

# Med23 Regulates Sox9 Expression during Craniofacial Development

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## Abstract

The etiology and pathogenesis of craniofacial birth defects are multifactorial and include both genetic and environmental factors. Despite the identification of numerous genes associated with congenital craniofacial anomalies, our understanding of their etiology remains incomplete, and many affected individuals have an unknown genetic diagnosis. Here, we show that conditional loss of a Mediator complex subunit protein, Med23 in mouse neural crest cells (*Med23<sup>fl/fl</sup>;Wnt1-Cre*), results in micrognathia, glossoptosis, and cleft palate, mimicking the phenotype of Pierre Robin sequence. *Sox9* messenger RNA and protein levels are both upregulated in neural crest cell–derived mesenchyme surrounding Meckel's cartilage and in the palatal shelves in *Med23<sup>fl/fl</sup>;Wnt1-Cre* mutant embryos compared to controls. Consistent with these observations, we demonstrate that Med23 binds to the promoter region of *Sox9* and represses *Sox9* expression in vitro. Interestingly, *Sox9* binding to  $\beta$ -catenin is enhanced in *Med23<sup>fl/fl</sup>;Wnt1-Cre* mutant embryos, which, together with downregulation of *Col2a1* and Wnt signaling target genes, results in decreased proliferation and altered jaw skeletal differentiation and cleft palate. Altogether, our data support a cell-autonomous requirement for Med23 in neural crest cells, potentially linking the global transcription machinery through Med23 to the etiology and pathogenesis of craniofacial anomalies such as micrognathia and cleft palate.

**Keywords:** neural crest cells, micrognathia, cleft palate, Mediator, Pierre Robin sequence, cleidocranial dysplasia

## Introduction

The craniofacial complex comprises specialized tissues and organs, including the primary sense organs, central and peripheral nervous systems, and musculoskeletal components of the head and neck. Anatomically, it is the most complex region of the human body and is developmentally prone to genetic and environmental insult, resulting in craniofacial anomalies. Jaw, palate, and skull malformations are among the most common human birth defects and have considerable functional, aesthetic, and social consequences. Despite advances in phenotyping and genotyping, many affected individuals have an unknown genetic diagnosis. Therefore, it is imperative to identify novel genetic and environmental factors and the cellular mechanisms they regulate during normal craniofacial development and in disease.

Neural crest cells (NCCs) comprise a migratory progenitor cell population essential for vertebrate development. Born in the neuroepithelium during early embryogenesis, NCCs undergo an epithelial to mesenchymal transition, delaminate, migrate, and differentiate into most of the cartilage, bone, and connective tissues of the head and face. Most craniofacial disorders are associated with defects in NCC development, but depending on which phase of NCC formation, migration, or differentiation is disrupted, distinct malformations such as micrognathia, cleft palate, or cleidocranial dysplasia can occur.

Mediator is a multiprotein complex that plays an indispensable role in regulating gene transcription (Conaway and Conaway 2013). Recently, Mediator has emerged as an integral coordinator of development and cell lineage determination with evidence for roles in development, metabolism, and cancer (Yin and Wang 2014). Mediator may, therefore, function as

a centralized hub that integrates multiple aspects of transcriptional regulation to ensure the proper pattern, timing, and intensity of global gene expression during development and in the etiology and pathogenesis of disease (Malik and Roeder 2000; Carlsten et al. 2013).

We previously described a novel allele in the Mediator complex subunit, *Med23*, termed *snouty (sn)* (Sandell et al. 2011; Dash et al. 2020). *Med23<sup>sn/sn</sup>* mutant mice exhibit craniofacial defects such as a shortened frontonasal prominence and hypoplastic pharyngeal arches, and they are embryonic lethal around E10.5. To bypass this lethality and demonstrate a cell-autonomous function for Med23 in NCC and craniofacial development, we generated a conditional knockout of *Med23*, which resulted in micrognathia, glossoptosis, cleft palate, and cleidocranial dysplasia. We subsequently determined Med23 to be a direct upstream repressor of *Sox9* in mice. Loss of Med23 results in increased *Sox9* expression and *Sox9*– $\beta$ -catenin protein interactions, which leads to decreased *Col2a1* and downstream components of Wnt signaling, as well as perturbation of NCC-derived palatal mesenchyme proliferation

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A supplemental appendix to this article is available online.

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and osteochondrogenitor differentiation. Thus, Med23 potentially links the global transcription machinery in NCC to cartilage and bone differentiation during craniofacial development.

## Materials and Methods

Details of the methods are provided in the Appendix.

## Results

### NCC-Specific Deletion of Med23 Results in Defects in Meckel's Cartilage Development and Cleft Palate

To investigate the cell-autonomous function of Med23, we generated NCC-specific mutants of Med23 by intercrossing *Med23<sup>fx/fx</sup>* mice with *Wnt1-Cre* mice. *Med23<sup>fx/fx</sup>;Wnt1-Cre* mutant embryos are morphologically indistinguishable from *Med23<sup>+/fx</sup>;Wnt1-Cre* control littermates until E12.5 (Fig. 1A, B). Furthermore, NCC migration and differentiation into neurons of the peripheral nervous system are comparable between mutant and control littermates at E10.5 as observed by *Sox10* in situ hybridization (Appendix Fig. 1) and Tuj1 immunostaining (Dash et al. 2020). However, by E14.5, *Med23<sup>fx/fx</sup>;Wnt1-Cre* embryos exhibit severe craniofacial defects, including micrognathia (small jaw) and glossoptosis (abnormally positioned tongue) (Fig. 1C, D). By postnatal day (P)0, the severity of micrognathia and glossoptosis is further evident, and difficulty in breathing and feeding causes neonatal lethality (Fig. 1E, F). To better define the cranioskeletal defects, we performed bone and cartilage staining beginning at E12.5 and observed a lack of Meckel's cartilage (MC) development in *Med23<sup>fx/fx</sup>;Wnt1-Cre* mutants compared to controls (Fig. 1G and H). By E14.5, *Med23<sup>fx/fx</sup>;Wnt1-Cre* mutants exhibit severely hypoplastic MCs (Fig. 1I, J), and because MC provides a scaffold for mandible development, its smaller size likely contributes to a smaller mandible at P0. In addition, *Med23<sup>fx/fx</sup>;Wnt1-Cre* mutants exhibit agenesis of the palatine bones and nasal cartilage and bones, together with abnormal development of the tympanic ring and skull bones, which result in cleidocranial dysplasia (Fig. 1K, L; Appendix Fig. 2).

*Med23<sup>fx/fx</sup>;Wnt1-Cre* embryos also present with defects in teeth development. While the molars in control embryos differentiate to the cap stage by E14.5 and late bell stage by E17.5, the molars in *Med23<sup>fx/fx</sup>;Wnt1-Cre* embryos are developmentally delayed at the bud stage at E14.5 and early bell stage at E17.5. Similarly, development of the upper incisors is also delayed in *Med23<sup>fx/fx</sup>;Wnt1-Cre* embryos having only reached the bell stage, compared to control embryos where the upper incisors are transitioning from the late bell to eruption stage. Furthermore, the lower incisors are also considerably smaller in *Med23<sup>fx/fx</sup>;Wnt1-Cre* mutants compared to controls (Fig. 1M–T).

The absence of the palatine bones in *Med23<sup>fx/fx</sup>;Wnt1-Cre* mutant embryos is indicative of cleft palate and is clearly observed by pseudo-scanning electron microscopy (SEM)

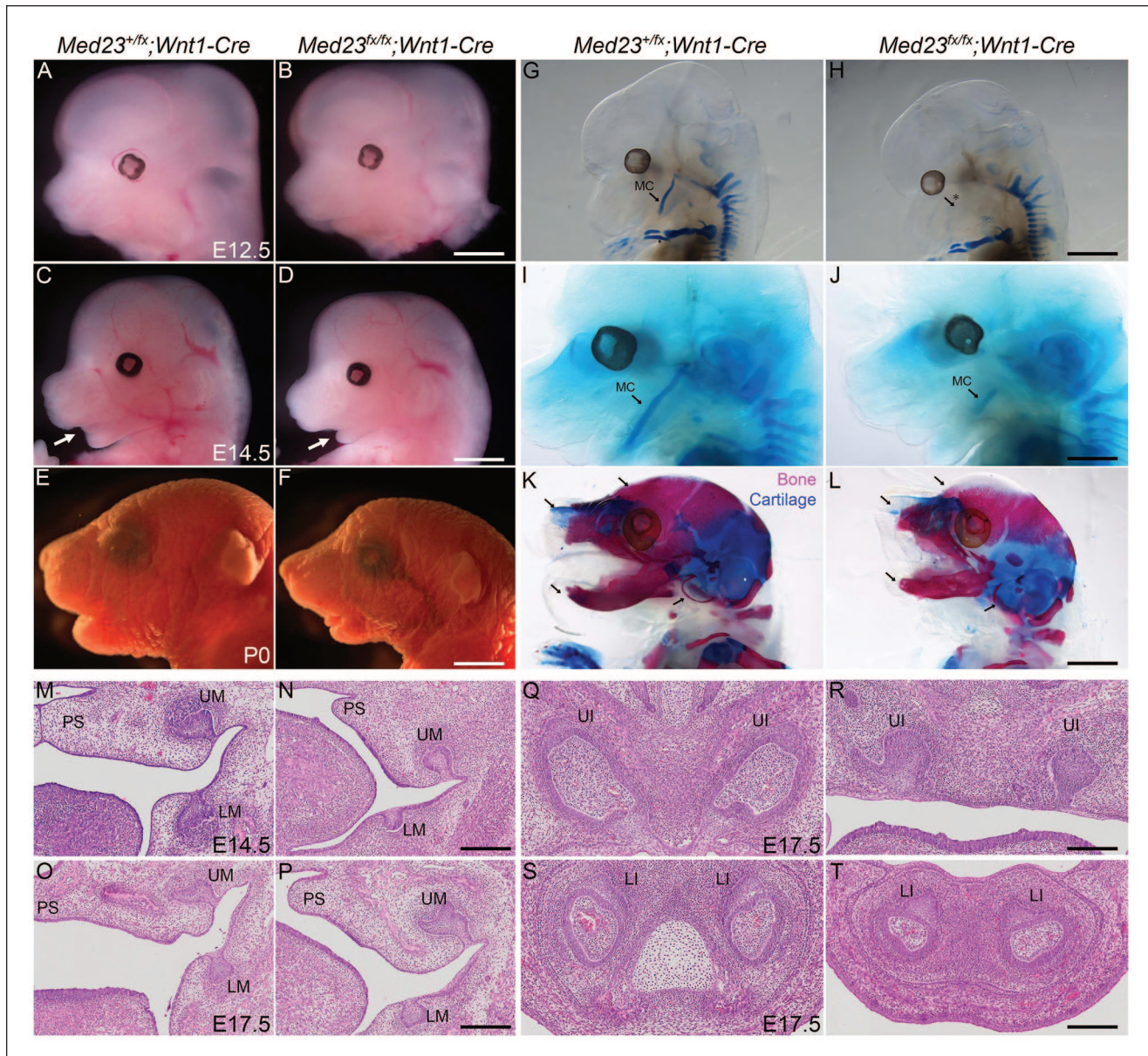
imaging (Sandell et al. 2012) in E14.5 embryos (Fig. 2A, B). The presence of cleft palate is also evident in histological sections wherein the palatal shelves remain separated by the tongue in association with apparent failure of palatal shelf elevation and fusion (Fig. 2C, D'). By E17.5, the palatal shelves in the anterior of mutant embryos have elevated above the tongue but still remain far apart. The posterior palatal shelves fail to elevate and remain well separated by the tongue (Appendix Fig. 3). This indicates that palatal shelf elevation is incomplete, which contributes to the failure of palatal shelf fusion and thus cleft palate in *Med23<sup>fx/fx</sup>;Wnt1-Cre* embryos.

### Failure of Palate Fusion in *Med23<sup>fx/fx</sup>;Wnt1-Cre* Embryos Is Independent of Tongue Development

We hypothesized that the presence of the tongue between the palatal shelves in *Med23<sup>fx/fx</sup>;Wnt1-Cre* embryos may serve as a physical impediment to palatal shelf fusion. To test this hypothesis, we dissected the maxillary apparatus from control and mutant embryos at E13.5 and cultured them ex vivo in roller culture. Prior to culture, the palatal shelves were not fused in either *Med23<sup>+/fx</sup>;Wnt1-Cre* control or *Med23<sup>fx/fx</sup>;Wnt1-Cre* mutant embryos (Fig. 2E, F). However, after 72 h culture, the control palatal shelves developed rugae and fused. In contrast, although rugae formed in *Med23<sup>fx/fx</sup>;Wnt1-Cre* embryos, the palatal shelves remained separated and unfused (Fig. 2G, H). This indicates that cleft palate in *Med23<sup>fx/fx</sup>;Wnt1-Cre* mutant embryos is independent of tongue development or its position.

### *Sox9* Is Upregulated in *Med23<sup>fx/fx</sup>;Wnt1-Cre* Palatal Shelf Mesenchyme

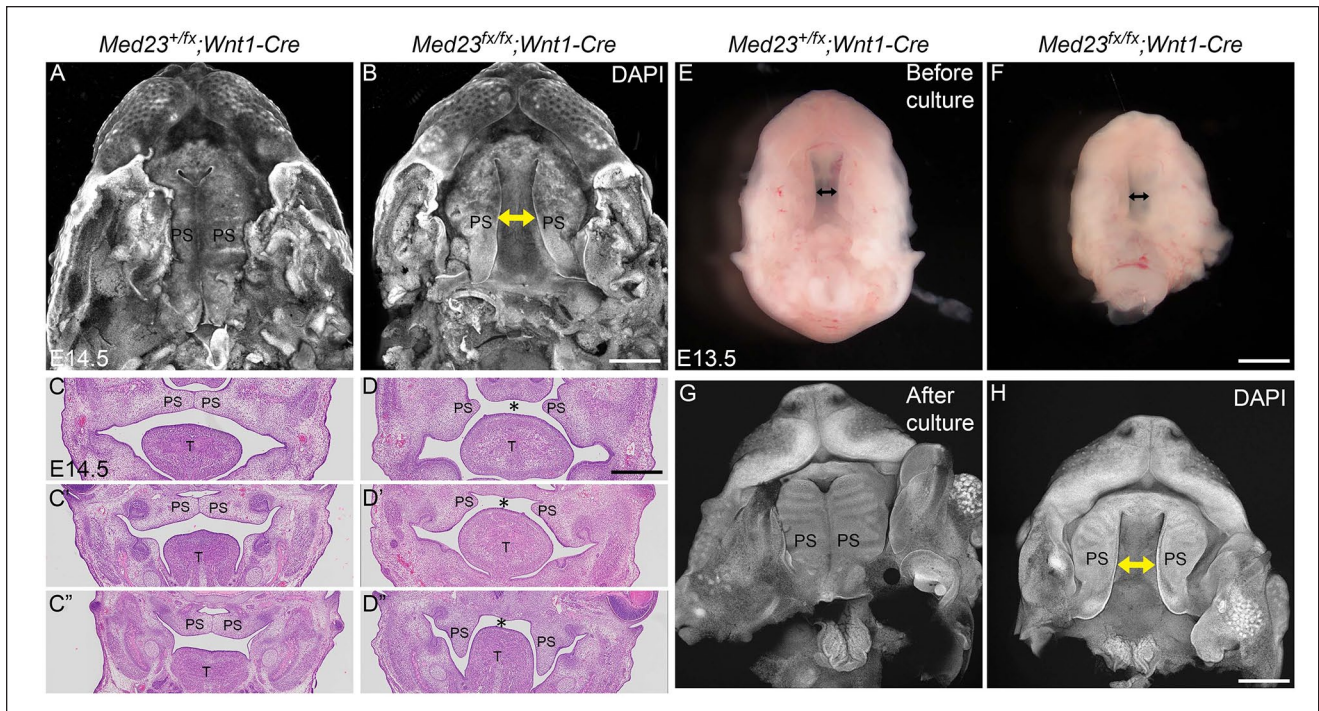
The micrognathia, cleft palate, and glossoptosis observed in *Med23<sup>fx/fx</sup>;Wnt1-Cre* mutants resemble the characteristics of Pierre Robin sequence (PRS) in humans, which is associated with mutations in an enhancer of *SOX9* (Benko et al. 2009; Selvi and Mukunda Priyanka 2013). *SOX9* is an early marker of migratory NCC and a master regulator of chondrogenesis (Lefebvre et al. 1997; Cheung and Briscoe 2003). *SOX9* directly binds to and activates chondrocyte-specific genes, including *COL2A1* (Bell et al. 1997; Lefebvre et al. 1997; Ng et al. 1997). Therefore, we examined the expression of *Sox9* in *Med23<sup>+/fx</sup>;Wnt1-Cre* and *Med23<sup>fx/fx</sup>;Wnt1-Cre* embryos by in situ hybridization and immunostaining, which revealed that both *Sox9* RNA and protein are upregulated in the mutants, especially in the frontonasal prominence, and NCCs colonizing the first pharyngeal arch between E9.5 and E11.5 (Appendix Fig. 4A–H). The upregulation of *Sox9* is also clearly evident at E12.5 and E14.5, when *Sox9* protein is ectopically expressed in the palatal shelves and in the region that condenses to form MC in *Med23<sup>fx/fx</sup>;Wnt1-Cre* embryos (Fig. 3A, B; Appendix Fig. 4I–L, O, P). Alcian blue counterstaining revealed that MC does not properly condense and is loosely packed in mutant embryos compared to controls (Appendix Fig. 4K', L').



**Figure 1.** Neural crest cell-specific deletion of *Med23* causes craniofacial defects. (A–F) Bright-field images of *Med23*<sup>+/fx</sup>;*Wnt1-Cre* and *Med23*<sup>fx/fx</sup>;*Wnt1-Cre* embryos indicate that the mutant embryos have no overt phenotype at E12.5. By E14.5, the mutant embryos have a smaller lower jaw. At P0, the lower jaw is still hypoplastic and the tongue is abnormally positioned in the mutants compared to controls. (G–L) Skeletal staining of *Med23*<sup>+/fx</sup>;*Wnt1-Cre* and *Med23*<sup>fx/fx</sup>;*Wnt1-Cre* embryos indicates that Meckel's cartilage development is delayed at E12.5 and hypoplastic at E14.5 in *Med23*<sup>fx/fx</sup>;*Wnt1-Cre* embryos. At P0, the mandible is smaller in mutants compared to controls. Furthermore, the incisors are underdeveloped, the nasal cartilage is absent, and the otic bones are hypoplastic in *Med23*<sup>fx/fx</sup>;*Wnt1-Cre* embryos (indicated by arrows). (M–T) Histology of *Med23*<sup>+/fx</sup>;*Wnt1-Cre* and *Med23*<sup>fx/fx</sup>;*Wnt1-Cre* embryos suggests that development of the upper and lower molars as well as upper and lower incisors is delayed in the *Med23*<sup>fx/fx</sup>;*Wnt1-Cre* embryos compared to *Med23*<sup>+/fx</sup>;*Wnt1-Cre* control embryos. LI, lower incisor; LM, lower molar; MC, Meckel's cartilage; PS, palatal shelf; UI, upper incisor; UM, upper molar. Scale bar for A, B is 200 mm; C, D is 250 mm; E, F is 350 mm; G–J is 200 mm; K, L is 300 mm; M, N is 70 mm; and O–T is 45 mm.

The upregulation of both *Sox9* transcript and protein in *Med23*<sup>fx/fx</sup>;*Wnt1-Cre* embryos suggests that *Med23* either directly or indirectly regulates *Sox9* transcription. To test this hypothesis, we performed chromatin immunoprecipitation (ChIP) on wild-type E13.5 palatal tissue using a *Med23*-specific antibody. We observed *Med23* binding to 2 regions

upstream of the *Sox9* transcription start site (TSS) (Fig. 3C). The –58-bp to –207-bp region upstream of the *Sox9* TSS has previously been identified to contain the minimal promoter of *Sox9* (Kanai and Koopman 1999). However, *Med23* also binds to –233 bp to –424 bp upstream of the *Sox9* TSS, which is an unannotated region. To identify if *Med23* binds to this region



**Figure 2.** *Med23<sup>fl/fl</sup>;Wnt1-Cre* embryos exhibit cleft palate. (A, B) Pseudo-scanning electron microscopy imaging of the palate of *Med23<sup>+/fl</sup>;Wnt1-Cre* and *Med23<sup>fl/fl</sup>;Wnt1-Cre* embryos at E14.5 shows that the palatal shelves in *Med23<sup>fl/fl</sup>;Wnt1-Cre* embryos remain far apart, which is indicative of cleft palate. (C, D') Histological analysis of *Med23<sup>+/fl</sup>;Wnt1-Cre* and *Med23<sup>fl/fl</sup>;Wnt1-Cre* palatal shelves at E14.5 shown anterior (C, D) to posterior (C', D') revealed that the palatal shelves in *Med23<sup>fl/fl</sup>;Wnt1-Cre* embryos are hypoplastic in the anterior region, while in the posterior region, palatal shelf growth is vertical beside the tongue. Asterisk denotes cleft palate. (E, F) Bright-field images of the palatal shelves at E13.5 prior to ex vivo culture illustrate that the palatal shelves are not fused at this stage in *Med23<sup>+/fl</sup>;Wnt1-Cre* and *Med23<sup>fl/fl</sup>;Wnt1-Cre* embryos. (G, H) Seventy-two hours after ex vivo roller culture, the palatal shelves of *Med23<sup>+/fl</sup>;Wnt1-Cre* have fused, while those of *Med23<sup>fl/fl</sup>;Wnt1-Cre* embryos remain unfused. Double-ended arrows indicate the unfused palatal shelves. PS, palatal shelf; T, tongue. Scale bar for A, B is 200  $\mu$ m; C–D' is 180  $\mu$ m; E–H is 200  $\mu$ m.

in a tissue that expresses Sox9, other than palatal mesenchyme, we performed ChIP on E13.5 limbs. We observed that Med23 binds only the minimal promoter of *Sox9* and not the  $-233$  to  $-424$  region (Fig. 3D), suggesting that binding to this region might be required for tissue-specific regulation of *Sox9* in palatal mesenchyme.

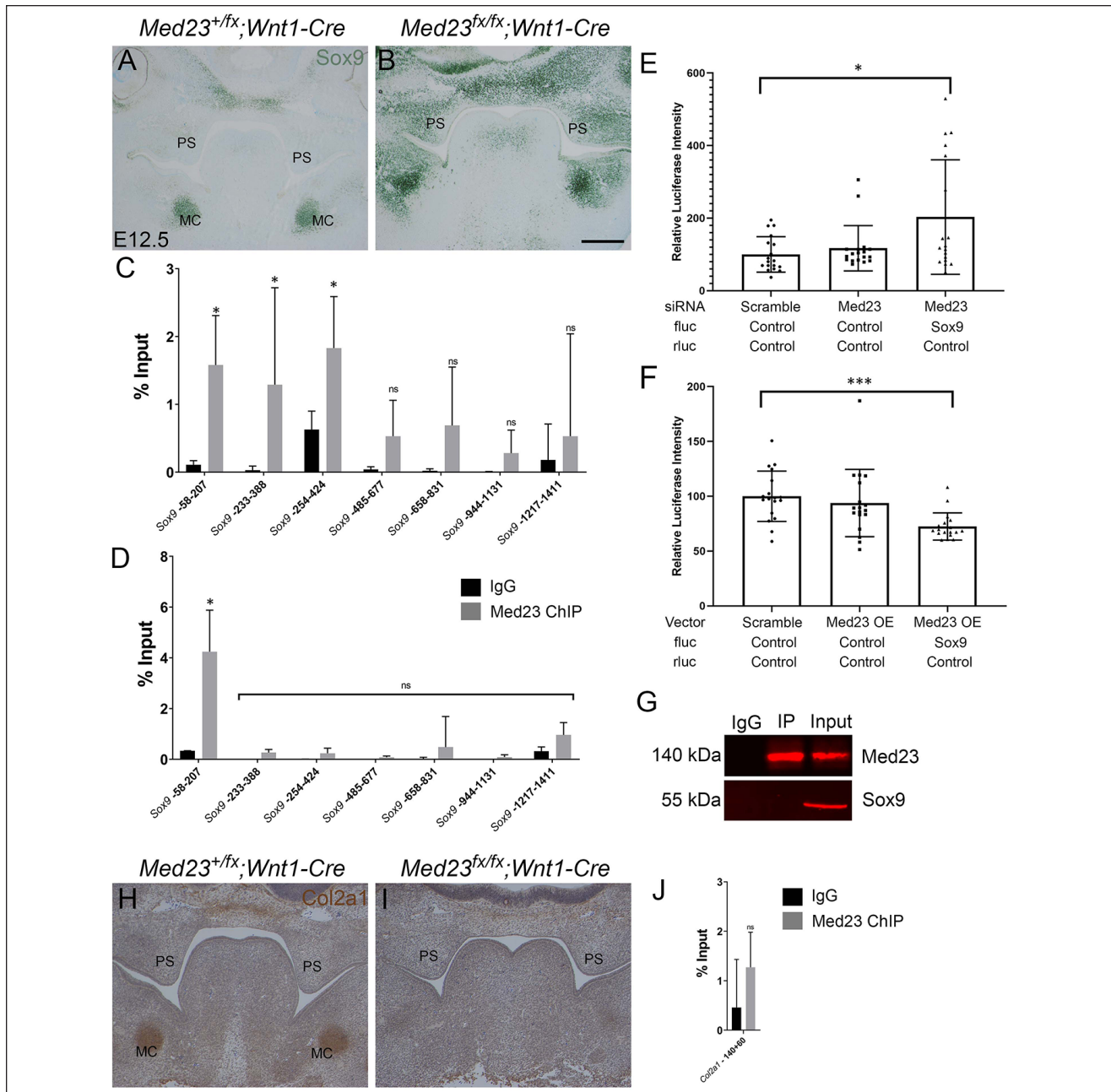
To determine if Med23 binding to the *Sox9* promoter region is functional, we performed a luciferase assay in Swiss-3T3 cells. When Med23 is knocked down by 50% using small interfering RNA (siRNA), luciferase activity under control of the *Sox9* promoter region increased (Fig. 3E). Conversely, a 50% increase in Med23 expression resulted in decreased luciferase activity (Fig. 3F). This suggests that Med23 binds to the promoter region of *Sox9* and represses Sox9.

Sox9 positively regulates *Col2a1* transcription (Bell et al. 1997; Lefebvre et al. 1997) and we, therefore, hypothesized that increased Sox9 expression should result in *Col2a1* upregulation. *Col2a1* is expressed in MC and nasal cartilage in E12.5 *Med23<sup>+/fl</sup>;Wnt1-Cre* embryos. However, *Col2a1* was surprisingly downregulated in the presumptive MC of *Med23<sup>fl/fl</sup>;Wnt1-Cre* mutants (Fig. 3H, I). The downregulation of *Col2a1* in the presence of elevated Sox9 is suggestive of an abrogation of Sox9 function and cranioskeletal maturation in *Med23<sup>fl/fl</sup>;Wnt1-Cre* embryos.

To determine if Med23 directly regulates *Col2a1* expression, we performed ChIP-quantitative polymerase chain reaction (qPCR) on the Sox9 binding region of *Col2a1* promoter but observed no binding between Med23 and the *Col2a1* promoter (Fig. 3J). We also performed coimmunoprecipitation of Med23 and Sox9 in E12.5 wild-type palatal shelves to determine if Med23 facilitated Sox9 binding at the *Col2a1* promoter but did not identify Sox9 to be a protein binding partner of Med23 (Fig. 3G). This suggests that Med23 is not directly required for Sox9-mediated transcriptional control of *Col2a1* and that the decrease in *Col2a1* occurs via another mechanism.

### Med23 Binds to Runx2 Protein but Does Not Regulate Its Expression

Our data revealed that Med23 loss of function in NCC results in micrognathia, cleft palate, and cleidocranial dysplasia. Runx2 is a master regulator of osteoblast differentiation (Ducy et al. 1997; Komori et al. 1997), and mutations in *RUNX2* are associated with cleidocranial dysplasia in humans (Mundlos et al. 1997). We therefore examined the differentiation of NCC-derived mesenchymal cells into primary osteoblasts at E12.5 via Runx2 immunostaining and observed the spatiotemporal



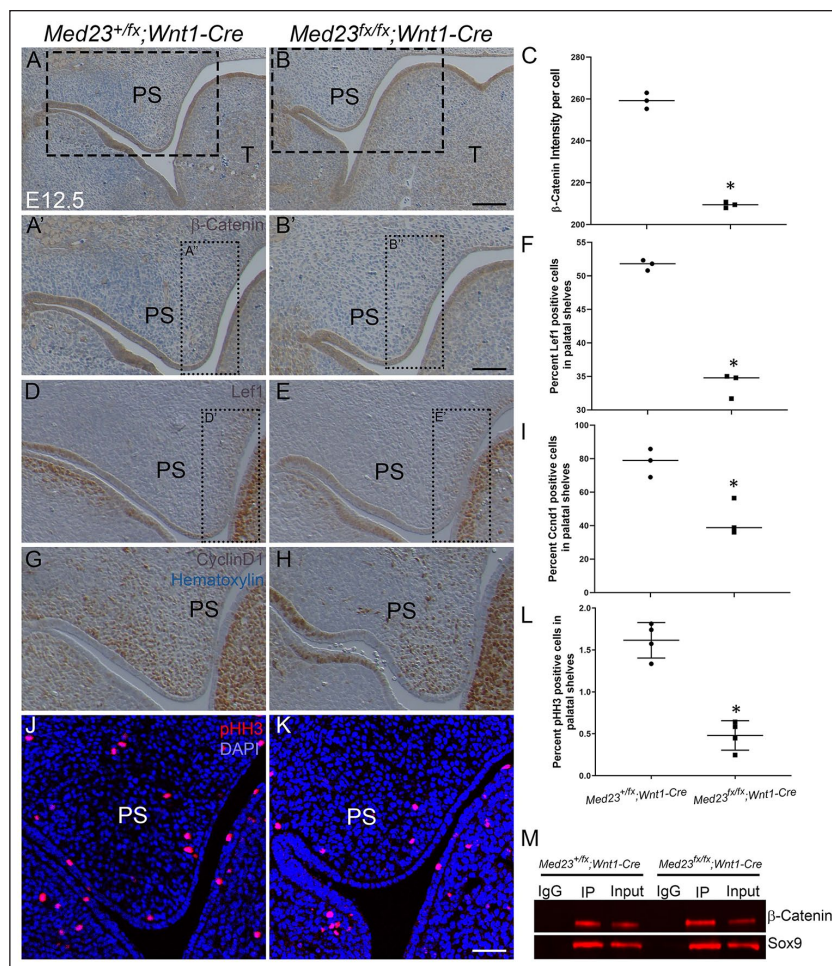
**Figure 3.** Sox9 is misexpressed in *Med23<sup>fx/fx</sup>;Wnt1-Cre* embryos. (A, B) Sox9 is overexpressed in the palatal shelves and presumptive Meckel's cartilages of *Med23<sup>fx/fx</sup>;Wnt1-Cre* embryos, as observed by immunostaining. (C) Chromatin immunoprecipitation performed on E12.5 wild-type palatal shelves using a Med23 antibody demonstrates that Med23 binds to the minimal promoter (-58 to -207) of *Sox9* as well as to a region -233 to -424 bp upstream of the *Sox9* transcription start site. (D) Chromatin immunoprecipitation performed on E13.5 wild-type limbs revealed Med23 binds to the minimal promoter of *Sox9* but not to the -233-bp to -424-bp region upstream of the *Sox9* transcription start site. (E) Luciferase activity is increased in 3T3-Swiss albino cells when the cells are cotransfected with small interfering RNA (siRNA) against Med23 and *Sox9* enhancer-*Luc* vector compared to controls that are cotransfected with scramble siRNA and *PGL3*-enhancer vector and siRNA against Med23 and *Sox9* enhancer-*Luc* vector. (F) On the other hand, luciferase activity is decreased when cells are cotransfected with *Sox9* enhancer-*Luc* vector and Med23 overexpression vector compared to controls. (G) Coimmunoprecipitation of Med23 with Sox9 shows that Med23 and Sox9 do not form a complex. (H, I) Col2a1 immunostaining in the palatal shelves demonstrates that Col2a1 is significantly reduced in Meckel's cartilages in *Med23<sup>fx/fx</sup>;Wnt1-Cre* embryos compared to *Med23<sup>+/fx</sup>;Wnt1-Cre* controls. (J) Chromatin immunoprecipitation performed on wild-type palatal shelves using a Med23 antibody, followed by quantitative polymerase chain reaction of the *Sox9* binding region of the *Col2a1* promoter, indicates that Med23 does not bind to *Col2a1* promoter. MC, Meckel's cartilage; PS, palatal shelf. Asterisk denotes statistical significance with  $P < 0.05$ . Scale bar for A-F is 120  $\mu$ m.

patterns of Runx2 expression to be similar in the mesenchymal cells surrounding MC in *Med23<sup>+/fx</sup>;Wnt1-Cre* and *Med23<sup>fx/fx</sup>;Wnt1-Cre* embryos (Appendix Fig. 5A–C). In addition, ChIP-qPCR performed on the P1 promoter of *Runx2* indicated that Med23 does not directly bind to the *Runx2* promoter (Appendix Fig. 5D) and thus does not directly regulate *Runx2* expression.

However, to further understand potential defects in osteoblast differentiation, we performed alkaline phosphatase staining and observed that while the mesenchyme had begun differentiating in E12.5 *Med23<sup>+/fx</sup>;Wnt1-Cre* controls, there were no differentiating cells observed in *Med23<sup>fx/fx</sup>;Wnt1-Cre* mutants (Appendix Fig. 5E, F). Reduced endogenous alkaline phosphatase staining was observed in the mesenchyme surrounding MC in E14.5 *Med23<sup>fx/fx</sup>;Wnt1-Cre* mutants compared to controls (Appendix Fig. 4K, L). This suggests that while *Runx2* expression is not affected in *Med23<sup>fx/fx</sup>;Wnt1-Cre* mutants, early osteogenic differentiation is perturbed. Because *Runx2* and Med23 proteins are known to interact in mouse preosteoblast cell lines (Liu et al. 2016), we performed Med23-Runx2 coimmunoprecipitation using E12.5 wild-type palatal shelves and observed that Med23 indeed binds to Runx2 (Appendix Fig. 5G). This suggests that Med23 may function as a cofactor for Runx2 in mediating transcriptional control of its target genes.

### *β-Catenin Is Downregulated in the Palatal Mesenchyme of Med23<sup>fx/fx</sup>;Wnt1-Cre Embryos*

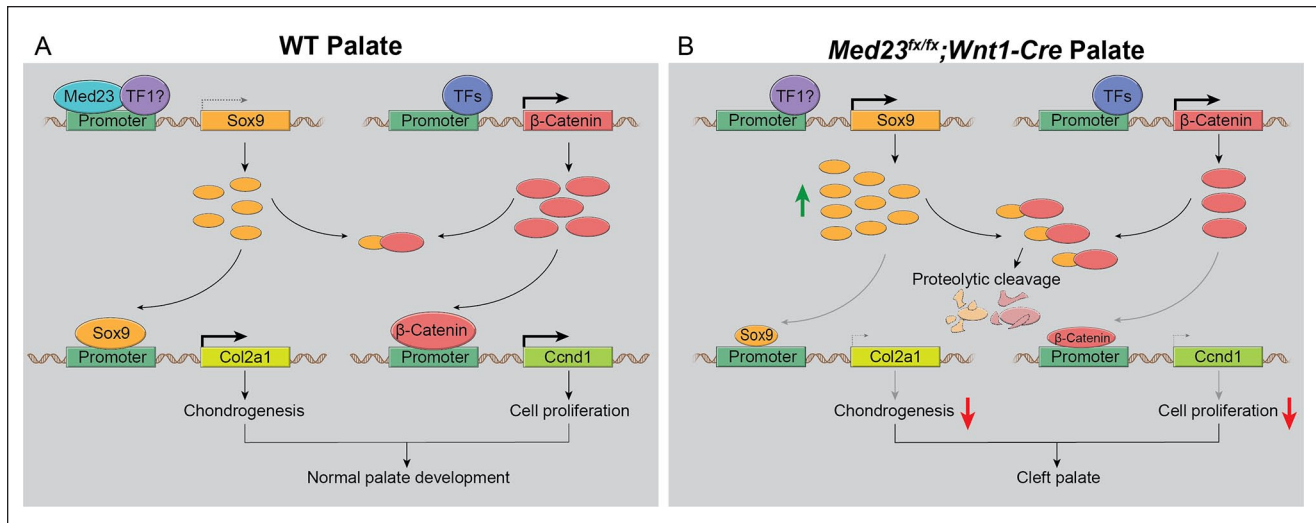
Sox9 upregulation has previously been shown to result in cleft palate in mice in association with downregulated Wnt signaling (Mori-Akiyama et al. 2003; Akiyama et al. 2004). Briefly, Sox9 overexpression leads to a dose-dependent increase in binding to  $\beta$ -catenin, which results in inactivation of both Sox9 and  $\beta$ -catenin, diminished Sox9 transcriptional activation of *Col2a1*, and downregulation of  $\beta$ -catenin target genes. We therefore immunostained sections of E12.5 *Med23<sup>+/fx</sup>;Wnt1-Cre* and *Med23<sup>fx/fx</sup>;Wnt1-Cre* embryos with  $\beta$ -catenin and observed that  $\beta$ -catenin is downregulated in the palatal mesenchyme in association with upregulated Sox9 expression (Fig. 4A–C, A', B'; Appendix Fig. 6A, B). Furthermore, we determined that *Lef1* and *Ccnd1*, which are downstream targets of  $\beta$ -catenin, were also downregulated in the palatal shelves of *Med23<sup>fx/fx</sup>;Wnt1-Cre* mutant embryos compared to



**Figure 4.**  $\beta$ -Catenin is downregulated in *Med23<sup>fx/fx</sup>;Wnt1-Cre* palatal shelves. (A–C)  $\beta$ -Catenin is downregulated specifically in the palatal shelves of *Med23<sup>fx/fx</sup>;Wnt1-Cre* embryos as observed by immunostaining with an antibody against  $\beta$ -catenin. The sections are counterstained with hematoxylin to visualize nuclei. Immunostaining with downstream targets of  $\beta$ -catenin, *Lef1* (D–F), and *Ccnd1* (G–I) demonstrates the number of palatal shelf mesenchymal cells expressing *Lef1* and *Ccnd1* is severely reduced in the *Med23<sup>fx/fx</sup>;Wnt1-Cre* embryos. (J–L) The number of proliferating cells is also significantly reduced in the *Med23<sup>fx/fx</sup>;Wnt1-Cre* palatal shelves compared to *Med23<sup>+/fx</sup>;Wnt1-Cre*. (M) Immunoprecipitation with Sox9 and immunoblotting with  $\beta$ -catenin using palatal shelves from E12.5 *Med23<sup>+/fx</sup>;Wnt1-Cre* and *Med23<sup>fx/fx</sup>;Wnt1-Cre* embryos indicates that the binding of Sox9 and  $\beta$ -catenin is higher in *Med23<sup>fx/fx</sup>;Wnt1-Cre* palatal shelves, despite  $\beta$ -catenin itself being downregulated as observed by band size in input lanes. The concentration of total protein in the input lanes is 10% of the concentration used for the immunoprecipitation experiment. PS, palatal shelf. Asterisk denotes statistical significance with  $P < 0.05$ . Scale bar for A, B is 160  $\mu$ m; A', B', D, E, G, H, J, K is 70  $\mu$ m.

controls (Fig. 4D–I; Appendix Fig. 6C, D). To test if Med23 directly regulates *Lef1* and *Ccnd1*, we performed ChIP-qPCR for known transcription factor binding sites of *Lef1* and *Ccnd1*. We observed no binding of Med23 to the promoters of *Lef1* and *Ccnd1* (Appendix Fig. 6E, F), suggesting that the downregulation of these genes is a consequence of diminished  $\beta$ -catenin activity independent of Med23.

Jaw, orofacial clefts, and skull vault disorders have each been attributed to mutations in WNT signaling component genes (Manocha et al. 2019; Reynolds et al. 2019). Since Wnt signaling is a critical regulator of cell proliferation through *Ccnd1*, we hypothesized that decreased proliferation may



**Figure 5.** Sox9 and β-catenin bind to each other in the palatal shelves. **(A)** In wild-type palatal shelves, Med23 represses the transcription of Sox9 (denoted by dashed arrow), probably in concert with other transcription factors. Sox9, when expressed in moderation, enables the transcription of its downstream targets such as Col2a1, thus regulating chondrogenesis. Meanwhile, β-catenin is also expressed in palatal shelf mesenchymal cells and enhances the expression of targets such as Ccnd1, thereby promoting cell proliferation. Together, Sox9 and β-catenin promote normal palate development. **(B)** In *Med23<sup>fx/fx</sup>;Wnt1-Cre* mutant palatal shelves, the absence of Med23 leads to upregulation of Sox9 (indicated by green arrow), which exhibits increased binding to β-catenin. Sox9 and β-catenin interactions can promote mutual proteolytic cleavage and inactivation of both proteins. However, Sox9 and β-catenin binding can also inhibit Sox9 from activating *Col2a1* transcription. Collectively, this leads to downregulation of downstream targets of both Sox9 and β-catenin, such as *Col2a1* and *Ccnd1*, respectively (indicated by dashed arrow), in the pathogenesis of cleft palate.

underpin the pathogenesis of cleft palate in *Med23<sup>fx/fx</sup>;Wnt1-Cre* mutant embryos. Phospho-histone H3 staining revealed fewer proliferating cells in the palatal shelves of E12.5 *Med23<sup>fx/fx</sup>;Wnt1-Cre* mutants compared to controls, further corroborating our β-catenin and *Ccnd1* immunostaining data (Fig. 4J–L). Although the number of proliferating cells was similar between controls and mutants at E14.5, by this stage, the palatal shelves of *Med23<sup>fx/fx</sup>;Wnt1-Cre* mutants were considerably smaller compared to controls, which affects their ability to meet in the midline (Appendix Fig. 7G–I). We also tested for apoptosis during palatogenesis via Cleaved-Caspase3 immunostaining and observed a similar number of apoptotic cells in controls and mutants (Appendix Fig. 7A–F). This suggests that reduced proliferation at E12.5 results in lower cell density by E14.5 and that fewer proliferating cells in the palatal mesenchyme limit the ability of the palatal shelves to grow and fuse to form a normal palate in *Med23<sup>fx/fx</sup>;Wnt1-Cre* embryos.

### Sox9 and β-Catenin Have Increased Interaction in *Med23<sup>fx/fx</sup>;Wnt1-Cre* Palatal Shelves

To test if Sox9 upregulation results in increased Sox9 and β-catenin interaction, we performed immunoprecipitation with a Sox9-specific antibody on E12.5 *Med23<sup>fx/fx</sup>;Wnt1-Cre* and *Med23<sup>fx/fx</sup>;Wnt1-Cre* palatal shelves. We found that β-catenin pull-down via Sox9 immunoprecipitation is higher in *Med23<sup>fx/fx</sup>;Wnt1-Cre* mutants compared to controls (Fig. 4M; Appendix Fig. 6G). Altogether, our data suggest that Med23 is required to repress Sox9 transcription, which helps to maintain optimal Sox9 expression in the palatal shelves. In the absence of

Med23, Sox9 expression increases, resulting in elevated Sox9–β-catenin interaction and decreased Wnt signaling. Collectively, this leads to perturbation of palatal shelf mesenchyme proliferation and osteoprogenitor differentiation, which underpins the pathogenesis of micrognathia and cleft palate in *Med23<sup>fx/fx</sup>;Wnt1-Cre* embryos.

## Discussion

Med23 is a tail subunit protein of the Mediator complex, which is required for enabling transcription by RNA polymerase II (Monté et al. 2018). Along with other tail complex proteins, Med23 connects the Mediator complex with sequence-specific transcription factors such as Elk1 (Stevens et al. 2002), Runx2 (Liu et al. 2016), and Irf7 (Griffiths et al. 2013). In addition, Med23 has been shown to function during transcript elongation (Wang et al. 2013), splicing (Huang et al. 2012), and chromatin modification (Yao et al. 2015). Here, we demonstrate that Med23 is required for craniofacial development, and its deletion in NCC results in micrognathia, cleft palate, glossoposis, teeth defects, and cleidocranial dysplasia.

In humans, mutations in the enhancer and exons of *SOX9* are associated with PRS (Jakobsen et al. 2007; Benko et al. 2009; Selvi and Mukunda Priyanka 2013). The *SOX9* enhancer region, denoted as HCNE-F2, can drive the expression of LacZ in craniofacial tissues in E11.5 transgenic reporter mouse embryos and contains binding sites for transcription factors such as MSX1, EN1, and ZNF628 (Benko et al. 2009). Since *SOX9* is required for chondrogenesis in the craniofacial and appendicular skeleton, mutations in this tissue-specific

enhancer region could explain craniofacial bone and cartilage defects without limb defects in patients with PRS.

Both overexpression and downregulation of Sox9 result in cleft palate and severe chondrodysplasia in mice, indicating that Sox9 expression needs to be tightly regulated for proper chondrogenesis (Mori-Akiyama et al. 2003; Akiyama et al. 2004). We hypothesize a similar mechanism in *Med23<sup>fx/fx</sup>; Wnt1-Cre* embryos where absence of Med23 lifts transcriptional repression of Sox9, resulting in its overexpression. Sox9, when overexpressed, has previously been shown to increase its binding with  $\beta$ -catenin, thereby reducing the pool of free Sox9 and  $\beta$ -catenin, resulting in reduced expression of their respective downstream genes such as *Col2a1* and *Cnd1*. Furthermore, the physical interaction of Sox9 and  $\beta$ -catenin triggers the ubiquitination/proteasome pathway, resulting in inactivation and degradation of both proteins (Akiyama et al. 2004). Similar to Sox9,  $\beta$ -catenin is essential for palatal development and growth, and it functions by interacting with cadherins that are required during chondrogenic mesenchymal condensation (Delise and Tuan 2002).  $\beta$ -Catenin controls cell proliferation, migration, and differentiation during embryonic development, and its ablation results in orofacial clefts (He et al. 2011). Thus, in *Med23<sup>fx/fx</sup>; Wnt1-Cre* embryos, a diminished pool of free  $\beta$ -catenin reduces the expression of downstream targets of  $\beta$ -catenin that are required for proliferation of palatal mesenchymal cells. Decreased cell proliferation at this crucial developmental stage results in smaller palatal shelves, contributing to the pathogenesis of cleft palate (Fig. 5).

While we expected upregulation of the Sox9 downstream target *Col2a1*, and consequently increased chondrogenesis in *Med23<sup>fx/fx</sup>; Wnt1-Cre* embryos, instead we observed downregulation of *Col2a1* and delayed chondrogenesis. Interestingly, Sox9- $\beta$ -catenin binding has been shown to diminish the amount of Sox9 freely available in progenitor cells, as well as inhibit Sox9's ability to transcriptionally activate *Col2a1*, which collectively can account for the reduction in *Col2a1* activity in *Med23<sup>fx/fx</sup>; Wnt1-Cre* embryos. However, we cannot rule out that Med23 may regulate other factors critical for *Col2a1* expression. Nonetheless, mutations in *COL2A1* result in Stickler syndrome in humans (Richards et al. 2000; Liberfarb et al. 2003; Hoornaert et al. 2010; Higuchi et al. 2017), which is characterized by cleft palate together with ocular, auditory, and skeletal defects. Thus, downregulation of *Col2a1* in *Med23<sup>fx/fx</sup>; Wnt1-Cre* mice, in addition to misregulation of Sox9 and  $\beta$ -catenin activity, is also consistent with the pathogenesis of cleft palate.

*Med23<sup>fx/fx</sup>; Wnt1-Cre* mutants exhibit cleidocranial dysplasia similar to the phenotypes observed following mesenchymal stem cell-specific knockout of *Med23* (*Med23<sup>fx/fx</sup>; Prx1-Cre*) (Liu et al. 2016) and in *Runx2<sup>+/-</sup>* mutant mice (Mundlos et al. 1997). Although Runx2 expression was not reduced in *Med23<sup>fx/fx</sup>; Wnt1-Cre* embryos, Med23 is known to physically associate with Runx2 and influence the expression of transcriptional targets of Runx2. Thus, in the absence of Med23, Runx2 transcriptional activity is diminished, contributing to reduced bone growth in association with micrognathia and cleidocranial

dysplasia. The Med23-Sox9 and Med23-Runx2 interactions, together with increased Sox9- $\beta$ -catenin binding and decreased transcription of Sox9 and  $\beta$ -catenin targets, molecularly account for the phenotypes observed in *Med23<sup>fx/fx</sup>; Wnt1-Cre* embryos and suggest that Med23 functions in concert with multiple transcription factors to regulate gene expression during craniofacial development.

In support of our findings, 2 other Mediator complex proteins, Med12 and Med25, have also been shown to bind to Sox9 protein and regulate craniofacial and limb development, respectively (Rau et al. 2006; Nakamura et al. 2011). This provides a precedent for the Mediator complex in regulating chondrogenesis as well as craniofacial development through Sox9. It is important to note, however, that in contrast to Med12, Med25, and Med31, which activate Sox9 activity in mice (Rau et al. 2006; Risley et al. 2010; Nakamura et al. 2011), Med23 represses Sox9. Collectively, this implies that regulation by Mediator is context dependent and that certain subunits such as Med23 may only be present as part of the complex at specific times to repress or activate osteochondrogenesis during craniofacial development. Therefore, it will be imperative in the future to identify the full repertoire of Med23 binding partners that govern its transcriptional regulation output.

### Author Contributions

S. Dash, P.A. Trainor, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; S. Bhatt, L.L. Sandell, contributed to conception, design, data acquisition, analysis, or interpretation, critically revised the manuscript; K.T. Falcon, contributed to data acquisition, analysis, and interpretation, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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### Declaration of Conflicting Interests

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