Beta-Catenin Signaling is Essential for Mammalian Larynx Recanalization and Establishment of Vocal Fold Progenitor Cells

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Summary statement

This study provides the first line of evidence demonstrating that \( \beta\text{-Catenin} \) signaling controls vocal fold morphogenesis by regulating proliferation and epithelial progenitor establishment. Disruption of these primary roles results in abnormal laryngeal recanalization.

Abstract

Congenital laryngeal webs result from failure of vocal fold separation during development in utero. Infants present with life-threatening respiratory problems at birth; extensive lifelong difficulties in breathing and voicing. The molecular mechanisms that instruct vocal fold formation are rarely studied. Here we show, for the first time, that conditional inactivation of \( \beta\text{-Catenin} \) in the primitive laryngopharyngeal epithelium leads to failure in separation of the vocal folds approximating the gross phenotype of laryngeal webbing. These defects can be traced to a series of morphogenesis defects, including delayed fusion of the epithelial lamina and formation of the laryngeal cecum, failed separation of the larynx and esophagus with reduced and disorganized cartilages and muscles. Parallel to these morphogenesis defects, inactivation of \( \beta\text{-Catenin} \) disrupts stratification of epithelial cells and establishment of p63+ basal progenitors. These findings provide the first line of evidence linking \( \beta\text{-Catenin} \) function to the cell proliferation and progenitor establishment during larynx and vocal fold development.
Introduction

Laryngeal webs are congenital malformations related to the incomplete recanalization of the laryngotracheal tube, when the vocal folds (VF) fail to separate and obstruct the entrance of lower airway structures. These anomalies result from different degrees of failure of the epithelial lamina resorption during intrauterine development and they may present at the moment of birth with life-threatening respiratory problems requiring immediate attention (Hartnick et al., 2000; Wyatt et al., 2005). They can cause difficulties in breathing, swallowing and voicing across the lifespan. Webs range from thin and membranous to thicker, high-grade webs, with associated mesenchymal abnormalities, such as aberrant shape of the cricoid cartilage (Cohen, 1985; Hartnick et al., 2000; Ahmad et al., 2007). Thickness and size of webbing determines the severity of the breathing complications (Cohen, 1985; Ahmad et al., 2007) (Fig. S1). Prevalence of laryngeal webbing at birth is 1 in 10,000 (Sacca et al., 2017). There is an association between anterior laryngeal webbing and velocardiofacial syndrome where laryngeal anomalies range from webs (Fokstuen et al., 1997) to complete atresia (Lipson et al., 1991) with a worldwide incidence estimated at 1 in 2,000 - 1 in 4,000 live births (Sacca et al., 2017). Despite the importance of a complete VF separation, little is known about how this is achieved during development.

Several studies have described laryngeal and VF embryonic development in humans (Sanudo and Domenech-Matteu, 1990; Zaw-Tun and Burdi, 1985; Hartnick et al., 2000; Wyatt et al., 2005; Ahmad et al., 2007) and rodent models (Lobcko et al., 1979; Henick, 1993). We have recently extended these findings and delineated five principal morphogenetic events that occur during murine VF development and provided gene expression pattern analysis that could serve as the foundation for studying mechanisms of normal and aberrant VF formation (Lungova et al., 2015). These developmental events include (a) initiation of the larynx and VFs with apposition of the lateral walls of the primitive laryngopharynx (LPh) at E (embryonic day) 10.5; (b) establishment of the epithelial lamina (EL) with fusion of the lateral walls of the primitive LPh (E11.5); (c) EL recanalization and separation of VFs that is synchronized with
(d) stratification of VF epithelium and development of laryngeal cartilages and muscles in the lamina propria (LP) (E13.5 – E18.5) and lastly (e) maturation of VF epithelium and LP during postnatal stages (Fig. S2). Based on these steps, we hypothesize that precisely controlled EL formation together with proper specification of VF epithelial progenitors are prerequisites for the accurate final VF separation.

The $\beta$-Catenin signaling pathway plays a critical role during development and adult stem cell maintenance of many organs (Stevens et al., 2003, Dessimoz et al., 2005; Apte et al. 2007) including lungs and trachea (Mucenski et al., 2003; Goss et al., 2009; Harris-Johnson et al., 2009; Woo et al., 2011). It regulates diverse cellular processes such as cell proliferation, apoptosis or cell fate determination. In the ventral anterior foregut endoderm, $\beta$-Catenin is required for the initiation of the respiratory lineage and its inactivation results in the absence of both trachea and lungs (Goss et al., 2009; Harris-Johnson et al., 2009). After lung specification, inactivation of $\beta$-Catenin in the lung epithelium leads to aberrant branching and proximal-distal patterning (Mucenski et al., 2003; Shu et al., 2005). Finally, inactivation of $\beta$-Catenin in the developing lung mesenchyme leads to decreased mesenchymal growth and disrupted endothelial differentiation (Yin et al., 2008; Li et al., 2008). In addition to its central role in Wnt signalling, $\beta$-Catenin is a component of adherence junctions connecting classical Cadherins through $\alpha$-Catenin to the actin cytoskeleton (Bienz, 2004; Lyashenko et al., 2011; Perez-Moreno et al., 2003). $\beta$-Catenin cell adhesion is particularly important for neuroepithelial and endoderm formation in embryoid bodies (Lyashenko et al., 2011), for directing coordinated cellular organization and movements within epithelia and for transmitting information from the environment to the interior of cells (Perez-Moreno et al., 2003).

In the present study, we report, for the first time, the requirement of $\beta$-Catenin function in VF and larynx morphogenesis. Conditional ablation of $\beta$-Catenin in the primitive LPh epithelium during EL formation leads to failed VF separation resembling human laryngeal webbing on a gross tissue level. The failure in VF separation is preceded by delayed EL fusion
and disrupted establishment of VF basal epithelial progenitors. Delayed EL fusion may be caused by cell cycle arrest in the LPh epithelium and surrounding mesenchyme, consistent with the role of β-Catenin in cell proliferation (Huang et al., 2007; Shutman et al., 1999; Rowlands et al., 2003). In more severe cases, inactivation of β-Catenin affected the EL integrity. Mesenchymal cells filled the gap between the EL fragments and precluded VF separation. Besides reduced cell proliferation, loss of β-Catenin also disrupted initial differentiation of VF basal progenitors into p63 expressing cells, consistent with its role in promoting progenitor identity (Goss et al., 2009; Harris-Johnson et al., 2009). Specification of basally positioned VF progenitors is necessary for their terminal conversion into functional basal cells giving rise to a suprabasal layer that disintegrates during VF separation. In summary, this study provides the first evidence linking β-Catenin function to VF morphogenesis by regulating proliferation and cell fate determination of epithelial and mesenchymal cell lineages during the EL formation and recanalization, shedding light on the etiology of poorly understood congenital laryngeal malformations.

Results

Inactivation of β-Catenin in developing VFs

To determine whether β-Catenin signaling is active in developing VFs, we examined the expression of Axin2, a reporter gene for WNT/β-Catenin activity. We found that during EL formation, Axin2 was expressed in the EL as well as in the surrounding LP (Fig. 1A). To investigate the requirements for β-Catenin signaling in VF and larynx morphogenesis, we disrupted β-Catenin function in the EL by conditional gene inactivation using ShhCre (Harris-
Johnson et al., 2009; Harfe et al., 2004). By mating $Shh^{Cre}$ mice to TdTomato reporter mice, we found that $Shh^{Cre}$ was robust at the EL during EL formation (at E11.0), the site of prospective VFs. This is on the contrary to the dorsal compartment of the primitive LPh, which gives rise to the esophagus (Fig. 1B - D). $Shh^{Cre}$ was also robust in epithelial cells in more advanced stages of VF formation during EL recanalization at E14.5 (Fig. 1E, J). $Shh^{Cre}$ lineaged cells were found at the EL, the LC and PD (Fig. 1E - J). Moreover, the $Shh^{Cre}$ lineage tracing analysis revealed that during EL formation $Shh^{Cre}$ lineage signal extended into adjacent, cytokeratin (K) 8 negative mesenchyme (Fig. 1C - F). By RNA in situ hybridization, we found that $Shh$ expression remained primarily in the epithelium (Lungova et al. 2015). These data suggest that the mesenchymal lineaged cells either arise from rare $Shh$ expressing cells beyond the sensitivity of RNA in situ detection, or from epithelial cells that have undergone epithelial-mesenchymal transition (EMT) and delaminated into mesenchyme of the LP (Fig. 1G - J). These cells intermix with SOX9 expressing chondrocytes or surround MF-20 expressing myoblasts (Fig. 1G - J). By mating $Shh^{cre}$ mice to mice carrying a conditional knockout allele of $\beta$-Catenin ($Ctnnb1^{tm2Kem}$), we generated $Shh^{cre/+}; Ctnnb1^{tm2Kem/tm2Kem}$ (hereafter referred to as $\beta$-Cat$^{cko}$, for conditional knockout) mutant embryos. In the epithelial cells of E11.5 embryos, we found that $\beta$-Catenin was severely reduced in the EL, at the site of prospective VFs, while it remained present in the dorsal compartment of the primitive LPh, as compared to control embryos (Fig. 1K, L). As $Shh^{cre}$ activity in the mesenchyme is rather minor compared to that in the epithelium, expression of $\beta$-Catenin in mesenchymal cells remained strong (Fig. 1L). These data indicate that $Shh^{cre}$ is an effective tool for $\beta$-Catenin inactivation in the epithelium of prospective VFs.

$\beta$-Cat$^{cko}$ mutants exhibit incomplete recanalization of the laryngotracheal tube

$\beta$-Cat$^{cko}$ mutants die at birth with multiple defects including agenesis of the lung (Harris-Johnson et al., 2009). We first analyzed VF phenotype at E18.5, shortly before birth. $\beta$-Cat$^{cko}$
mutants failed to completely disintegrate the EL whereby resembling the phenotype described in laryngeal webbing at a gross tissue level. Unlike in control embryos (Fig. 2A, B) unseparated mutant VFs obstruct the entrance into the trachea in both the milder (Fig. 2D, E) and more severe (Fig. 2G, H) cases. Upon close examination, we found, that in milder cases, mutant VFs, at the site of EL persistent fusion, are mostly lined with a single epithelial cell layer on each side. In contrast, there are two cell layers on each side in fully separated VFs in control embryos (Fig. 2B, E insets). Our previous characterization of the wild-type (WT) VFs suggests that transition from one layer into two layers precedes successful VF separation (Lungova et al., 2015). In more severe cases, a large portion of the EL is replaced by mesenchymal cells that have migrated into the space between VFs, suggesting that VFs have completely grown together (Fig. 2H). Ventral from the VFs is the LC (Fig. 2E, H) that in control embryos becomes a part of the glottis once VFs separate (Fig. 2B). The dorsal region opens into the posterior glottis (Fig. 2C). In the mutant, the dorsal extent of the glottis is not as clearly defined, due to the absence of the septum (Fig. 2F, I). In more severe cases, unseparated VFs occluded more than 50% of the glottal region, and the posterior glottis was significantly narrowed, as compared to milder cases (Fig. 2F, I). These phenotypes in the severe cases are consistent with high-grade thick laryngeal webs in human (Wyatt et al., 2005; Ahmad et al., 2007). We sought to further characterize the cause for the phenotypes in mice to understand the etiology and progression of laryngeal webbing.

**β-Cat**

mutant laryngeal phenotype can be traced to an early defect of incomplete EL fusion

We found that aberrant VF development in **β-Cat**

mutants was already apparent at E11.5 during EL formation. In control embryos at E11.5, lateral walls of the primitive LPh obliterated the ventral lumen and formed the EL (Fig. 3A, B). More caudally, the trachea was already separated from the esophagus (Fig. 3C). In **β-Cat**

mutant embryos at E11.5, while
the cranial part of the primitive LPh fused normally to establish the EL (Fig. 3D), the caudal region with the prospective VFs remained open (Fig. 3E). More caudally, the trachea was not separated from the esophagus (Fig. 3F). At the cranial level of the larynx and esophagus in the control, dorsal and ventral patterning was delineated by SOX2 expression dorsally and NKX2-1 expression ventrally (Fig. S3A). In the mutant, SOX2 expression domain was extended ventrally at the expense of NKX2-1 (Fig. S3B). This is similar to failed patterning at the more caudal trachea and esophagus level as shown before (Minoo et al., 1999; Que et al., 2007; Domyan et al., 2011).

Two days later at E13.5, in control embryos, three morphogenetic events occurred. First, the LC appeared as a T-shaped lumen extending caudally along the ventral border of the EL indicating the start of EL recanalization, while remaining epithelial cells at the EL were still fused (Fig. 3G, H). Second, a septum developed between the esophagus and VFs, and the cricoid cartilage formed in this space (Fig. 3G, I). Third, in the fused EL near the cricoid, a cavity could be detected, indicating the start of recanalization into the PD (Fig. 3G, I). In \( \beta\)-Catenin \( \alpha\) mutants at E13.5, a portion of the lateral walls finally fused to establish a shortened EL (Fig. 3J, K). However, all three subsequent morphogenetic events were disrupted. First, there is a delay in LC expansion (Fig. 3J, K). Second, no septum or cricoid cartilage separated the esophagus and future larynx with VFs (Fig. 3J, L). Third, because there is no septum delineating the dorsal portion of the EL, there is no apparent PD (Fig. 3J, L).

At E16.5, in control embryos, with continuing dilation of the LC and PD (Fig. 3M, N), the EL gradually disintegrates to open the laryngotracheal tube by E18.5. Meanwhile, epithelial cells lining the developing VFs acquired a two-cell layer morphology (Fig. 3N), suggesting that these events may precede final VF separation at E18.5. In \( \beta\)-Catenin \( \alpha\) mutants at E16.5, the LC finally expands (Fig. 3O, P, Q, R). However, in milder mutants, EL is lined by a single epithelial cell layer that fails to stratify, and a longer portion of the EL remain fused compared to control (Fig. 3P). In more severe mutants, the ventral and dorsal EL are interrupted by mesenchymal cells that run through (Fig. 3Q, R). Staining for \( \beta\)-Catenin indicates that the mesenchymal cells
that disrupt the EL is a mixture of $\beta$-Catenin$^+$ and $\beta$-Catenin$^-$ cells (Fig. S3 E, F). As the overall $\beta$-Catenin expression is low at this stage, the $\beta$-Catenin$^-$ cells could still be wild type in genotype. This more severe gross phenotype resembles severe cases of human laryngeal webs.

To elucidate the origin of severe laryngeal webbing, we stained epithelial cells with cytokeratin (K) 8. In a subset of the more severe $\beta$-Cat$^{cko}$ mutants, at E13.5, the ventral tip of the EL pinched off from the remaining cells of the EL, in contrast to controls (Fig. 4A - D) and the LC was formed in this small cluster of pinched off epithelial cells (Fig. 4D). At E16.5, in the control, the LC gradually expands and may drive the separation of the remainder of the EL as they are still connected (Fig. 4E, F). In $\beta$-Cat$^{cko}$ mutants, however, the delayed LC expansion cannot drive the separation of the remainder of the EL as the two epithelial regions are now separated by mesenchymal cells (Fig. 4G, H). Our findings demonstrate that the defects in EL formation preceded the failure of VF separation.

**Cellular mechanism underlying aberrant EL formation**

To address whether $\beta$-Catenin plays a role in cell survival and/or proliferation, we examined embryos at E11.5 using TUNEL assay for cell death and EdU incorporation in S-phase for cell proliferation. We found no increased cell death in the mutant EL or adjacent LP as compared to control mice (Fig. S4 A, B). In contrast, there was a statistically significant decline of cell proliferation in the EL and LP, in the caudal, but not cranial region of the developing larynx in $\beta$-Cat$^{cko}$ mutants (EL caudal region: $p = 0.0001$; LP caudal region: $p = 0.0320$) (Fig. S4 C - G). This reduction may contribute to the failed epithelial morphogenesis that follows, such as the expansion of the LC.

Previous studies have shown that $\beta$-Catenin regulates cell proliferation via its interaction with Cyclin D1 (Shutman et al., 1999; Rowlands et al., 2003; Atanasoski et al., 2001). Cyclin D1 also interacts with cell cycle inhibitors, such as p27$^{kip}$ and p21$^{cip}$ genes, that control the exit of cells from the cell cycle (Geng et al., 2001; Lee et al., 2006).
Immunofluorescent staining revealed that Cyclin D1 was strikingly absent in the mutant EL and was also reduced in surrounding LP as compared to control (Fig. 5A - F). In contrast, expression of p27^Kip increased in both the EL and LP during EL formation (Fig. 5G – L). Low Cyclin D1 levels and high p27^Kip expression is consistent with the outcome that a majority of cells have withdrawn from the cell cycle and are arrested at a G1 checkpoint. Statistically significant differences in Cyclin D1 and p27 expression levels were also confirmed by quantitative analysis using the Student’s t-test (EL Cyclin D1: p = 0.0009, LP Cyclin D1: p = 0.0428; EL p27: p = 0.0002, LP p27: p = 0.0008) (Fig 5M, N).

To elucidate whether reduced cell proliferation is responsible for the mutant phenotypes, we stimulated proliferation by introducing p27^Kip mutation into the β-Catenin mutant (Shh-Cre/+; Ctmnb1F/F; p27−/−), and addressed, if it rescues EL fusion in the mutant. At E13.5, inactivation of p27^Kip gene from the genotype of β-Catcko mutants rescued a septum between the larynx and esophagus, the EL ventral to this appears fused and is connected to the LC (Fig. 5O - R). However, the size of the EL remains shorter than in control embryos, as confirmed by K8 staining (Fig. 5P, R) possibly due to the expansion of the mesenchymal cells between the esophagus and the larynx (Fig. 5O, Q). This ectopic expansion of the mesenchyme may be due to global knockout of p27^Kip, which led to increased proliferation in the mesenchyme independent of its interaction with β-Catenin in the epithelium. These results demonstrate that inactivation of p27^Kip led to a partial rescue of the β-Catenin mutant morphogenesis defect, suggesting that epithelial cell proliferation defects contribute to, but are not the sole reason of the morphogenesis phenotypes in the mutant.

β-Catenin inactivation causes defects in differentiation of mesenchymal cells in the LP

Abnormalities of mesenchyme-derived structures such as cartilage and muscle are common features in high-grade laryngeal webs (Hartnick et al., 2000; Ahmad et al., 2007). Accompanying epithelium defects, we also found that β-Catcko mutants exhibited defects of
the cartilaginous and muscular structures in the mesenchyme (Fig. 2D - I). The middle portion of the thyroid cartilage and the shape of arytenoids were also altered (Fig. 2A, D, G), affecting the attachment of the thyroarytenoid (TA) muscle (Fig. 2B, E, H). To more precisely define the mesenchymal defects revealed by histological observation, we outlined cell types with anti-SOX9 antibody for laryngeal cartilage and anti-myosin heavy chain MF-20 antibody for developing myoblasts. In control embryos at E11.5, there were intensely stained SOX9 expressing cells subjacent to the ventral tip, and these will give rise to the middle portion of the thyroid cartilage (intermediate lamina, IL) (Fig. 6A, B). Intensely stained cells lateral to the larynx will give rise to lateral laminae (LL) of the thyroid cartilage (Fig. 6A). Lightly stained cells subjacent to the dorsal EL (at the site of the arytenoid swelling) will give rise to the arytenoids (Fig. 6A). In \( \beta\text{-Cat}^{\text{kox}} \) mutants at E11.5, SOX9 expressing cells were detected principally at the sites of the arytenoid swellings and developing lateral lamina, while SOX9 expression around the ventral tip of the EL was much reduced (Fig. 6C, D). In \( \beta\text{-Cat}^{\text{kox}} \) mutants at E13.5, the cluster of SOX9 expressing cells ventrally from the EL was much smaller as compared to controls, resulting in an underdeveloped intermediate lamina (Fig. 6E, F). While arytenoids formed, the cricoid did not develop, likely due to absence of a septum (Fig. 6F).

Concurrent with differentiation of laryngeal cartilages, other mesenchymal cells initiated differentiation into laryngeal muscles. At E11.5 and 13.5, in \( \beta\text{-Cat}^{\text{kox}} \) mutants, accompanying a decrease in SOX9 expression, there were slightly more MF20 expressed mesenchymal cells, especially in the ventral laryngeal region as compared to controls (Fig. 6A - F). At E16.5 in the control, MF20 expressing cells have given rise to three principal groups of laryngeal muscles. Ventrally there is the thyroarytenoid muscle that is a paired muscle attached to the intermediate lamina (Fig. 6G, H). Dorsally there are the laryngeal muscles, which consist of the interarytenoid muscle connecting the posterior surfaces of arytenoid cartilages and posterior cricoarytenoid muscles that connect arytenoid cartilages with the cricoid (Fig. 6G, I). The thyroarytenoid muscle functions in phonation and acts as a sphincter that tightens and narrows the laryngeal inlet during swallowing. The muscle is brought in
parallel together to close the glottis by the contraction of interarytenoid muscles. On the other hand, the posterior cricoarytenoid muscle opens VFs for breathing. Both muscles, interarytenoid and posterior cricoarytenoid, are located in the septum (here referred to for simplicity as dorsal laryngeal muscles) (Fig. 6G, I). \( \beta \)-Catenin ablation in developing VF epithelium disrupts the attachment and function of all these muscles. In \( \beta \)-Cat\( ^{\text{k}0} \) mutants, the thyroarytenoid muscle inserts into the lateral laminae instead of the intermediate lamina of the thyroid, and its mass seems to be reduced (Fig. 6J, K). This defect may lead to insufficient glottal closure significantly affecting swallowing and voicing. Concurrently, the dorsal laryngeal muscles are disconnected due to the absence of the septum and cricoid cartilage (Fig. 6J, L), they cannot function together to approximate the VFs or abduct them. These data suggest that epithelial cells interact with the surrounding mesenchyme starting from early stages of VF morphogenesis during EL formation. Primarily loss of \( \beta \)-Catenin in the epithelium leads to profound disorganization of the adjacent mesenchymal cells.

**Inactivation of \( \beta \)-Catenin affects remodeling of VF basement membrane**

Next, we characterized changes in shape and cellular organization of VF including remodeling of their basement membrane during EL formation. Using images from transmission electron microscopy and expression analysis of laminin alpha 5 (Lam 5), we confirmed that in control embryos, during EL fusion at E11.5, prospective basal cells had a well-defined basement membrane (Fig. S5A - J). Cells that lost contact with the lumen, e.g. cells at the ventral tip of the EL, also lost apical characteristics such as filopodia (Fig. S5). Cells at the EL tip formed basal protrusions that pointed towards the surrounding mesenchyme (Fig. S5 C, E). In \( \beta \)-Cat\( ^{\text{k}0} \) mutants, cells at the ventral tip of the EL retained the original elongated cell shape with apical filopodia and tight junctions (Fig. S5 G, H, I, arrowheads). Their Lam 5 (Fig. S5 F) positive basement membrane was smooth without protrusions (Fig. S5 H, J). During EL recanalization at E16.5, compared to the continuous Lam 5-positive
basement membrane in control embryos (Fig. S5 K), there is fragmentation of Lam 5 expression between the interrupted EL segments (Fig. S5 L, M), suggesting a breakdown of basement membrane. Despite these changes, E-Cadherin expression remains robust in the remainder of the EL in the mutant (Fig. S5 N, O), suggesting that $\beta$-Catenin loss does not have a direct effect on adherence junctions.

$\beta$-Catenin inactivation in the EL disrupts establishment of VF epithelial basal progenitors

To understand whether $\beta$-Catenin inactivation affects specification of prospective VF basal progenitors and their subsequent differentiation, we assayed for the expression of p63 and cytokeratins K8 and K5. p63 is a nuclear marker of basal cells, and at E11.5 it was expressed in all epithelial cells along with a simple epithelial cell marker K8 (Fig. 7A - D). p63+ K8+ cells were cuboidal and perfectly organized in two single cell layers that were fused along the midline (Fig. 7A - D). In the $\beta$-Cat$^{cko}$ mutant, in epithelial layers that have not yet fused, while K8 was expressed in all cells, p63 was detected in a mosaic fashion (Fig. 7E - H). Interestingly, even though p63+ cells are cuboidal just like in the control, p63- cells were slightly taller. (Fig. 7E). Negative staining for Alcian blue shows that p63 negative cells in $\beta$-Cat$^{cko}$ mutants did not adopt a different fate (Fig. S3 C, D). At E13.5 in control embryos, p63+ cells increased in number along the EL midline (Fig. 7 I, J). K8 expression in these cells became weaker than in cells lining the PD or LC (Fig. 7K). These p63+ cells with downregulated K8 expression represent basal cell progenitors for the stratified squamous VF epithelium. In $\beta$-Cat$^{cko}$ mutants, the prospective LC cells in the ventral tip of the EL remained p63- K8+, while cells at the EL expressed p63 and K8 (Fig. 7L - N). More dorsally, there is large domain of p63- cells expressing K8, which are columnar (Fig. 7M, N). At E18.5 after EL separation in the wild-type and control, expression of cytokeratin 5 (K5), a stratified epithelial marker, begins to be detected (Lungova et al., 2015). The epithelium is composed of two cell
layers, the basal p63+, K5+ and K8-low cell layer and apical p63-low, K5+ and K8+ cell layer (Fig. 7O – S). This expression of cytokeratins is unique for VFs within the foregut (Lungova et al., 2015). In comparison, in β-Catcko mutant mice, fused VFs were lined with a single layer of p63+ and K8+ epithelial cells, most of which, but not all, also expressed K5 (Fig. 7T - X). A few basal cells were p63-, K8+ suggesting that these cells either did not initiate p63 expression or did not maintain it (Fig. 7V, W). In the dorsal compartment near the PD region, the previously observed columnar cells remain, and were p63-, K5- and K8+ (data not shown). In summary, these data suggest that inactivation β-Catenin disrupts the normal formation of p63+, K5+ and K8- basal progenitor cells.

Discussion

The larynx is a vitally important organ, sitting at the cross roads between the gastrointestinal and respiratory tracts whereby, orchestrating swallowing, breathing, coughing and, in humans, voice. Loss of a functioning airway is life-threatening, as is loss of airway protection from ingested food and drink. Impaired voice production holds significant implications for individual health and wellness, social and occupational function; and societal productivity. Molecular mechanisms that cause congenital laryngeal webbing have not been elucidated and there is also little histological data that further characterize tissue from patients. In humans with laryngeal webbing, fibrous tissue covers the larynx (Cohen, 1985; Hartnick et al., 2000; Wyatt et al., 2005; Ahmad et al., 2007). This gross phenotype has been modeled for the first time in the β-Catcko mutant. The described phenotype in β-Catcko mutants ranges from milder to severe laryngeal webs that occlude more than 50% of the glottis; this range on a gross tissue level is similar with clinical observations (Cohen, 1985; Ahmad et al., 2007). In this mouse model, we traced the morphological defects to surprisingly early events of VF morphogenesis, when epithelial cells fuse to establish the EL and initiate their differentiation into VF basal epithelial progenitors. Our finding illustrates that a thorough understanding of
the origin of defects as demonstrated through animal models, can provide important insights into the etiology of human congenital laryngeal malformations.

Genetic evidence from β-Cat^Δκο mutants establishes β-Catenin as a key player involved in early larynx and VF morphogenesis. Consistent with the dual role of β-Catenin it regulates expression of genes involved in cell proliferation, likely mediated by Cyclin D1, and simultaneously, genes that are involved in specification of VF basal epithelial progenitors. Moreover, as a part of cell adherent junctions, β-Catenin can also affect cell adhesion and EL integrity. Inactivation of β-Catenin in the epithelium and/or in a small number of cells in the mesenchyme also disrupts normal patterning of laryngeal cartilages and muscles.

Our data stipulate a combination of mechanical and genetic control that drive EL recanalization and VF separation (Fig. S2). This mechanism is unique for the VFs and has not been described in any other tissues. Mechanical forces are generated within the epithelium, as the cavities, the LC and PD, expand and exert pressure on the epithelial walls, and in the mesenchyme by initial contraction of the dorsal laryngeal muscles that pull on the VFs (Fig. S2). Recent research has shown that the epithelial wall adapts to enable the expansion of the lumen (Andrew & Ewald, 2010; Hoijman et al., 2015). Besides cell division, which increases the number of cells in the epithelial layer, mitotically active epithelial cells remodel their shape and contract apicobasally as shown during Drosophila tracheal or Zebrafish inner ear morphogenesis (Nelson et al., 2012; Hoijman et al., 2015). Both mechanisms contribute to lumen expansion and simultaneously determine its shape (Hoijman et al., 2015). Therefore, cell cycle arrest in β-Cat^Δκο mutants may delay the LC expansion and thus affect EL recanalization [Fig. 8 (1, 2)]. Our results also indicate that the cells at the ventral tip of the EL may guide the LC expansion. Prior to the LC expansion, these cells send finger-like projections into the surrounding mesenchyme. These projections of the basement membrane can have endocytic or exocytic properties (Hexige et al., 2015) or can work as mechanosensors (Murphy and Courtneidge, 2011). However, because the LC appears in this region two days later, we hypothesize that these basal cell progenitors may coordinate extracellular matrix degradation
with the basal cell membrane extension, as the LC expands. Absence of these protrusions in the mutant EL can be associated with the delay in LC extension in \( \beta\text{-Cat}^{cko} \) mutants. In the more severe cases of failed EL separation, when the EL loses its integrity and mesenchymal cells fill the space in-between, they block the EL separation, despite the pressure exerted from LC expansion [Fig. 8 (3, 4)].

The \( \beta\text{-Catenin} \) mutant also exhibit a number of mesenchymal defects including cartilage and muscle malformation. While \( Shh^{\text{Cre}} \) activity is detected in a few of the mesenchymal cells in addition to robust signal in the epithelium, the mesenchymal cells do not make major contribution to either the cartilage or muscle. This suggests that the cartilage and muscle defects are not due to direct loss of \( \beta\text{-Catenin} \) in these cells. Secondary causes could include earlier cellular defects. For examples, failed formation of the laryngeal septum would lead to the lack of cricoid cartilage. The mesenchymal defects could also be due to altered signals from the epithelium following inactivation of \( \beta\text{-Catenin} \) in the epithelium. Prior research has shown that changes in signals such as SHH and BMP would affect cartilage or muscle formation (Murtaugh et al., 1999; Zeng et al., 2002). The defects in mesenchyme could also feedback to regulate epithelial formation. For example, dorsal laryngeal muscles connect arytenoid cartilages and arytenoids with the cricoid cartilage, and together they contribute mechanical force to EL recanalization. In the \( \beta\text{-Cat}^{cko} \) mutant, the altered attachment of the dorsal laryngeal muscles, misshapen thyroid and arytenoid cartilages and absence of cricoid may significantly reduce the force necessary to pull VFs apart.

In the \( \beta\text{-Catenin} \) mutant, failure in VF separation leading to anterior laryngeal webbing is preceded by delayed EL fusion and aberrant specification of VF basal epithelial progenitors (Fig. 8). Our data obtained from \( \beta\text{-Cat}^{cko} \) mutants supported previous observations that EL fusion coincides with intensive cell proliferation (Lobsko et al., 1979; Muller et al., 1985; Lungova et al., 2015). In \( \beta\text{-Cat}^{cko} \) mutants, low Cyclin D1 levels and high p27\(^{\text{Kip}}\) expression in epithelial and mesenchymal cells in the LP show that during early stages of VF morphogenesis, the majority of cells withdraw from the cell cycle and arrest at a G1
checkpoint. The lateral walls remain closely juxtaposed and fail to fuse at the midline at the appropriate time. Eventually, the ventral part of the EL fuses [Fig. 8 (2)]. In the control, during EL formation, the juxtaposed epithelium is a single layer where all cells are p63+K8+. Two days later, they start to convert into two layers: a basal p63+K8- VF progenitor layer, and a suprabasal p63-K8+ cell layer. The basal layer represents a reservoir of stem cells for epithelial regeneration and tissue repair (Yu et al., 2005; Mou et al., 2016). In the mutant, large patches of the epithelium are p63- K8+, suggesting that these cells may have failed to establish their basal cell state, and became K8 suprabasal only. For the p63+ cells that are present, they failed to transition into the bilayer structure with P63+ K8- mature progenitors in the basal layer. Future studies may focus on the role of in regulation of VF basal cell differentiation during development and/or postnatally during VF epithelial regeneration.

Advances in the understanding of morphogenetic processes during VF embryonic development will promote greater insight into the pathophysiology of vocal fold congenital disorders. We have shown, for the first time that loss of β-Catenin during early stages of VF morphogenesis are associated with congenital laryngeal malformations resembling laryngeal webbing. Characterization of the regulatory mechanisms controlling the establishment and expansion of VF progenitors will help in devising therapeutic strategies for targeting these disorders and optimizing surgical techniques for their corrections. Suggested role of β-Catenin in differentiation of VF basal cell population also opens a new field for function of β-Catenin in postnatal VF epithelial regeneration and tissue repair.
Material and Methods

Generation of $\beta$-Catenin mutants and embryo isolation. Mice carrying a conditional loss-of-function allele of $\beta$-Catenin (Ctnnb1$^{tm2Kem}$) were mated to mice carrying Shh$^{Cre}$ allele to generate Shh$^{Cre/+}$; Ctnnb1$^{tm2Kem/+}$ ($\beta$-Cat$^{cko}$, for conditional knockout) (Harris-Johnson et al., 2009; Harfe et al., 2004; Brault et al., 2001). Offspring were genotyped using the following primer pairs for Cre: 5’-TGATGAGGTTCGCAAGAACC-3’ and 5’-CCATGAGTGAACGAACCTGG-3’, product size 420bp; for Ctnnb1$^{tm2Kem}$: 5’-AAGGTAGAGTGATGAAAGTTGTT-3’ and 5’-CACCATGTCCTCTGTCTATTC-3’, product sizes 324bp from the Ctnnb1$^{tm2Kem}$ allele and 221bp from the wild-type allele. Timed pregnant females were dissected and mutant and control animals studied at the indicated embryonic stages, following regulations of protocols approved by the University of Wisconsin Animal Care and Use Committee. Shh$^{Cre/+}$; Ctnnb1$^{tm2Kem/-}$ embryos were used as controls.

Phenotype analyses of $\beta$-Cat$^{cko}$ mutants. To assay for Cre activity through TdTom expression, the R26R reporter line was introduced into the background of the Shh$^{Cre}$ line (Harris-Johnson et al. 2009; Muzumdar et al., 2007). TdTom activity was detected using standard IF staining protocol on paraffin embedded transversal sections using anti-Red Fluorescent Protein at E11.0 and E14.5. Whole mount in situ hybridization with digoxigenin-labeled probes for Axin2 was performed on transversal 120um thick vibratome sections at the level of developing larynx and vocal folds at embryonic stage E11.5 as described by Neubuser (Neubuser et al., 1997)

Generation of $\beta$-Cat$^{cko}$; p27 -/- double mutants and embryo isolation. Shh$^{Cre/+}$; Ctnnb1$^{tm2Kem/+}$; Cdkln1b$^{tm1Mlf/+}$ males were crossed to Ctnnb1$^{tm2Kem/+}$ ; Ctnnb1$^{tm2Kem/-}$; Cdkln1b$^{tm1Mlf/+}$ females to generate Shh$^{Cre/+}$; Ctnnb1$^{tm2Kem/+}$; Cdkln1b$^{tm1Mlf/-}$ double mutants (here referred as $\beta$-Cat$^{cko}$; p27 -/- double mutants). Offspring were genotyped using the following primer pairs for Cre (as mentioned above); Ctnnb1$^{tm2Kem}$ (as mentioned above) and Cdkln1b$^{tm1Mlf}$: 5’- GATGGACGCCAGACAAGC and 5’ - CTC GTGCCATTCGTATCTGC for the
wild-type allele, product size 190bp; 5’ – CTTGGGTGGAGAGGCTATTC and 5’ – AGGTGAGATGACAGGAGATC for the mutant allele, product size 280bp. Timed pregnant females were dissected and mutant and control animals studied at the indicated embryonic stages.

**Tissue collection for IF staining.** Pregnant females were sacrificed at E11.5, E13.5, E16.5 and E18.5 (for β-Cat<sup>cko</sup> mutants), at E11 and E14.5 (for ShhCre lineage tracing experiment), and at E13.5 (for double β-Cat<sup>cko</sup>, p27−/− mutants) following regulations of protocols approved by the University of Wisconsin Animal Care and Use Committee. For each experimental group at least three different mouse embryos were collected for analysis. We included nine WT embryos for characterization of Axin2 expression and lineage tracing experiments (E11 and E14.5); eighteen control mouse embryos and eighteen β-Cat<sup>cko</sup> mutants were used for characterization of the β-Cat<sup>cko</sup> mutant phenotype. In double mutant mice, only two embryos were collected for analysis due to the low frequency for a double mutant (1:16) (total number of mouse embryos = 47). Mouse neck regions were dissected and immediately fixed in 4% paraformaldehyde in phospho-buffered saline at 4°C/overnight, dehydrated in a gradient series of ethanol, treated with xylene and embedded in paraffin. Paraffin blocks were cut into serial sections (5 μm), dewaxed and rehydrated, heated to boiling in 10mM citrate buffer pH=6 for antigen retrieval and treated with 0.5% triton in PBS for cell membrane permeabilization. Sections were then stained using standard IF protocol. For each individual at stage E11.5 and E13.5 two transversal sections (one cranial and one caudal section) of developing VFs were characterized, accounting for a very small size of the larynx during early stages of VF formation. In more advanced stages of VF development four transversal sections (two cranial and two caudal) were characterized. Each experiment was replicated two times in the laboratory. Primary antibodies used are listed in the Table 1. They were applied overnight at 4°C degrees. Secondary antibodies used are listed in the Table 1, they were applied 1h and 30 min at room temperature (RT). Slides were mounted using Vectashield with DAPI (Vector Laboratories; Peterborough, UK). Images were taken with a Nikon Eclipse E600 with a camera
Olympus DP71, images were adjusted for brightness using the installed DP 71 software, Olympus Corporation. Figure panels were created using MS Powerpoint 2010. Alcian blue staining was performed using the Alcian blue 1% 2.5 pH stain kit, Newcomer Supply (Catalogue number 9102A), according the manufacturer’s protocol (Newcomer Supply, Middleton, Wi, USA).

**TUNEL Assay.** TdT-mediated dUTP-biotin nick end labelling (TUNEL, Chemicon-Millipore; Billerica, USA) was employed to detect apoptosis. After rehydration, sectioned samples were treated with proteinase K at 20 μg/ml at room temperature for 15 minutes (EMD Millipore; Billerica, MA, USA). After equilibration buffer, the reaction mixture was prepared as per manufacturer instructions and applied at 37°C for 50min. After anti-digoxigenin-peroxidase reaction 30 min/RT, positive cells were visualized by chromogen substrate diaminobenzidine (DAB kit, Vector Laboratories, Peterborough, UK)) and slides were counterstained with hematoxylin.

**Cell Proliferation Assay, cell quantification and statistical analysis.** Pregnant females received an intraperitoneal injection of 100 μg EdU (Sigma Aldrich; St. Louis, MO, USA) per gram bodyweight 1 hour before sacrifice. Embryos were fixed, processed to form paraffin-embedded sections. EdU detection was performed according to the manufacturer’s protocol (Invitrogen; Carlsbad, CA, USA). IF labeled cells in the EL and LP were manually counted using Image J. For each experiment at E11.5 three separate mutant-control littermate pairs were analyzed. For each mutant and control sample, two sections were quantified - cranial and caudal sections (three control embryos, n=6 sections and three mutant embryos, n=6 sections). The percentage of EdU+ nuclei was compared using General Linear Model procedure with Repeated Measures Analysis of Variance. Results are reported as mean±std, and were considered statistically significant at p≤0.05.

**Quantification of Cyclin D1 and p27 expression and statistical analysis.** Similar to quantification of EdU positive cells, Cyclin D1 and p27 IF labelled cells were manually counted
using Image J. For each experiment at E11.5 three separate mutant-control littermate pairs were analyzed. For each mutant and control sample, one section was quantified at the caudal region of the developing VFs (three control embryos, n=3 sections and three mutant embryos, n=3 sections). The percentage of Cyclin D1+ or p27+ nuclei were compared using the Student's t-test. Results are reported as mean±std, and were considered statistically significant at p≤0.05.

**Author contributions:**


**Acknowledgements**

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**Competing interests**

The authors have declared that no conflict of interest exists.
References


### Table 1: Lungova et al.

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**Figure 1: Inactivation of β-Catenin in the primitive LPh leads to a failure in VF separation.** (A) Axin 2 expression as determined by RNA *in situ* hybridization in vibratome transversal sections at the level of developing VFs at E11.5. (B) Anti-TdTom (red) IF staining in ShhCre; R26R embryos at E11.0 during EL formation. (C – F) Double IF staining for anti-TdTom (red) and anti-cytokeratin K8 (green) in ShhCre; R26R embryos at E11.0 (C, D) and E14.5 (E, F). (G, H) Double IF staining for anti-TdTom (red) and anti-SOX9 (green) in ShhCre; R26R embryos at E14.5. (I, J) Double IF staining for anti-TdTom (red) and anti-MF-20 (green) in ShhCre; R26R embryos at E14.5. Bracketed regions in the panels of E, G, and I are
magnified in F, H, and J respectively. (K, L) IF anti-β-Catenin staining in transverse sections of the EL at E11.5, when EL is established. An arrow in L indicates diminished β-Catenin activity in the EL of the β-Cat<sup>cko</sup> mutants. For each experimental at least three different individuals were collected for analysis. For each individual at least two transversal sections from cranial and caudal VF regions were characterized. The experiment was replicated two times in the laboratory. Abbreviations: EL, epithelial lamina; LC, laryngeal cecum; LP, lamina propria; LPh, laryngopharynx; PD pharyngoglossic duct; CC, cricoid cartilage; dLM, dorsal laryngeal muscles.
Figure 2: Inactivation of β-Catenin in the primitive LPh leads to laryngeal webs. (A – C) H&E staining in transversal sections demonstrating morphology of the VFs in control embryos at E18.5. Bracketed regions in the A panel are magnified in B and C respectively. A bracketed region in the B panel is magnified in the same panel in the upper right corner. A solid black arrow denotes the two-layered VF epithelium in control embryos. (D – F) H&E transversal sections demonstrating morphology of the VFs in β-Catcko mutants at E18.5 in milder cases. Bracketed regions in the D panel are magnified in E and F respectively. A bracketed region in the E panel is magnified in the same panel in the upper right corner. A dashed black arrow denotes aberrant stratification of VF epithelium in mutant VFs. (G – I) H&E transversal sections demonstrating morphology of the VFs in β-Catcko mutants at E18.5 in severe cases. Bracketed regions in the G panel are magnified in H and I respectively. For each experimental
group at least three different individuals were collected for analysis. For each individual at least two transversal sections from cranial and caudal VF regions were characterized. The experiment was replicated two times in the laboratory. Abbreviations: AC, arytenoid cartilage; CC, cricoid cartilage; EL, epithelial lamina; Es, esophagus; dLM, dorsal laryngeal muscles; G, glottis; LC, laryngeal cecum; PG, posterior glottis; spt, septum; TA, thyroarytenoid muscle; TC, thyroid cartilage; VF, vocal fold.
Figure 3: Inactivation of β-Catenin in the primitive LPh leads to a delayed and aberrant EL formation and recanalization. (A - F) H&E transversal sections demonstrating morphology of the VFs in control embryos and β-Cat<sup>cko</sup> mutants at E11.5 and at E13.5 (G – L). Bracketed regions in the panels of G, J are magnified in H, K and I, L respectively. (M - R) H&E transversal sections demonstrating morphology of the VFs in control embryos (M – N),
\(\beta\text{-Cat}^{\text{cko}}\) mutants at E16.5, in milder cases (O – P) and severe cases (Q – R). Bracketed regions in the panels of M, O, Q are magnified in N, P, R. Black solid arrows in the panel N denote expansion of the LC and PD respectively, an arrowhead points to the two-layered VF epithelium. A black solid arrow in the P panel denotes expansion of the LC. Arrowheads in the panels of P, R denote a flat single layer of VF basal cells in milder (P) and severe cases (R) of laryngeal webbing. For each experimental group at least three different individuals were collected for analysis. For each individual at least two transversal sections from cranial and caudal VF regions were characterized. The experiment was replicated two times in the laboratory. Abbreviations: AC, arytenoid cartilage; CC, cricoid cartilage; EL, epithelial lamina; Es, esophagus; dLM, dorsal laryngeal muscles; LC, laryngeal cecum; LPh, primitive laryngopharynx; PD, pharyngoglottic duct; PG, posterior glottis; spt, septum; TA, thyroarytenoid muscle; TC, thyroid cartilage; Tr – trachea; VF – vocal fold.
Figure 4: Inactivation of β-Catenin affects the EL integrity. (A - H) Anti-K8 staining of cells at the EL in green at 13.5 (A - D) and E16.5 (E - H). (A, B) Anti-K8 staining in control embryos, a bracketed region in the A panel is magnified in the B panel. (C, D) Anti-K8 staining in β-Cat<sup>cko</sup> mutants, a bracketed region in the C panel is magnified in the D panel. A white dashed arrow in the panel D denotes the process of separation of the tip of the EL from the remaining cells at the EL. (E - H) Anti-K8 staining of cells at the LC, EL and PD in green at E16.5 in control embryos β-Cat<sup>cko</sup> mutants (G, H). For each experimental group at least three different
individuals were collected for analysis. For each individual at least two transversal sections from cranial and caudal VF regions were characterized. The experiment was replicated two times in the laboratory. Abbreviations: EL, epithelial lamina; Es, esophagus; LC, laryngeal cecum; PD, pharyngoglottic duct; VF – vocal fold.
Figure 5: Inactivation of β-Catenin in the EL leads to downregulation of Cyclin D1 and upregulation of p27Kip. (A – F) Red anti-Cyclin D1 staining in control embryos (A – C) and β-Catcko mutants (D – F) at E11.5. Bracketed regions at the panels of A and D are magnified at B, E respectively. White dashed arrows in E, F denote cyclin D1 negative cells in the EL. (G - L) Red anti-p27Kip staining in control embryos (G - I) and β-Catcko mutants (J - L) at E11.5. White solid arrows in K, L denote p27 positive cells at the EL. (M, N) Statistical analysis of quantitative distribution of Cyclin D1 (M) and p27 (N) positive cells. The Student T-test was performed to compare Cyclin D1 and p27 expressions at the EL and LP in control embryos and β-Catcko mutants. For each experimental group at least three different individuals were collected for analysis. For each individual at least two transversal sections from cranial and caudal VF regions were characterized. The experiment was replicated two times in the laboratory (O, Q) H&E transversal sections demonstrating morphology of VFs in control (O) and double mutant embryos (Q) at E13.5. (P, R) Anti-K8 green staining in control embryos (P) and double mutants (R). Bracketed regions in the panels of O, Q are magnified in P, R respectively showing the size of the EL. For a double mutant embryo only two individuals were collected for analysis due to the low frequency for a double mutant. For each individual at least two transversal sections from cranial and caudal VF regions were characterized. The experiment was replicated two times in the laboratory. Abbreviations: Con, control embryos; EL, epithelial lamina; Es, esophagus; LC, laryngeal cecum; LPh, laryngopharynx; Mut, β-Catcko mutant embryos; PD, pharyngoglottic duct; spt, septum.
Figure 6: Inactivation of \(\beta\)-Catenin at the EL leads to defective differentiation of mesenchymal cells.  

(A – D) Green anti-SOX9 and red anti-Myosin MF-20 staining in transversal sections of control embryos (A, B) and \(\beta\)-Cat\(^{\text{cko}}\) mutants (C, D) at E11.5. Bracketed regions in the panels of A, C are magnified in B, D respectively.  

(E, F) Green anti-SOX9 and red anti-Myosin MF-20 staining in transversal sections of control embryos (E) and \(\beta\)-Cat\(^{\text{cko}}\)
mutants (F) at E13.5. (G, J) H&E transversal sections demonstrating morphology of the VFs at E16.5 in control embryos (G) and β-Cat"cko" mutants (J). Bracketed regions are magnified in H, K (H – control; K - β-Cat"cko" mutants), and I, L (I – control embryos; L - β-Cat"cko" mutants) respectively. Green anti-SOX9 staining shows differentiation of cells into cartilages, red anti-MF-20 staining shows differentiation of mesenchymal cells into muscles. For each experimental group at least three different individuals were collected for analysis. For each individual at least two transversal sections from cranial and caudal VF regions were characterized. The experiment was replicated two times in the laboratory. Abbreviations: AC, arytenoid cartilage; AS, arytenoid swelling; CC, cricoid cartilage; EL, epithelial lamina; Es, esophagus; dLM, dorsal laryngeal muscles; IL, intermediate lamina; LC, laryngeal cecum; LL, lateral lamina; LPh, laryngopharynx; PD, pharyngoglottic duct; spt, septum; TA, thyroarytenoid muscle.
Figure 7: Inactivation of β-Catenin at the EL leads to defective specification of VF basal progenitors. (A – H) Red anti-p63 and green anti-K8 staining in control embryos (A - D) and
mutants (E- H) at E11.5. A bracketed region in the E panel is magnified in the lower right corner. Bracketed regions in panels of B, F are magnified in C, D and G, H respectively. White solid arrows in the panels of B, C, F, G denote p63+ cells, white dashed arrows in the panels of F, G denote p63- cells. (I – K) Red anti-p63 and green anti-K8 staining in control embryos at E13.5. A solid arrow in the J panel denotes p63+ cells and a dashed white arrow in the panel K denotes K8 downregulation in the EL. (L - N) Red anti-p63 and green anti-K8 staining in mutants at E13.5. Bracketed regions in the L panel are magnified in M and N showing dorsal regions with ventral regions magnified in the left upper corners. Dashed arrows denote p63-cells (M), solid arrows denote K8+ expression in mutants (N). (O, T) H&E staining of fully separated VFs in control embryos (O) and fused VFs in mutants, in milder cases (T). A bracketed region in the O panel is magnified in P – S. (P, Q) demonstrate red anti-p63 staining, (P, R) green anti-K8 staining, (S) green anti-K5 staining. A bracketed region in the T panel is magnified in U - X. (U - W) demonstrate anti-p63 (red) and anti-K8 (green) staining, (X) demonstrates green anti-K5 staining. Arrowheads in the panels of V and X denote p63- and K5- cells respectively. For each experimental group at least three different individuals were collected for analysis. For each individual at least two transversal sections from cranial and caudal VF regions were characterized. The experiment was replicated two times in the laboratory. Abbreviation: AC, arytenoids; CC, cricoid; EL, epithelial lamina; Es, esophagus; dLM, dorsal laryngeal muscles; G, glottis; LC, laryngeal cecum; LPh, laryngopharynx; PD, pharyngoglottic duct; TC, thyroid cartilage; TA, thyroarytenoid muscle; VF, vocal fold.
Figure 8: Schematic illustration demonstrating the EL fusion and recanalization in control embryos and $\beta$-Cat$^{kco}$ mutants, in milder and more severe cases of laryngeal webbing. (1 A - B) Formation of the EL in control embryos at E11.5 (A). Formation of LC (red cells) and PD (yellow cells) in control embryos at E13.5 (B). Epithelial cells in the EL express p63 (grey nuclei), proliferate and accumulate along the EL midline. Meanwhile mesenchymal cells differentiate into laryngeal cartilages. (2 C) Inactivation of $\beta$-Catenin in $\beta$-Cat$^{kco}$ mutants leads to the failure in the EL fusion at E11.5 and disrupted initial differentiation of VF basal progenitors. Some VF epithelial cells remain columnar and fail to upregulate p63 (green nuclei). (2 D) The delay in the EL fusion leads to the delay in LC expansion (in red) and the
absence of the PD and the septum with the cricoid cartilage. (3 - 4) Failure in specification of basally positioned of VF progenitors versus a suprabasal cell layer may prevent EL recanalization in milder web cases. (3 E, F). (4) In more severe cases of laryngeal webs, inactivation of \( \beta \)-Catenin in the epithelium affects the EL integrity. After delayed EL fusion, cells at the tip of the EL can pinch off from the remaining EL enabling mesenchymal cells to migrate into the space in-between. It is also possible that some epithelial cells undergo aberrant epithelial-mesenchymal transition and contribute to laryngeal web formation (4 G, H).

Abbreviations: AS, arytenoid swelling; AC, arytenoid cartilage; CC, cricoid cartilage; EL, epithelial lamina; IL, intermediate lamina; LC, laryngeal cecum; PD, pharyngoglottic duct.
Supplemental materials

Figure S1: Laryngeal webs in humans. Unseparated VFs block the entrance into the trachea.

Directions: (V) ventral or (A) anterior; (D) dorsal or (P) posterior. Abbreviations: AC, arytenoid cartilage; CC, cricoid cartilage, TC, thyroid cartilage; Tr, trachea; VF, vocal fold.
Figure S2: Schematic illustration demonstrating the effect of mechanical forces acting on the EL recanalization in control embryos. VFs initiate their development at E10.5, when the lateral walls of the primitive LPh are closely juxtaposed. At E11.5, the lateral walls fuse together and create the EL. At E13.5 the EL initiates recanalization, due to the expansion of the LC and PD. The LC extends into the larynx from the cranial to caudal direction (red arrow), the PD is continuous with the subglottis and trachea (green arrow). The activity of dorsal laryngeal muscles affects primarily the final stages of the EL recanalization, when the laryngeal cartilages and muscles complete their formation (blue arrows). Abbreviations: EL, epithelial lamina; Es, esophagus; LPh, primitive laryngopharynx; LC, laryngeal cecum; PD, pharyngoglottic duct; Tr, trachea; VF, vocal folds.
Figure S3: Differentiation of epithelial cells at the EL at E11.5 in control embryos and β-
Cat<sup>cko</sup> mutants and anti-β-Catenin staining of mesenchymal cells in the disrupted EL in β-
Cat<sup>cko</sup> mutants. (A, B) Anti-SOX2 (red) and anti-NKX2-1 (green) double staining at the EL, at
E11.5, in control embryos (A) and mutant mice (B). SOX2 expression domain is extended
ventrally at the expense of NKX2-1 in β-Cat<sup>cko</sup> mutants. (C, D) Negative Alcian blue staining of
VF epithelial progenitors in β-Cat<sup>cko</sup> mutants at E11.5. (E, F) Anti-β-Catenin staining of
mesenchymal cells in the disrupted EL in β-Cat<sup>cko</sup> mutants at E18.5. A bracketed region in the E
panel is magnified in the F panel showing that mesenchymal cells that migrate between the EL
fragments is a mixture of β-Catenin positive and β-Catenin negative cells. Abbreviations:AC,
arytenoid cartilage; EL, epithelial lamina; Es, esophagus; LP, lamina propria; LPh, primitive
laryngopharynx; TA, thyroarytenoid muscle; TC, thyroid cartilage.
Figure S4: Assessment of cell proliferation and apoptosis during EL formation. (A, B) TUNEL assay detecting apoptotic cells in the EL and LP in control and $\beta$-Cat$^{cko}$ mutant embryos at E11.5. Black arrows denote positive apoptotic cells in brown. (C – F) EdU proliferation assay shows green proliferating cells in the EL and LP in controls and $\beta$-Cat$^{cko}$ mutants in the cranial (C, E) and caudal regions (D, F). (G) Statistical analysis of quantitative distribution of EdU positive cells with p-values. Abbreviations: EL, epithelial lamina; LP, lamina propria; LPh, primitive laryngopharynx.
**Figure S5: Assessment of cellular and structural changes during EL formation and recanalization** (A, F) Anti-Laminin 5 (basement membrane marker) staining (in red) demonstrates that epithelial cells retain basal features during EL formation. (B - E, G - J) Transmission electron microscope (TEM) images demonstrate EL fusion in control embryos (B) and failure in EL fusion in β-Cat\(^{cko}\) mutants (G) at E11.5. Bracketed regions in the panels of B, G are magnified in the panels of C (for control embryos) and H (for β-Cat\(^{cko}\) mutants), respectively. Bracketed regions in the panels of C, H are magnified in the panels of D, E, and I, J showing details of the apical regions (D,I) and the basement membrane (E,J). Solid black arrows denote the basement membrane with protrusions in control embryos, dashed black arrows denote smooth basement membrane without protrusions in β-Cat\(^{cko}\) mutants. Arrowheads in the panels of H and I denote filopodia and tight junctions in the apical surface of epithelial cells in β-Cat\(^{cko}\) mutants. (K) Anti-Laminin 5 staining (in red) in control embryos during EL recanalization at E16.5. (L, M) Anti-Laminin 5 staining (in red) in β-Cat\(^{cko}\) mutants during EL recanalization at E16.5 in more severe cases of laryngeal webs. A bracketed region in the L panel is magnified in M showing disintegration of the EL in severe cases of laryngeal webs. A dashed arrow denotes the dorsal fragment of the EL with basal cells that downregulate Lam 5 expression. (N,O) IF staining for ECadherin in β-Cat\(^{cko}\) mutants in more severe cases of laryngeal webs. E-Cadherin expression remains robust in the remainder of the EL in the mutant, suggesting that β-Catenin loss does not have a direct effect on adherence junctions.” Abbreviations: BM, basement membrane; EL, epithelial lamina; LC, laryngeal cecum; LPh, primitive laryngopharynx.