in 95% ethanol overnight. The samples were stained with 0.03% Alcian blue (*Sigma-Aldrich, A3157*) solution overnight, washed with 95% ethanol for 3h, and cleared with 2% KOH for 12h. The embryos were then stained with 0.01% Alizarin red (*Sigma- Aldrich, A5533*) solution overnight and cleared in 1% KOH/20% glycerol for 48h. Specimens were stored and imaged in 1:1 glycerol/95% ethanol solution. Leica M165FC microscope and Leica Application Suite software was used for imaging.

3.3 HEMATOXYLIN & EOSIN (H&E) STAINING

7µm thick sections of paraffin-embedded 129svev mouse heads were deparaffinized with xylene and rehydrated. The tissues were stained with Harris Hematoxylin solution (*Thermo Fisher Scientific*) and counterstained with Eosin Y solution (*Thermo Fisher Scientific*). The stained tissues were then dehydrated serially with ethanol and placed in xylene. The samples were mounted in mounting medium (*Richard-Allan Scientific*).

3.4 FLUORESCENT IMMUNOHISTOCHEMISTRY

Paraffin-embedded tissue samples from C57BL/6J WT and *Trps1*^{-/-} mouse heads fixed in either 4% paraformaldehyde (for detection of *Trps1* and *Tgfb3*) or Carnoy's solution (for detection of CSPG, β -catenin and *Twist1*) were obtained from our laboratory's previous work. Heat-induced epitope retrieval was performed in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween20, pH=6.5). The tissues were blocked with 10% bovine serum albumin (BSA) in 1xPBS and incubated with primary antibody at 4°C overnight. The primary antibodies used were 1:50 rabbit anti-*TRPS1* (*Abnova*, *PAB17465*), 1:200 anti-chondroitin sulfate proteoglycan (*Sigma*, *C8035*), 1:100 rabbit anti-*TGF β 3* (*Abcam*, *ab15537*), 1:250 rabbit anti- β -catenin (*Abcam*, *ab50581*), and 1:100 rabbit anti-*Twist1* (*Abcam*, *ab32572*). After rinsing, a secondary antibody conjugated with anti-rabbit AlexaFluor 488 (*Thermo Fisher Scientific*) was used for detection and incubated at room temperature for 30 minutes. The stained tissues were mounted in Molecular Probes ProLong Gold Antifade Mountant with DAPI (*Thermo Fisher Scientific*). The

slides were stored at 4°C and images were taken with Zeiss AxioCam on a Zeiss Axioskop A1 microscope and ZEN software.

3.5 CRANIOFACIAL MEASUREMENTS OF WHOLE MOUNT STAINING SAMPLES

Total head length was measured from the tip of nasal cartilage to the most posterior aspect of the supraoccipital bone. The nasal length was measured from the tip of nasal cartilage to the frontonasal suture. The nasal angle was determined from the images by drawing a plane from the tip of the nasal cartilage to the superior border of supraoccipital bone and a second plane tangent to the nasal bone surface. The angle was then measured between the two planes using a protractor. Mandible length was measured from the most posterior point at the condylar process to the most anterior point at the symphysis. Lengths of the head, nose and mandible were measured with calipers.

3.6 STATISTICAL ANALYSIS

Non-parametric Wilcoxon rank sum test was used to determine a difference between WT and *Trps1*^{-/-} mice head, nose and mandible measurements with $\alpha=0.05$. The statistical analysis was performed using StataSE software.

4.0 RESULTS

4.1 SKELETAL AND CRANIOFACIAL PHENOTYPE OF *TRPS1*^{-/-} MICE

TRPS1 haploinsufficiency in humans results in characteristic features that indicate a disturbance in skeletal development. The severity of skeletal deformation in mice with a homozygous *Trps1* mutation is further increased, specifically within the thoracic spine and ribs, which ultimately leads to neonatal respiratory failure and death (Malik et al. 2002). For this reason, analysis of the *Trps1*^{-/-} mouse skeletal elements in this study was performed on E18.5 embryos shortly before birth. Comparisons of whole mount skeletal preparations of E18.5 WT and *Trps1*^{-/-} mice demonstrated apparent bowing of the forelimb long bones, specifically the ulna and radius, and an overall underdevelopment of the sternum in *Trps1*^{-/-} mice (Figure 4.1A,B). Closer inspection of the *Trps1*^{-/-} mouse sternums revealed that the ossification of the xiphoid process was especially reduced compared to the WT group (Figure 4.1C,D). Analysis of the thoracic region showed delayed ossification of the cervical vertebrae and abnormal shape of the rib cage in *Trps1*^{-/-} mice (Figure 4.1A,B; E,F).

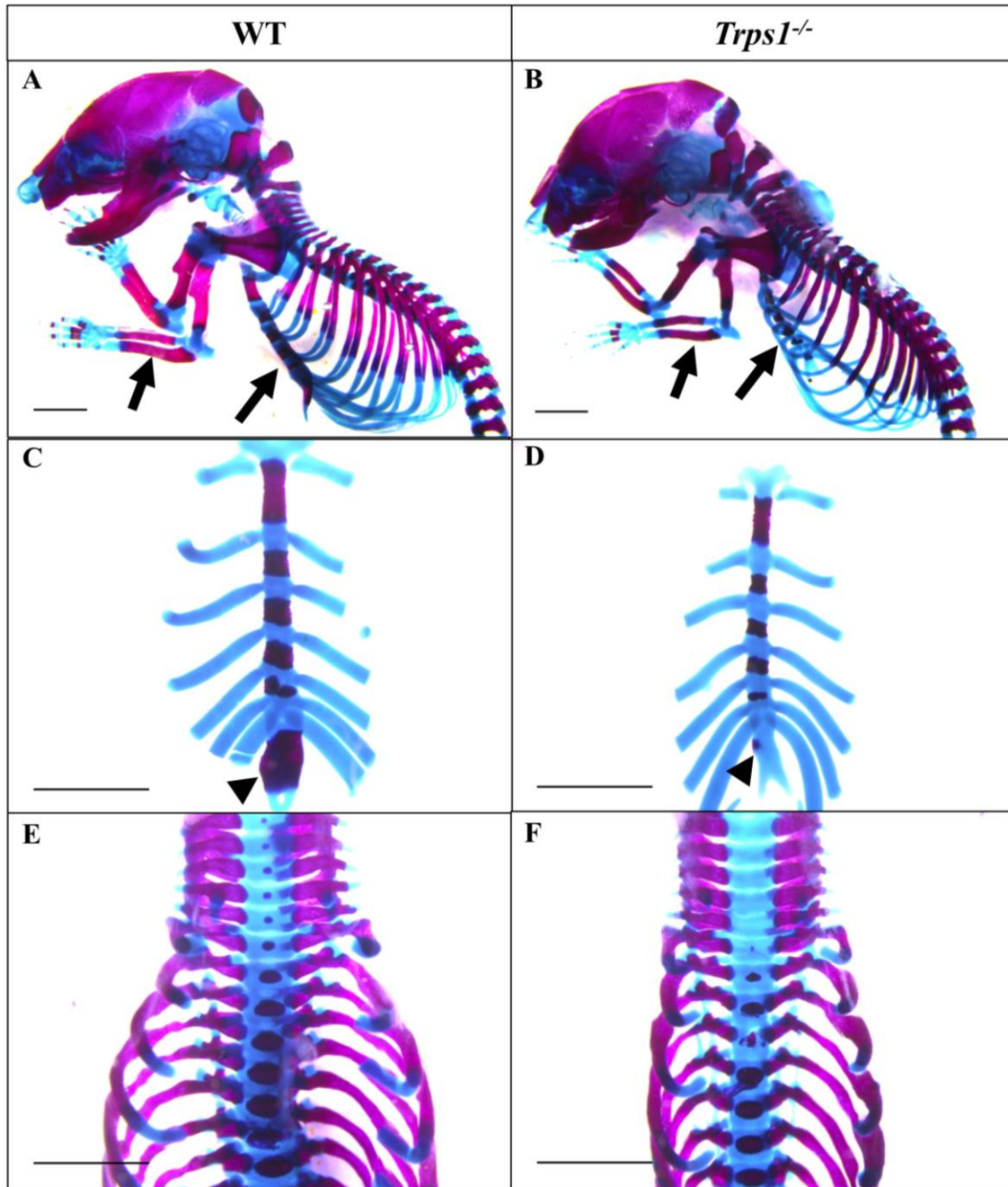


Figure 4.1. Appendicular and axial skeleton abnormalities in *Trps1*^{-/-} mice. Alcian blue (cartilage) and Alizarin red (ossified bone) staining of E18.5 WT (left) and *Trps1*^{-/-} (right) mouse skeletons. A,B) Side view of the embryo demonstrating increased bowing of ulna and radius, and underdevelopment of the sternum (arrows) in *Trps1*^{-/-} mice. C,D) Magnified view of the sternum illustrating reduced ossification especially at the xiphoid process in *Trps1*^{-/-} mice (arrowheads). E,F) Frontal view of the thoracic vertebrae and ribs showing delayed vertebral ossification in *Trps1*^{-/-} mice. Scale bar 2mm.

The skeletal analysis was then focused on the craniofacial region. No statistically significant difference was found between the mean head length of *Trps1*^{-/-} mice (9.4mm ± 0.3mm) and WT mice (9.9mm ± 0.4mm) (Figure 4.2/4.3, Table A1). However, the mean nasal length of *Trps1*^{-/-} mice (2.1mm ± 0.1mm) was 15% shorter than the WT mice (2.5mm ± 0.1mm) ($p < 0.05$) (Figure 4.2/4.3, Table A1). The nasal angle of the *Trps1*^{-/-} mice ($31.1^\circ \pm 1^\circ$) was on average 18% steeper than the WT mice ($26.4^\circ \pm 2.8^\circ$) ($p < 0.05$) (Figure 4.2/4.3, Table A1). No apparent differences in the nasal cartilage were detected.

Multiple differences between WT and *Trps1*^{-/-} mandibles were also detected. The *Trps1*^{-/-} mutant phenotype includes unerupted incisors, reduced mineralized regions as well as less cartilage in the coronoid, condylar and angular processes (Figure 4.2B). We also found that the average *Trps1*^{-/-} mandible (4.6mm ± 0.1mm) was 17% shorter than the average WT mandible (5.6mm ± 0.1mm) ($p < 0.05$) (Figure 4.2/4.3, Table A1). Meckel's cartilage and clearly demarcated molar crypts were present in both genotypes (Figure 4.2B). In the midface region, the zygomatic arches were less developed in *Trps1*^{-/-} mice in comparison with the WT littermates (Figure 4.4A,B), which is consistent with the craniofacial skeletal phenotype of adult *Trps1*^{+/-} mice (Malik et al. 2002). Additionally, analysis of the skeletal preparations revealed underdeveloped vomer bones in *Trps1*^{-/-} mice (Figure 4.4C,D). Finally, we observed cranial base abnormalities in *Trps1*^{-/-} mice, where the basisphenoid was smaller and the presphenoid bone was absent (Figure 4.4E-H). However, there was no difference in the basioccipital and cranial vault bones between the two genotypes (Figure 4.4E-J). These results suggest that *Trps1* plays a vital role in the development of many thoracic, appendicular and craniofacial skeletal elements.

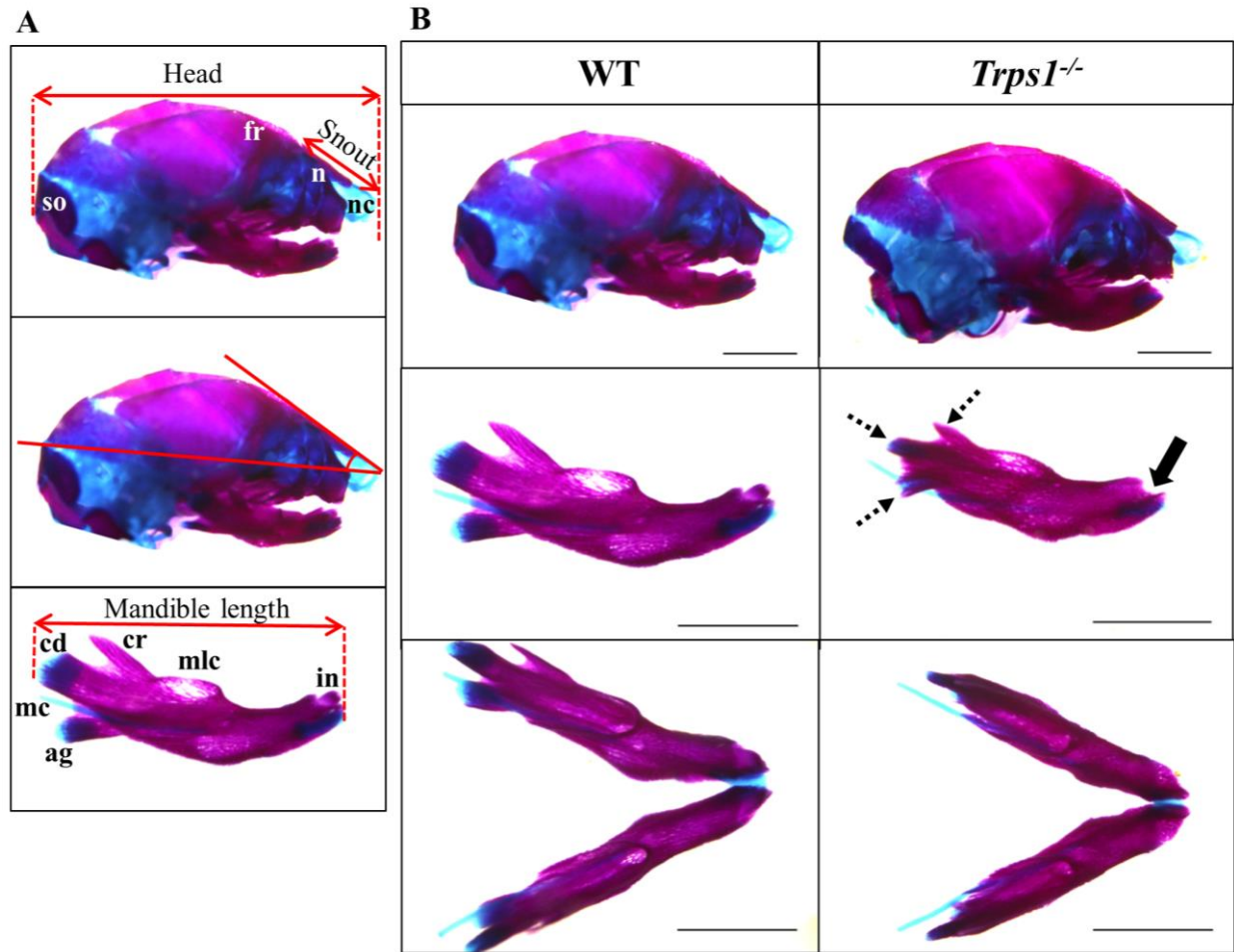


Figure 4.2. Craniofacial abnormalities in *Trps1*^{-/-} mice. Alcian blue (cartilage) and Alizarin red (ossified bone) staining of E18.5 WT and *Trps1*^{-/-} mouse heads. A) Landmarks for head and nasal length (top), nasal angle (middle) and mandibular length (bottom). B) Side view of the head skull (top) and mandible (middle); top view of mandible (bottom): Coronoid process, condylar process/cartilage, and angular process/cartilage of *Trps1*^{-/-} mice were hypoplastic (dotted arrows). The mandibular incisors have not erupted in *Trps1*^{-/-} mandible (block arrow). Meckel's cartilage and molar crypts were clearly visualized in both WT and knockout phenotypes. *Scale bar 2mm.* *ag-* angular process, *cr-* coronoid process, *cd-* condylar process, *fr-* frontal bone, *in-* incisor, *mc-* Meckel's cartilage, *mlc-* molar crypt, *n-* nasal bone, *nc-* nasal cartilage, *so-* supraoccipital.

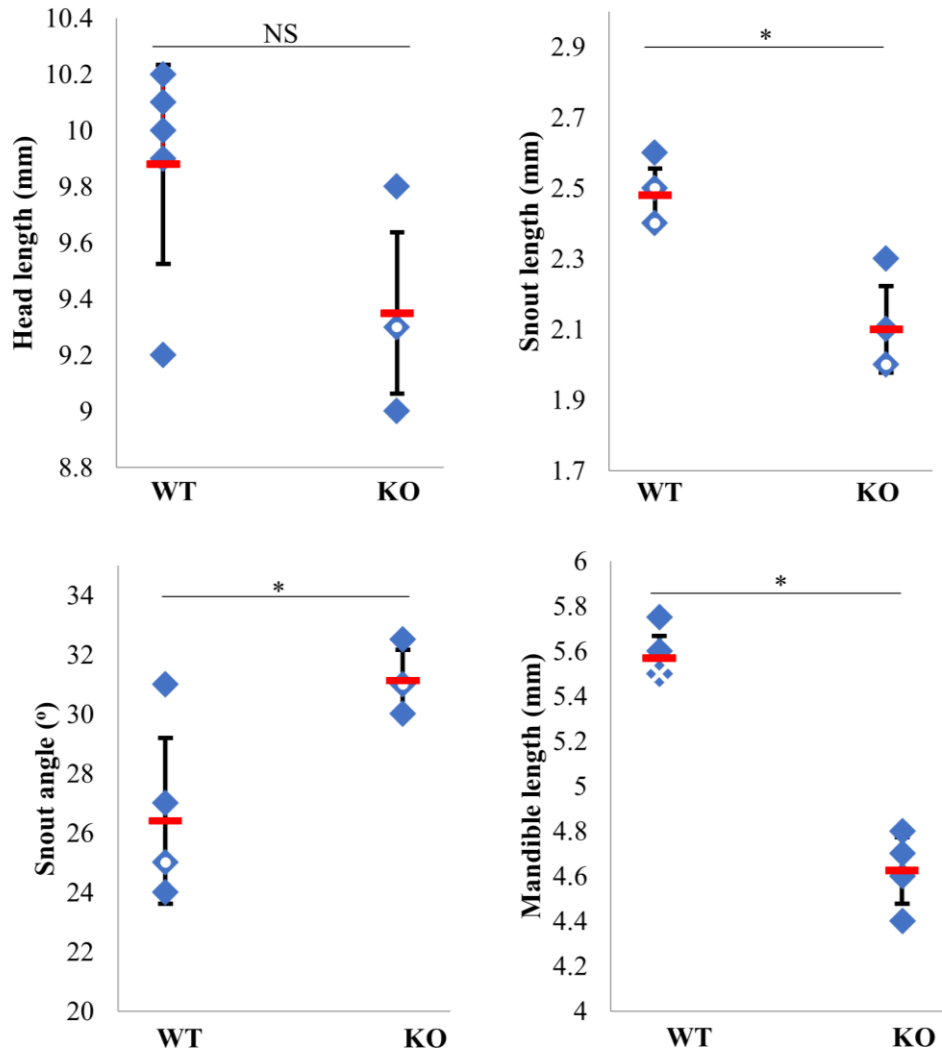


Figure 4.3. Comparative quantitative analysis of WT and *Trps1*^{-/-} mice craniofacial dimensions. There was no significant difference in total length of the head between the two groups but the nose was significantly shorter and downward-sloping in the *Trps1*^{-/-} mice. The *Trps1*^{-/-} mandible was also significantly shorter than WT. Red dashes represent the mean angle value in each data set. Solid data points represent individual samples, data points with white circle within indicate 2 samples with the same measurement and data points with white cross within indicate 3 samples with the same measurement. Error bars indicate the standard deviation of the sample. * indicates statistically significant differences between the WT and *Trps1*^{-/-} groups ($p < 0.05$).

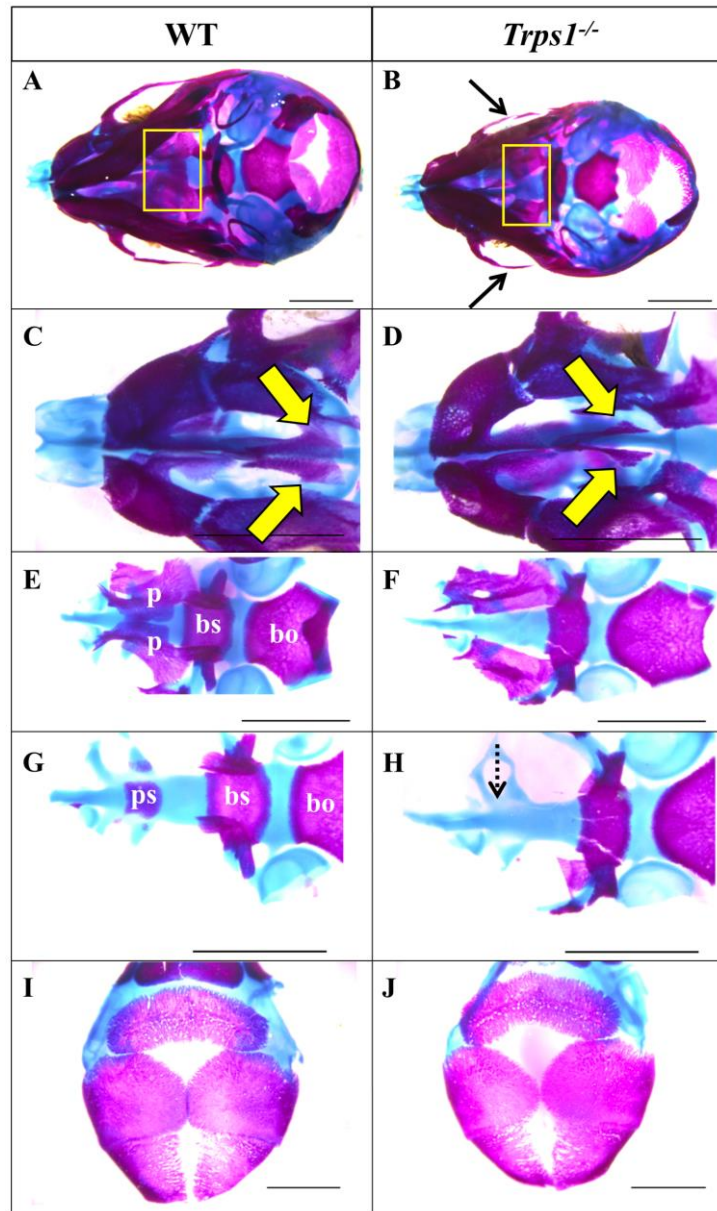


Figure 4.4. Midface and cranial base abnormalities in *Trps1*^{-/-} mice. Alcian blue (cartilage) and Alizarin red (ossified bone) staining of E18.5 WT (left) and *Trps1*^{-/-} (right) mouse heads. A,B) Inferior view of skull demonstrating hypoplastic zygomatic process (solid arrows) and palatal shelf separation (boxed area) in *Trps1*^{-/-} mice. C,D) Magnified view of the nasal area illustrating underdeveloped vomer bones in *Trps1*^{-/-} mice (black arrows). E,F) The basioccipital bone is unaffected in *Trps1*^{-/-} mice, while the basisphenoid bone is smaller and the cleft palate is evident with the unfused palatal shelves. G,H) Removal of palatine bones revealed the absence of presphenoid bone in *Trps1*^{-/-} mice (dotted arrow). I,J) Cranial vault bones showed no apparent differences between the two groups. Scale bar: 2mm. *bo*- basioccipital, *bs*- basisphenoid, *p*- palatine bone, *ps*- presphenoid.

4.2 EXPRESSION OF *TRPS1* IN WILDTYPE MOUSE PALATAL SHELVES

Due to the clear presence of cleft palate in *Trps1* mutant mice as demonstrated by the skeletal staining experiments, our next objective was to investigate how *Trps1* might affect palatal development by delineating its expression using immunohistochemistry. Previous studies have shown that *Trps1* has a wide range of expression during mouse embryogenesis and is strongly expressed in the subcutaneous region of the snout, maxilla and mandible (Kunath et al. 2002). Here, we focused our study on the expression of *Trps1* during different stages of palatogenesis. We found that *Trps1* was generally expressed in the mesenchyme of the nasal septum and maxillary process, developing intrinsic muscles of the tongue, and in the tips of the palatal shelves. During palatal outgrowth (E12.5), *Trps1* protein was detected in the nasal region of the maxillary process (Figure 4.5). *Trps1* was also found in palatal shelf mesenchyme and epithelium, including the epithelium surrounding the palatal shelves at the future fusion sites (Figure 4.5C). As development progressed (E13.5), the presence of *Trps1* became more widespread in the palatal shelves, mandible and tongue, especially in the posterior region of the oral cavity (Figure 4.6C,D). When the palatal shelves were in the adhesion/fusion stage (E14.5), *Trps1* was still expressed in the nasal septum, maxillary mesenchyme, palatal shelf epithelium and tongue (Figure 4.7). *Trps1* expression was evident in the seam of the MEE in both anterior and posterior sections of the palate (Figure 4.7E,F), suggesting that *Trps1* may play a role in palatal fusion.

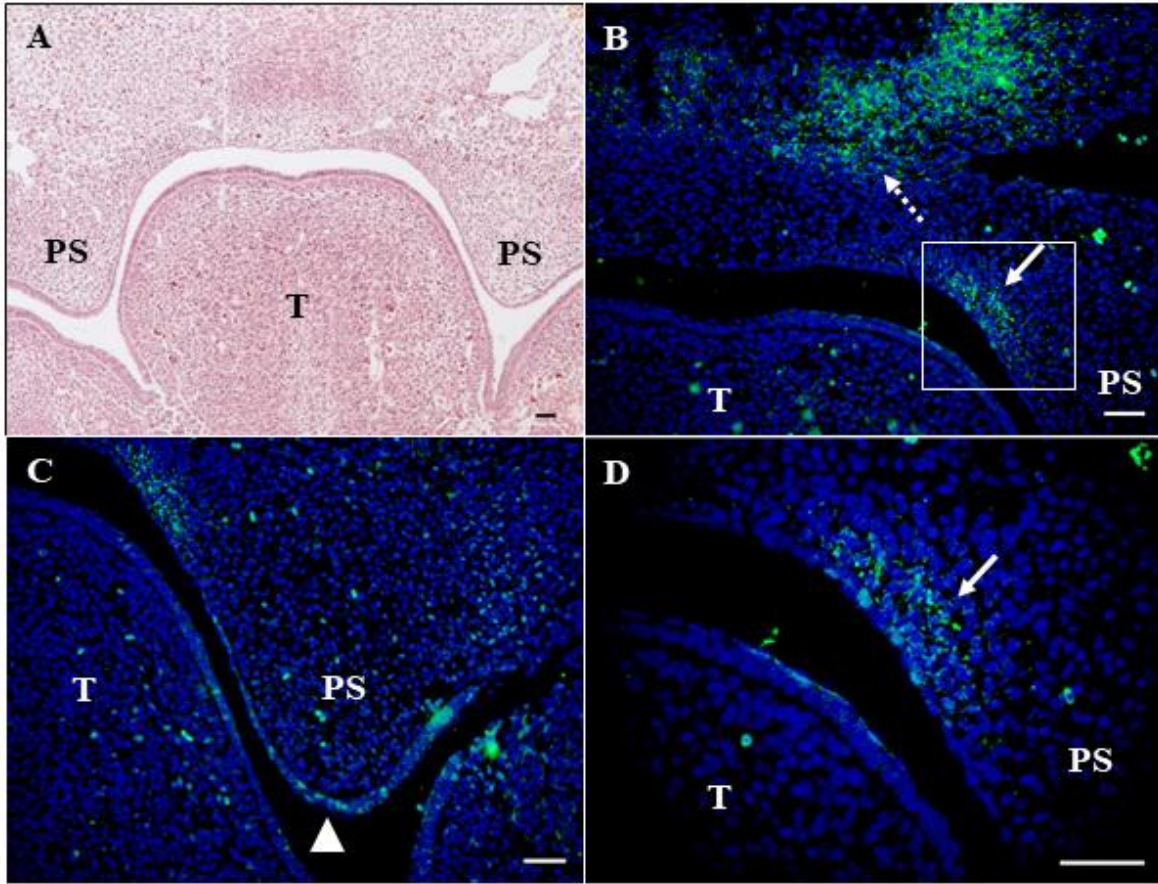


Figure 4.5. Trps1 protein expression pattern during mouse palatal development at E12.5. A) H&E staining of a WT frontal head section of the oral cavity at E12.5. B) Immunofluorescent staining for Trps1 protein (green) showing that Trps1 was expressed in a skeletogenic condensation region (dotted arrow) and the nasal region of the maxillary process (arrow). C) Trps1 was detected in the MEE of the palatal shelf (arrowhead). D) Magnified view of the boxed area in B showing Trps1 expression in a localized mesenchymal area within the nasal region of the maxillary process (arrow) and the adjacent epithelium. *Scale bar 50 μ m. PS- palatal shelf, T- tongue.*

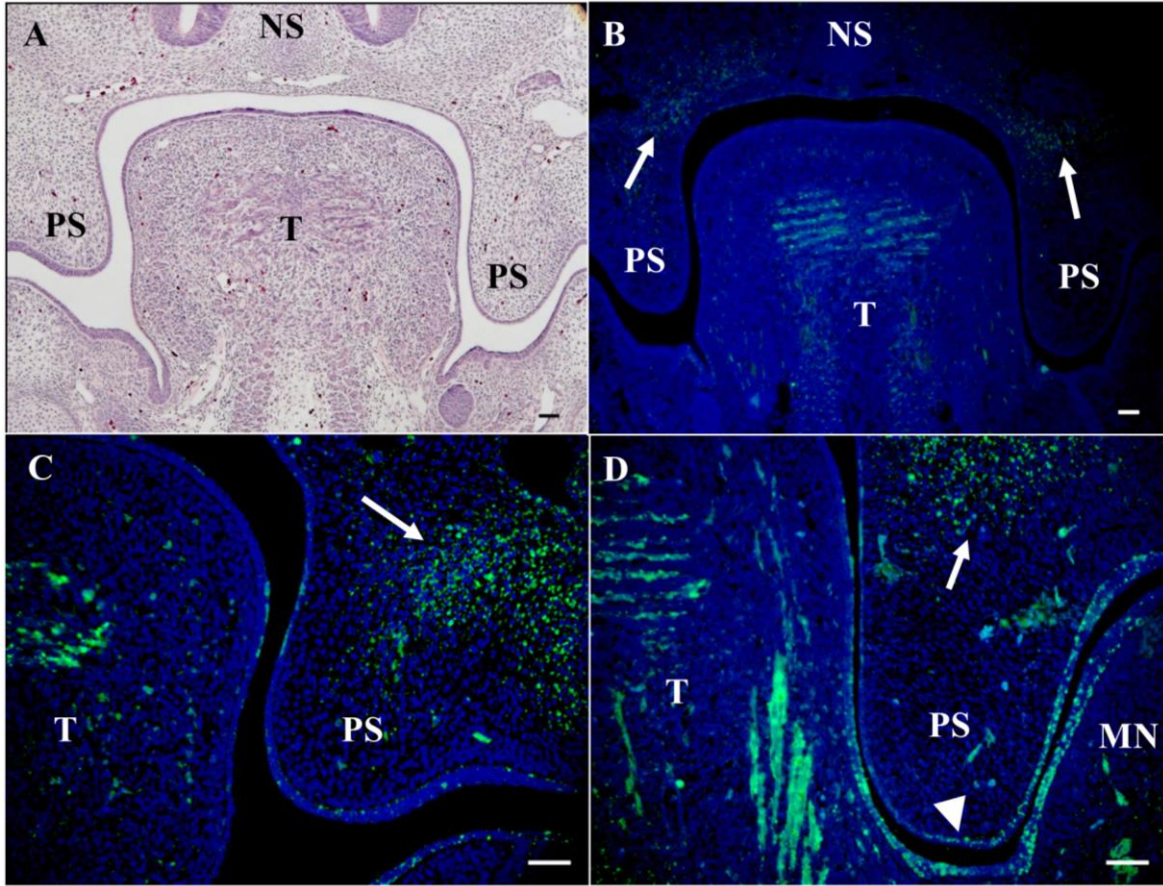


Figure 4.6. Trps1 protein expression pattern during mouse palatal development at E13.5. A) H&E staining of a WT frontal head sections of the oral cavity at E13.5. B) Immunofluorescent staining for Trps1 protein (green) showing Trps1 expression in maxillary process mesenchyme (arrows) and developing muscles of the tongue (T). C) Anterior section of the oral cavity showing Trps1 expression in the palatal shelf, maxillary mesenchyme (arrow) and tongue (T). D) Posterior section of the oral cavity showing Trps1 in maxillary mesenchyme (arrow), palatal shelf epithelium (arrowhead) and tongue (T). Scale bar 50 μ m. MN- mandible, NS- nasal septum, PS- palatal shelf, T- tongue.

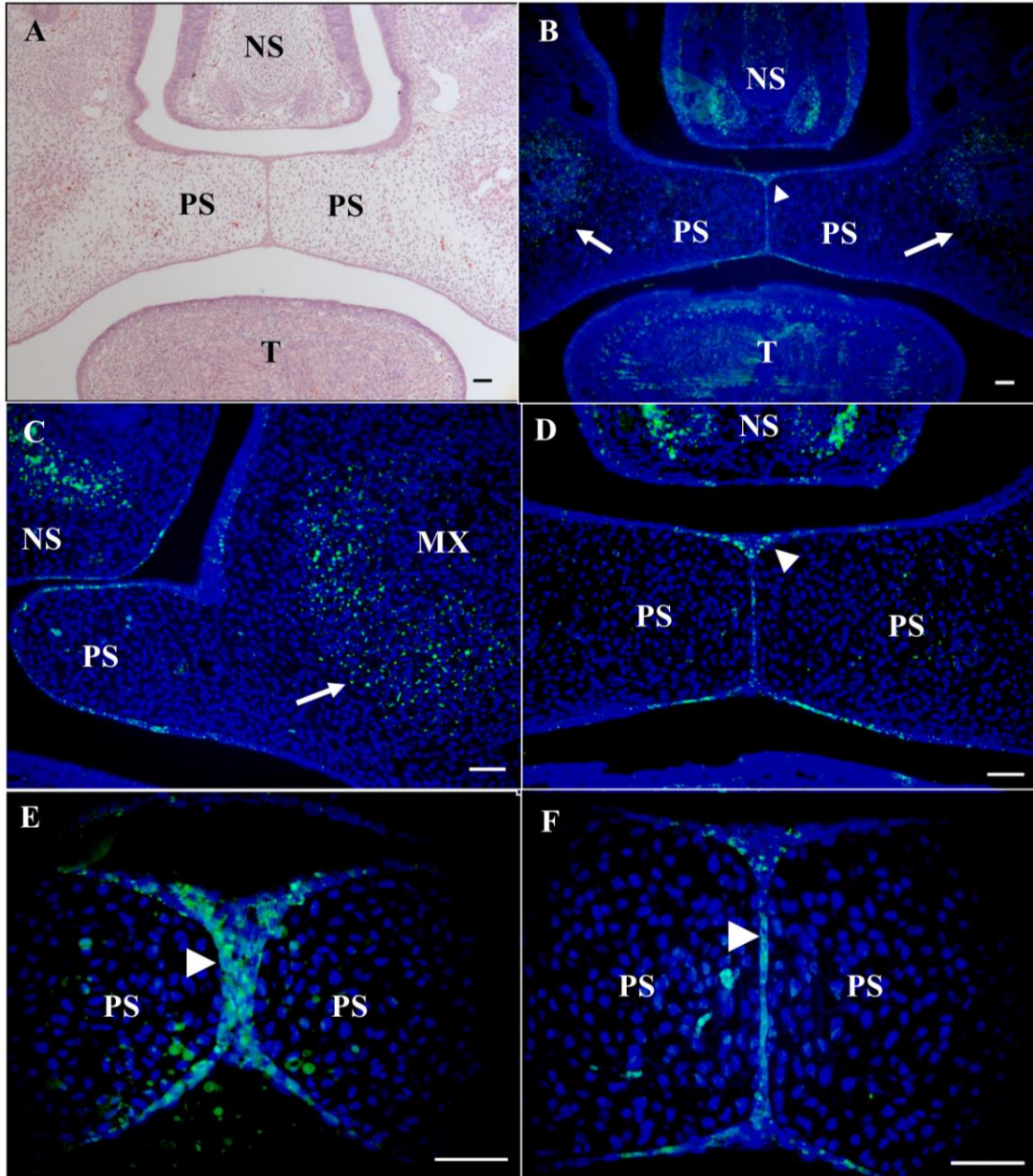


Figure 4.7. *Trps1* protein expression pattern during mouse palatal development at E14.5. A) H&E staining of a WT frontal head sections of the oral cavity at E14.5. B) Immunofluorescent staining for *Trps1* protein (green) showing *Trps1* within the nasal septum (NS), palatal shelf epithelium (arrowhead), maxillary mesenchyme (arrows) and tongue (T). C,D) Higher magnification of anterior (C) and posterior (D) palatal shelf sections illustrating *Trps1* presence in the maxillary mesenchyme laterally (arrow) and palatal shelf epithelium (arrowhead). E,F) Increased magnification of the anterior (E) and posterior (F) sections where the palatal shelves meet demonstrating clear *Trps1* expression at the MEE (arrowheads). Scale bar 50µm. MX- maxilla, NS- nasal septum, PS- palatal shelf, T- tongue.

4.3 DOWNREGULATION OF *TGFβ3*, *β-CATENIN* AND *TWIST1* EXPRESSION IN *TRPS1*^{-/-} PALATES

The association between *TGFβ3* mutation and cleft palate in both humans and animal studies highlights the importance of this signaling molecule in palatogenesis (Proetzel et al. 1995; Carinci et al. 2007; Rienhoff et al. 2013). Given that epithelial expression of the cell adhesion molecule CSPG is lost in *Trps1*^{-/-} palatal shelves (unpublished data) and that accumulation of CSPG at the fusion surfaces depends on Tgfβ3 (Gato et al. 2002; Krauss 2011), we hypothesized that the observed absence of CSPG is related to a disruption in Tgfβ3 signaling. Immunohistochemical staining revealed that *Tgfβ3* follows a very similar expression pattern to *Trps1* in WT mice where they are both strongly expressed in the maxillary mesenchyme and palatal shelf MEE (Figure 4.8). However, like CSPG, *Tgfβ3* expression was lost in the MEE of *Trps1* knockout palatal shelves (Figure 4.9). This suggests that CSPG deficiency on the fusion surface is a consequence of the loss of *Tgfβ3* expression in *Trps1*^{-/-} palatal shelf epithelium.

The absence of Tgfβ3 in the *Trps1*^{-/-} epithelium led us to investigate other proteins within the palatal shelf epithelium that are essential for palatal fusion and may be involved in *Trps1* regulatory networks. We analyzed *Twist1*, which is the key EMT regulator required for palatal fusion and functions downstream of Tgfβ3, and β-catenin, which has been implicated in both *Trps1* and Tgfβ3 signaling (Yu et al. 2008; He et al. 2011; Fantauzzo and Christiano 2012). We discovered that both *Twist1* and β-catenin proteins were primarily present in the epithelium of the WT E14.5 palatal shelves, but their expressions were undetectable in *Trps1*^{-/-} palatal shelves (Figure 4.9). Therefore, it seems that *Trps1* is required for the epithelial expression of several proteins critical for palatal shelf fusion.

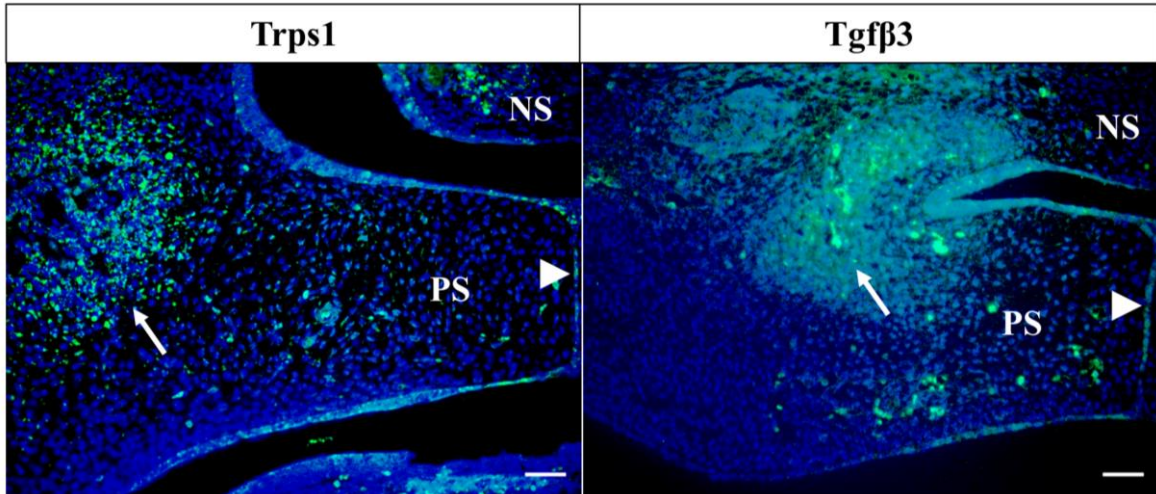


Figure 4.8. Similarities between the expression patterns of Trps1 and Tgfβ3. Both Trps1 (left) and Tgfβ3 (right) are expressed in maxillary mesenchyme (arrows) and palatal shelf epithelium (arrowheads). Scale bar 50μm. NS- nasal septum, PS- palatal shelf.

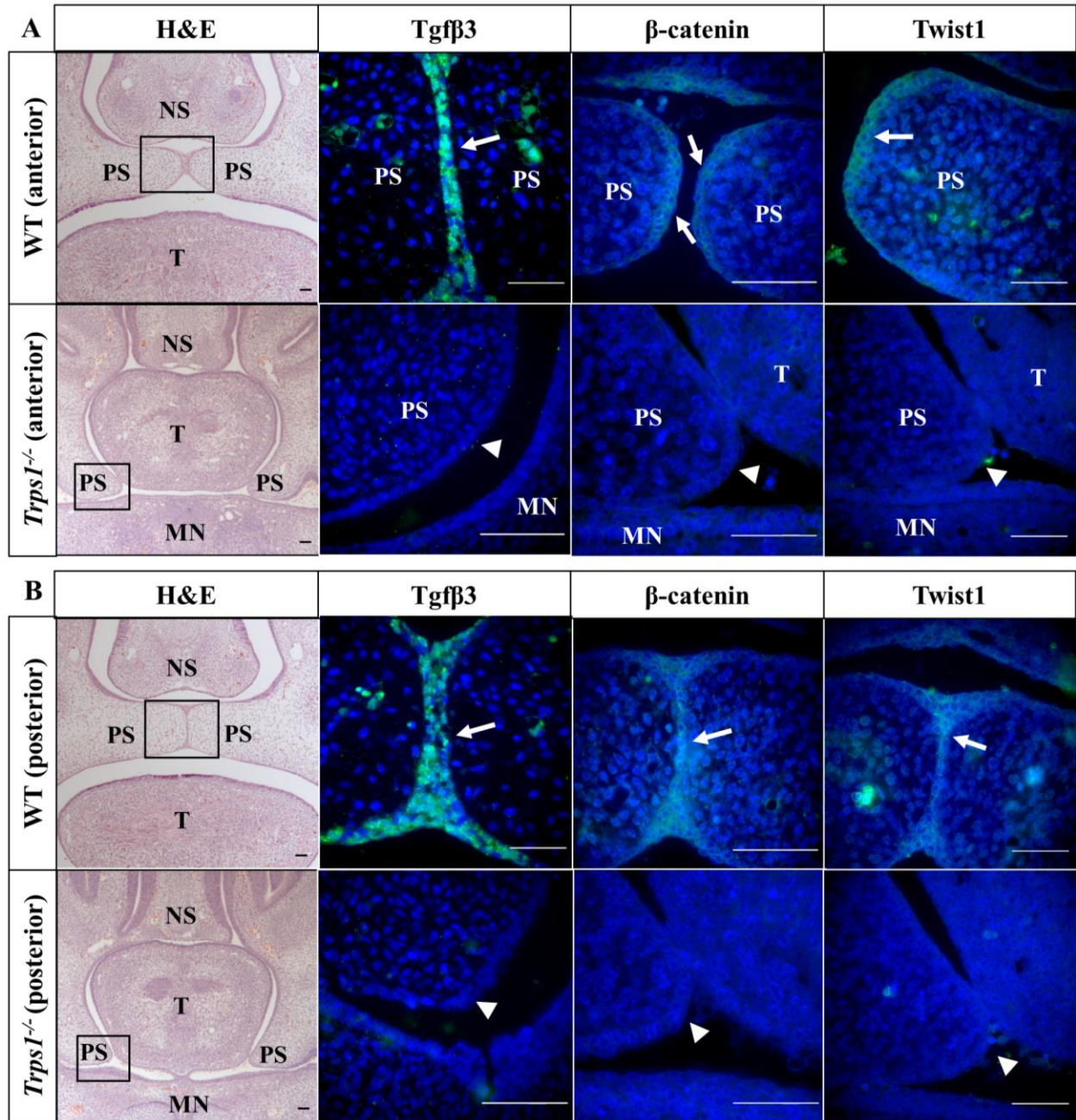


Figure 4.9. Expression of Tgfb3, β -catenin and Twist1 were decreased in *Trps1*^{-/-} mouse palatal shelf epithelium. Immunofluorescent staining for Tgfb3, β -catenin and Twist1 (green) were performed on E14.5 WT and *Trps1*^{-/-} mice and imaged within the boxed area marked on the H&E images. Anterior (A) and posterior (B) sections of palatal shelves demonstrated positive Tgfb3, β -catenin and Twist1 signal in the MEE of WT mice (arrows) but not in *Trps1*^{-/-} palatal shelf epithelium (arrowheads). Scale bar: 50 μ m. MN- mandible, NS- nasal septum, PS- palatal shelf, T- tongue.

5.0 DISCUSSION

TRPS1 is crucial for proper skeletal and craniofacial development, as evident by the characteristic phenotype of TRPS patients. This is supported by other studies that have demonstrated that *Trps1* is expressed in the precursor cells of long bones, ribs, vertebrae and facial bones (Kunath et al. 2002). The skeletal phenotype of *Trps1*^{-/-} mice seen in this study is similar to what Malik et al. (2002) and Suemoto et al. (2007) described in regards to the abnormal ribcage anatomy and underdeveloped forelimbs, sternum and vertebrae. Here, we also noted that the shape of the forelimb long bones displayed a bowed appearance compared to the WT mice. Interestingly, the thoracic defects observed in *Trps1*^{-/-} mice are analogous to the phenotype of TRPS patients who display pectus carinatum, a chest deformity featuring a protrusive sternum due to abnormal costal cartilage growth (Beals 1973; Felman and Frias 1977). Therefore, it appears that *Trps1* is highly involved in endochondral bone formation within both the appendicular and axial skeleton.

Disruption in endochondral bone development within the craniofacial region of TRPS patients has also been described before. Cephalometric studies performed by King and Frias (1979) revealed a shortening of the posterior cranial base in TRPS patients. In this study of mice with *Trps1*^{-/-} mutation, we detected abnormalities within the anterior cranial base instead, most notably the complete absence of the presphenoid bone in E18.5 *Trps1*^{-/-} mice. Other craniofacial abnormalities of *Trps1*^{-/-} mice identified here include a short and downward sloping nose,

hypoplastic zygomatic process of the maxilla, and micrognathic mandible with underdeveloped bony processes. These features are consistent with the phenotype of adult *Trps1*^{+/-} mice (Malik et al. 2002) and also draws resemblance to the bulbous nose and hypoplastic jaws typically seen in TRPS patients (Ludecke et al. 2001; Griffiths et al. 2016). Further examination of the mandible also revealed a reduction in the cartilage component of the condylar and angular processes in *Trps1*^{-/-} mice. This is consistent with previous studies identifying *Trps1* as a regulator of chondrocyte proliferation and differentiation in appendicular skeleton and TMJ (Suemoto et al. 2007; Napierala et al. 2008; Wuelling et al. 2009; Michikami et al. 2012). It has been proposed that the condylar hypoplasia seen in *Trps1* mutant mice may be due to cellular disruption of the condylar chondrocytes resulting in premature chondrocyte maturation and decreased proliferation (Michikami et al. 2012). In terms of dental abnormalities, we noted delayed tooth eruption in E18.5 *Trps1*^{-/-} mice, a feature that is also common in TRPS patients. Finally, we also detected the presence of cleft palate in *Trps1*^{-/-} mice (Figure 4.4F), which has been previously reported by Kantaputra et al. (2008). Despite the dysplastic cranial base and jaws in *Trps1* mutant mice, it appears that the cranial vault was largely unaffected. The craniofacial characteristics of *Trps1* homozygous mutant mice are highly comparable to the phenotype of patients with TRPS1 haploinsufficiency but with increased severity, indicating the dose-dependent effect of *Trps1*-deficiency.

To investigate the role of *Trps1* during palatal development, we delineated its expression pattern at various embryonic stages. To review, the key steps of palatogenesis are initiation of palatal outgrowth from the maxillary process, vertical growth, palatal shelf elevation, horizontal growth, adhesion and fusion. Interference at any of these stages can result in cleft palate. We demonstrated that *Trps1* is expressed in the oral cavity during palatal development, specifically

in the mesenchyme of the maxilla, mandible, tongue and nasal septum. This suggests that a mutation in the *Trps1* gene may affect palatogenesis at various stages, but the specific mechanism remains unknown. For example, the presence of Trps1 in palatal shelf mesenchyme opens up the possibility of Trps1 playing a role in palatal shelf elevation as this process is thought to be guided by internal forces (Li et al. 2017). This possibility is supported by the fact that by E14.5, WT palatal shelves have elevated above the tongue while the *Trps1*^{-/-} palatal shelves were still oriented vertically (Figure 4.9). The expression of *Trps1* in the developing intrinsic muscles of the tongue suggests that it could also play a role in tongue descent (Figure 4.6). In other craniofacial disorders such as Pierre Robin sequence, cleft palate results from obstructed tongue descent secondary to a micrognathic mandible (Levi et al. 2011). Thus, the small mandible of *Trps1*^{-/-} mice could also be a contributing factor to the tongue's inability to descend. Finally, the clear expression of *Trps1* within the palatal shelf epithelium suggested that cleft palate in *Trps1*^{-/-} mice is due to a failure of palatal shelf fusion. This was confirmed by our laboratory's unpublished data showing that *Trps1*^{-/-} palatal shelves were not able to initiate the fusion process, which is most likely caused by a failure in palatal shelf adhesion secondary to the loss of CSPG on the epithelial surface (Figure 1.4). Our finding that Tgfb3 was absent in *Trps1*^{-/-} palatal epithelium strongly suggests that this CSPG-deficiency is a consequence of the loss of Tgfb3. In summary, *Trps1* is required for the initiation of palatal shelf fusion and for the epithelial expression of several other proteins critical for this process.

Trps1 has been shown to interact with the Tgfb and canonical Wnt signaling pathways in kidney and hair follicles, respectively (Gai et al. 2009; Fantauzzo and Christiano 2012). However, the mechanism by which a Trps1-deficiency decreases the expression of *Tgfb3*, *β-catenin* and *Twist1* in the MEE remains elusive. Most studies have shown that Trps1 primarily

functions by inhibiting expression of its target genes (Malik et al. 2001; Napierala et al. 2008). However, some evidence shows that *Trps1* can also act as a transcriptional activator in hair follicle progenitors (Fantauzzo and Christiano 2012). This opens up the possibility that expression of *Tgfβ3*, *β-catenin* or *Twist1* might be directly activated by *Trps1* in palatal shelf epithelium (Figure 5.1).

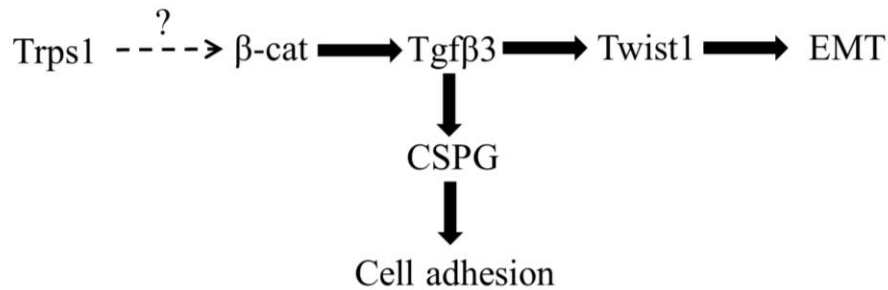


Figure 5.1. Proposed mechanism by which *Trps1* regulates *Tgfβ3*, *β-catenin* and *Twist1* expression during palatal fusion. The direct relationship between *Trps1* and *β-catenin* in palatal shelves is unclear.

The regulation of palatogenesis requires elaborated communication between different signaling molecules including *Tgfβ3*, *β-catenin* and *Twist1*. As illustrated in Figure 5.1, *β-catenin* is necessary for *Tgfβ3* expression in palatal shelf epithelium, which in turn promotes *Twist1* expression (Yu et al. 2008; He et al. 2011). Both *Tgfβ3* and *Twist1* are essential for palatal fusion due to their role in EMT stimulation (Yu et al. 2009; Ke et al. 2015). Interestingly, *Trps1* has instead been associated with the reverse process of EMT, known as MET, in tumor and liver cells and positively correlated with E-cadherin levels (Su et al. 2014; Zhe et al. 2015; Huang et al. 2016). Some proposed mechanisms include *Trps1* downregulating the expression of the EMT promoting protein *Zeb2* and *Trps1* directly activating the expression of an EMT antagonist *Foxa1* (Stinson et al. 2011; Huang et al. 2016). On the other hand, *Twist1* is able to

silence *Foxa1* expression in cancer cells to promote EMT (Xu et al. 2016). These pathways are summarized in Figure 5.2. While these data from malignant tissues support the role of *Trps1* in MET, the developmental functions of *Trps1* may also stem from its regulation of other genes such as *Tgfb3*, β -*catenin* and *Twist1* to promote cell adhesion or EMT in palatal shelves (Figure 5.1).

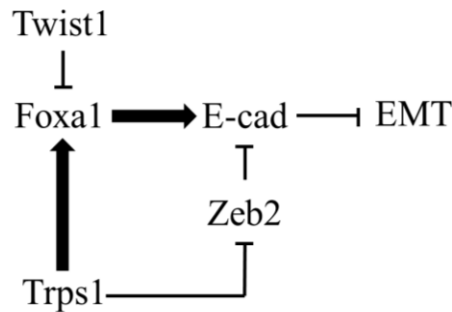


Figure 5.2. Relationship between *Trps1* and EMT found in cancer cell studies.

Our study highlights the importance of *Trps1* in skeletal and craniofacial development, especially palatal fusion. However, further studies should be carried out to investigate whether exogenous *Tgfb3*, β -*catenin* or *Twist1* can rescue the cleft palate phenotype of a *Trps1*-deficiency and to examine other proteins in related pathways that may be dysregulated as well. This would allow us to advance our understanding of the molecular mechanisms underlying the cleft palate pathogenesis in TRPS. Here, we have examined the processes of palatal shelf adhesion and fusion but future studies should also delve into how *Trps1* may affect other stages of palatogenesis, including tongue descent, palatal shelf proliferation, elevation, apoptosis or EMT.

6.0 CONCLUSIONS

In summary, *Trps1* is required for proper axial, appendicular and craniofacial skeleton, including the anterior cranial base and jaws. During development, *Trps1* is expressed in the epithelium and mesenchyme of the palatal shelves and is necessary for the initiation of palatal fusion. Additionally, the expressions of other proteins essential for palatal fusion, such as Tgf β 3, β -catenin and Twist1, are dependent on *Trps1*.

APPENDIX

CRANIOFACIAL DIMENSIONS FROM WHOLE MOUNT SKELETAL STAINING

Table A1. Comparison of craniofacial measurements between wildtype (WT) and *Trps1*^{-/-} mice. * indicates a statistical significant difference between the two groups.

	Head length (mm)	Snout length (mm)	Snout angle (°)	Mandible length (mm)
WT 1	10.1	2.5	31	5.5
WT 2	10	2.4	25	5.5
WT 3	9.9	2.6	27	5.75
WT 4	9.2	2.5	24	5.5
WT 5	10.2	2.4	25	5.6
<i>Trps1</i> ^{-/-} 1	9.3	2.1	30	4.7
<i>Trps1</i> ^{-/-} 2	9.8	2	31	4.6
<i>Trps1</i> ^{-/-} 3	9.3	2	32.5	4.8
<i>Trps1</i> ^{-/-} 4	9	2.3	31	4.4
Average WT	9.88 ± 0.35	2.48 ± 0.07	26.4 ± 2.79	5.57 ± 0.10
Average <i>Trps1</i>^{-/-}	9.35 ± 0.29	2.1 ± 0.12	31.13 ± 1.03	4.63 ± 0.15
% difference	5%	15%	18%	17%
<i>p</i> -value	0.08	0.01*	0.04*	0.01*

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