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Notum attenuates Wnt/βcatenin signaling to promote tracheal cartilage patterning

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Abstract

Tracheobronchomalacia (TBM) is a common congenital disorder in which the cartilaginous rings of the trachea are weakened or missing. Despite the high prevalence and clinical issues associated with TBM, the etiology is largely unknown. Our previous studies demonstrated that *Wntless* (*Wls*) and its associated Wnt pathways are critical for patterning of the upper airways. Deletion of *Wls* in respiratory endoderm caused TBM and ectopic trachealis muscle. To understand mechanisms by which Wls mediates tracheal patterning, we performed RNA sequencing in prechondrogenic tracheal tissue of $Wls^{f/f};Shh^{Cre/wt}$ embryos. Chondrogenic *Bmp4*, and *Sox9* were decreased, while expression of myogenic genes was increased. We identified *Notum, a* deacylase that inactivates Wnt ligands, as a target of *Wls* induced Wnt signaling. *Notum*'s mesenchymal ventral expression in prechondrogenic trachea overlaps with expression of *Axin2*, a Wnt/β-catenin target and inhibitor. *Notum* is induced by Wnt/β-catenin in developing trachea. Deletion of Notum activated mesenchymal Wnt/β-catenin and caused tracheal mispatternning of trachealis muscle and cartilage as well as tracheal stenosis. Notum is required for tracheal morphogenesis, influencing mesenchymal condensations critical for patterning of tracheal cartilage and muscle. We propose that *Notum* influences mesenchymal cell differentiation by generating a barrier for Wnt ligands produced and secreted by airway epithelial cells to attenuate Wnt signaling.

Introduction

Tracheobronchomalacia (TBM) is a common congenital malformation in which the walls of the conducting airways lack adequate cartilage causing airway obstruction. Airway malacias are observed in 1/2000 live births (Boogaard et al., 2005; Kenny AP, 2013); Tracheal sleeves and complete tracheal rings (CTR) are rare conditions, but accompanied by severe clinical problems including respiratory distress as well as cardiovascular anomalies (Hewitt et al. 2016). Despite their prevalence and clinical importance, the etiology of these conditions is largely unknown and the mechanisms underlying the abnormal patterning of the conducting

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airways is poorly understood (Fausett and Klingensmith, 2012). Treatment of severe structural abnormalities of the proximal conducting airways are limited to invasive surgery and palliative care. Understanding the mechanisms regulating tracheal cartilage and muscle formation is critical for developing new diagnostic and treatment strategies for tracheal-bronchial malformations.

While an increasing wealth of information is known about morphogenesis of the respiratory tract in embryonic development, our understanding of how the upper airways are patterned remains poorly understood. Upper airways are organized across the cephalic-caudal and dorsal-ventral axes where cartilage alternates with muscle. How this pattern is established remains unknown; however, perturbations in normal patterning of the tracheal mesenchyme causes TBM and CTR. In previous studies, we demonstrated that deletion of *Wls,* a cargo receptor mediating secretion of Wnt ligands, from respiratory and digestive endoderm caused lack of tracheal cartilage and abnormal smooth muscle patterning in developing trachea (Snowball et al., 2016; Snowball et al., 2015b). To understand the mechanisms underlying the patterning of developing trachea we performed an unbiased analysis of gene expression in $Wls^{f/f};Shh^{Cre/wt}$ tracheal tissue and identified novel Wnt responsive genes in respiratory tract. Among them, *Notum was identified* as a novel *Wls-promoted Wnt/β-catenin* target expressed in developing trachea.

Notum is a target and modulator of the Wnt/β-catenin signaling that attenuates Wnt signaling (Giraldez et al., 2002). Notum encodes an extracellular deacylase that removes the covalent bound lipid moieties of Wnt ligands; without this lipid modification, Wnt ligands are inactive. Studies in invertebrates, fish and frog indicate that Notum plays a critical role in cephalic development by repressing Wnt/β-catenin (Ayers et al., 2012; Flowers et al., 2012; Roberts-Galbraith and Newmark, 2013; Zhang et al., 2015). TCF binding sites are present in the *Notum* promoter as similarly seen in the *Axin2* promoter. *Notum* is a direct target of Wnt/β-catenin, that serves in a negative feedback loop required for attenuation of the strength of Wnt/β-catenin signaling (Kakugawa et al., 2015; Zhang et al., 2015).

Wnt signaling activity requires precise regulation, as increased and decreased activity leads to abnormal development and disease (Freese et al., 2010; Konigshoff and Eickelberg, 2010; Mohinta et al., 2007; Van Scoyk et al., 2008). Several studies have shown that Notum attenuates Wnt signaling in *in vitro* and *in vivo* functional assays. Notum is increased in several Wnt driven cancer such as gastric, hepatic and colorectal cancer (De Robertis et al., 2015). In these pathologies, high levels of Notum could be detected in circulation via ELISA constituting a molecular biomarker for diagnosis of cancer (Madan et al., 2016). Notum may contribute to osteoporosis as deletion of *Notum* in mouse causes increased bone mass (Tarver et al., 2016). Currently, it is unknown how abnormal levels of Notum affect the Wnt/β-catenin signaling during mammalian development or in respiratory tract. In vitro studies suggest that Notum attenuates Wnt/β-catenin independent signaling; however, it is unknown whether *Notum* has a direct impact on Wnt/β-catenin independent signaling in vivo (Zhang et al., 2015).

In order to understand the role of Notum in respiratory tract we generated a novel Crispr-Cas9 *Notum knockout (KO)* mouse. We observed defects in tracheal development,

characterized by abnormal cartilage smooth muscle and stenosis. Our studies determined that Notum is required for formation of mesenchymal condensations. We proposed that Notum likely interacts with Axin2 and is regulated by epithelial Wls-mediated signaling, to attenuate Wntβ-catenin signaling in the developing trachea promoting cartilage formation.

Methods

Mouse breeding and genotyping

Animals were housed in a pathogen-free environment and handled according to the protocols approved by CCHMC Institutional Animal Care and Use Committee (Cincinnati, OH, USA). Generation of the *Wntless* (*Wls*) conditional knock-out (CKO) mouse has been described (Carpenter et al., 2010). $Wls^{f/f}; Shh^{Cre/wt}$ embryos were obtained by breeding $Wls^{f/f}$ mice with Shh^{Cre} mice and rebreeding the resulting mice with $Wls^{f/f}$ mice (Harfe et al., 2004). Crispr-Cas9 *Notum* mouse was generated by the Transgenic Animal and Genome editing core (CCHMC) (See Fig. 4 for details). Breeding *Notum founder* mouse with wild type mice generated $Notum^{150/Wt}$ mice and rebreeding generated $Notum^{150/150}$. The presence of the mutation was confirmed by sequencing analysis (See below). $Axin^{LacZ/Wt}$ mice were crossed with $Notum^{150/Wt}$ to generate $Notum^{150/Wt}$; $Axin^{LacZ/Wt}$ mice. $Ror2^{f/f};Dermo1^{Cre/wt}$ mice were obtained by breeding $Ror2^{f/f}$ with $Dermo1^{Cre/wt}$ mice and mating resultant mice to $Ror2^{f/f}$ mice (Ho et al., 2012a; Sosic et al., 2003). *Sox9EGFP* mice were previously described (Gong et al., 2003). To generate $Ctnnb^{\Delta 3};Dermo1^{Cre/wt}$ embryos, $Ctnnb^{\Delta 3}$ mice were crossed to $Dermo1^{cre/wt}$ mice (Harada et al., 1999). Genotypes of transgenic mice were determined by PCR using genomic DNA isolated from mouse-tails or embryonic tissue. Primers utilized for genotyping are provided as Supplementary material (Supplementary table 1).

Transcriptomic analyses

RNA-sequencing was performed on pooled (n=8), isolated, tracheas from Control and $Wls^{f/f}; Shh^{Cre/wt}$ E11.5 and E13.5 embryos. RNA was isolated using trizol according to manufacturer's instructions. RNA sequencing was performed by Cincinnati Children's Hospital Medical Center's Gene Expression Core utilizing the Ovation RNA-Seq System v2 (NuGEN) and Nextera XT DNA Sample Preparation kits (Illumina Technologies). RNA-seq FASTQ files were aligned using Bowtie to mouse genome version mm9 (Langmead et al., 2009). Differential gene expression was determined using Ingenuity Pathway Analysis (www.qiagen.com/ingenuity). Genes were deemed differentially expressed with an unpaired T test p-value<0.05, fold change >1.5 and read density>1 in both replicates of at least on condition. Heatmap of differentially expressed genes were z-score normalized and generated using Partek Genomics Suite (http://www.partek.com/pgs). ToppGene's ToppFun was used to identify functional enrichment hits of significantly altered RNAs in either RNA-seq analysis (Chen et al., 2009). Sequencing data has been deposited in GEO repository under GSE97445.

DNA constructs

Plasmid utilized for transfection were pGL2 Top Flash, pGL2 Fop Flash (Sinner et al, 2004), pCMVsport6ΔNNotum GE, pcDNA3 ΔCNotum, pcDNA3 Full Length Notum, (GenScript),

pGL2ATF2 (a gift from Dr. Niehrs) (Ohkawara and Niehrs, 2011), pcDNA6 active Wnt5a, pcDNA6 active Wnt3a (Addgene) and pCS2+ Wnt5a GFP (a gift from Dr. Gradl) (Wallkamm et al., 2014).

Sequencing protocol

DNA obtained from tail was subject to amplification by PCR using specific primers to detect the region of interest where Crispr-Cas9 generated mutation was expected to occur. PCR product was isolated using a gel isolation kit following manufacturer's instructions. Isolated DNA was submitted for DNA sequencing. Sequences obtained after PCR amplification were compared to wild type Notum sequence using MacVector (NM_175263.4).

Measurements of embryonic trachea

Image J was utilized to measure lumen of the trachea, mesenchyme, cartilage, trachealis smooth muscle, and total area of the trachea. Each of these measurements were taken as close as possible to the same level, middle region of the trachea and same magnification. The images were measured with a freeform drawing instrument, tracing the desired area of measurement. Measurements were normalized to the total area of the trachea.

Histology, immunohistochemistry and immunofluorescence staining

Embryonic tissue was fixed and embedded in paraffin or frozen using OCT. Sections (6um) were processed for H&E or DAB staining as described (Mucenski et al., 2003). For immunofluorescence, antigen retrieval was performed, when necessary, using 10 mM citrate buffer, pH 6, before slides were blocked for 2 hours in TBS containing 10% Donkey serum and 1% BSA. Following blocking, slides were incubated, overnight, at 4° in primary antibody diluted accordingly in blocking solution. After removal of unbound primary antibody, slides were incubated with secondary antibody at a dilution of 1:200 for one hour. Slides were then washed and cover-sliped using Vecta Shield mounting media with or without Dapi. Fluorescent staining was visualized and photographed using an automated fluorescence microscope (Zeiss). Source and dilution of primary antibodies used for this study has been provided as Supplementary material (Table S2).

Cell Proliferation

E11.5 pregnant mice were injected with BrdU at concentration 100ug/g of body weight. Embryos were isolated at day E12.5 or E13.5. Embryonic sections were labeled with BrdU, Sox9 and SMA antibodies to determine mitotic cells in tracheal epithelium and mesenchyme. Labeled cells and total cells were counted per each field photograph at 20X, and ratios of proliferating cells to total cells, defined as number of Sox9 and αSMA stained cells, were calculated. Average mitotic index was determined for five different samples.

Embryonic whole Tracheal-lung culture

Embryonic tracheas were harvested at E11.5 or E13.5 and cultured at air-liquid interphase as described (Hyatt et al., 2004). LiCl (20mM and 40mM) or XAV (1uM) was added to the media one hour after initiation of culture. Explants were photograph every 24 hour and harvested after 72 hour and processed for lectin, alcian blue staining or RNA isolation.

RNA extraction and RT-PCR

Gene expression was determined by quantitative RT-PCR. RNA was isolated from embryonic tracheal explants using a commercially available kit (RNAeasy mini kit or micro kit, Qiagen-Promega). Reverse transcription was performed according to manufacturer instructions (Verso Fisher Sci), and Taqman probes were utilized to detect differential expression using a StepOnePlus RT-PCR System.

In-Situ Hybridization

Procedure was performed according to a protocol developed by Advanced Cell Diagnostics (ACD) (Wang et al., 2012). In situ probes were designed by ACD. In brief, slides were baked and deparaffinized. In situ probes were added to the slides and hybridization was performed for 2 hours at 40°C followed by several rounds of amplification steps. Signal was detected by chromogenic reaction using DAB and slides were counter stained with Hematoxylin. After mounting with permanent mounting media, slides were photographed using a wide field Nikon i90 microscope.

Lectin Staining

Samples were fixed overnight in PFA and then, rinsed multiple times in PBS. Afterwards, samples were incubated for 1 hour in blocking buffer comprised of 2% Goat Serum, 1% BSA, .3% Triton X and PBS. After removing blocking buffer, PNA lectin solution (in 10% normal goat serum and PBS) was added to the samples at a final concentration of 10 ug/ml. Samples were covered in aluminum foil and incubated overnight at 4C. Samples were rinsed with PBS multiple times and photographed using a Leica fluorescence dissecting microscope.

Transfections and luciferase assay

NIH3T3 cells were cultured in 12 well plate, at 37 degrees C and 5% CO_2. Cells 60%–70% confluent were transfected with a total of 2ug of plasmidic DNA using Lipofectamine 3000 according to manufacturer's instructions. Cells were harvested 48 hours posttransfection, washed with PBS and lyzed using a passive lysis buffer reagent. Luciferase activity was determined over 10 s integration time using a luminometer.

Alcian Blue staining

Embryonic tracheas were placed in 3% Acetic Acid for 1 hour, followed by Alcian Blue staining for another 1 hour. Samples were cleared in 100% Methanol and then rehydrated in 70% Ethanol, 50% Ethanol and PBS for 10 minutes each. After washes, the tracheas were fixed in 4% PFA for 15 minutes and then bleached for 1–2 hours in bleaching solution (30% H_2O_2 and 0.5% KOH). The tracheas were cleared in 0.5% KOH at 4°C and then dehydrated to 70% Ethanol with a series of washes (PBS, 30% Ethanol, 50% Ethanol) and stored in 70% Ethanol.

Whole mount X-galactosidase staining

Embryonic lungs were dissected and fixed in 4% paraformaldehyde (PFA) in PBS for 30 minutes, then washed in PBS and stained two to three hours in X-gal staining solution. To

stop the reaction, explants were washed in 3% dimethyl sulfoxide-PBS, rinsed in PBS, washed and stored in 70% ethanol. Explants were dehydrated in a graded series of ethanol and processed for paraffin embedding and sectioning. Slides were depparaffinized and counterstained with fast red.

Tracheal mesenchymal cell Isolation and culture

Embryos were harvested at E10.5 to isolate mesenchymal cells that will contribute to the tracheal mesenchyme (trMEF). Respiratory tract regions of at least five embryos of the same genotype (mT/mG Dermo1$^{Cre/+}$) were isolated, washed in PBS, minced in trypsin and incubated for 10 minutes at 37C. After incubation, tissue was pipetted until cell suspension formed. Cells were seeded in flasks containing MEF tissue culture media composed of DMEM, 1% penicillin/streptomycin and 20% non-heat inactivated FBS. (Supplementary Fig. 1).

Migration assay and cell tracking

Tracheal mesenchymal cells were seeded as monolayer in 24 well plates. Using a 200ul pipet tip, a wound was induced in the monolayer. Culture media were removed and replaced with media containing low percentage of non-heat inactivated FBS and Wnt5a, Wnt3a, and Notum conditioned media. Images were acquired at 0, 7, 11 and 24 hours post-scratch using an Olympus inverted microscope. Migration was determined as the ratio of covered surface to initial wound surface using Image J (NIH) software. Live imaging of migrating cells was obtained using confocal microscopy. Cell tracking was performed using NIS Elements tracking module and path speed was determined for approximately 50 cells/treatment over 10 hour. Path speed is measured by calculating the total path traveled by the cells divided by the total amount of time elapsed.

Statistics

Quantitative data were presented as mean ± standard error. Experiments were repeated at least twice with a minimum of three biological replicates for each group. Statistically significant differences were determined by paired T-test or one-way ANOVA followed by post hoc pairwise multiple comparison procedures (Tukey's multiple comparisons test). Significance was set at $P<0.05$.

Results

Tracheal mesenchymal genes are differentially modulated by epithelial Wnt signaling

Our previous studies determined that deletion of *Wls* from tracheal epithelium impaired expression of Sox9 in tracheal and bronchial mesenchyme and caused tracheobronchomalacia (Snowball et al., 2015b). Epithelial deletion of Wls caused ectopic and abnormally organized smooth muscle extending into the ventrolateral aspect of the trachea (Fig. 1A). Since gene expression profile during tracheal formation is largely unknown, we performed an unbiased analysis of the RNAs expressed in control and *Wls*$^{f/f;}$ *Shh*$^{Cre/wt}$ trachea (Lange et al., 2015). RNA was isolated at prechondrogenic stages: E11.5 when trachea and esophageal tube are fully separated and E13.5 when mesenchymal condensations occur. We identified genes differentially regulated after epithelial deletion of

Wls at both developmental stages. *Bmp4, Tbx4, Tbx5, and Tmem16*, genes required for tracheal cartilage development, were differentially expressed. *Sox9* critical for chondrogenesis, *Lef1*, *Tcf7* both direct targets of Wnt/β-catenin signaling (Tebar et al., 2001), were downregulated. A number of *genes were differentially regulated after epithelial deletion of Wls that were not previously identified in developing trachea, including Wnt/β-catenin modulators Notum (*Giraldez et al., 2002; Kakugawa et al., 2015; Zhang et al., 2015*), Tiki2 (Trabd2b), and genes involved in neural homeostasis Lrrc7 and Lgi3 (*Gu et al., 2005; Lee et al., 2006) (Fig. 1B,C). Several genes mediating muscle development were upregulated in $Wls^{f/f}; Shh^{Cre/wt}$ trachea, at both developmental stages analyzed (Fig. 1A, D).

Since positional information may play a key role in determining the dorsal-ventral patterning of the trachea, we analyzed the expression pattern of novel tracheal genes and compared to expression of genes known to regulate tracheal and lung development, including *Bmp4*, the epithelially expressed *Wnt7b* and mesenchymally expressed *Wnt5a. Notum* and *Lgi3* were restricted to the ventral and dorsal mesodermal aspect of the trachea, suggesting a potential role for these genes in cartilage and muscle formation respectively (Fig. 2A). *Lrrc7* was seldom detected in control trachea tissue. Interestingly, in $Wls^{f/f}; Shh^{Cre/wt}$ tracheas, as predicted by the RNAseq. *Notum* and *Lgi3* were downregulated, while *Myh11* (a muscle marker) and *Lrrc7* were increased and ectopically expressed circumferentially in the tracheal mesenchyme (Fig. 2B). *These data support the concept that Wnt ligands secreted by the tracheal epithelium influence the levels and spatial expression pattern of genes modulating tracheal cartilage development.*

Notum, a Wnt/β-catenin target gene, is expressed in distinct regions of the respiratory tract and throughout the mouse embryo

Notum RNA is restricted to the ventral mesenchyme of the trachea suggesting its potential role in chondrogenesis. Notum is expressed in a complementary pattern to *Wnt7b* and *Wls*, both detected in the circumference of the tracheal epithelium. *Notum* is expressed in the ventral subepithelial tracheal mesenchyme adjacent to epithelial *Wnt7b and to mesenchymal Wnt5a* (Fig. 2A, 3A). *Wnt5a and Bmp4*, which are involved in tracheal cartilage development, are also expressed in the ventral mesenchyme. As development of the respiratory tract progresses, *Notum* RNA becomes segmented and restricted to the periphery of mesenchymal condensations where cartilage develops (Fig. 3B,C). This expression pattern overlaps with regions of high *Wntβ-catenin* activity as determined by the X-gal staining of E13.5 tracheal tissue using the reporter mouse $Axin2^{LacZ}$ (Fig. 3F). *These data support the concept that ligands secreted by the tracheal epithelium influence the spatial expression pattern of Notum.* We also examined *Notum* RNA in other systems of the embryo during early organogenesis. In the lung, we detected transcripts in subepithelial mesenchyme surrounding airways and acinar tubules (Supplementary Fig. 2). In the spinal cord, *Notum* is detected between developing vertebral bodies. *Notum* is also expressed in the epithelium of the stomach, but excluded from the mesenchyme. We detected expression of Notum in anterior region of mesonefros, in complementary expression pattern to Wnt4, and in the epithelium of the stomach. *Notum* was also present in anterior mesenchyme of jaw and limbs (Supplementary Fig. 3). These data suggest that *Notum* influences patterning and formation in various organs.

Wnt/β-catenin signaling modulates *Notum* in the respiratory tract

We tested whether Wls mediated induction of *Notum* depends upon *β-catenin* signaling. Wnt/β-catenin activity was inhibited by incubating E12.5 control trachea-lung tissue in air-liquid interphase cultures in the presence of the tankyrase inhibitor XAV939, a drug that stabilizes Axin2, causing degradation of β-catenin. Inhibition of Wnt/β-catenin in control tracheas decreased *Notum* and *Lef1,* a known target of β-catenin (Fig. 3G). Incubation of E10.5 tracheal mesenchymal cells in presence of Wnt3a conditioned media induced *Notum*, while Wnt4 and Wnt5a had no effect on *Notum* expression. *Axin2* was induced by Wnt3a conditioned media. Similarly, as observed for Notum, Wnt4 and Wnt5a did not induce *Axin2* (Fig. 3H). Taken together, these data identify *Notum* as a target of Wnt/β-catenin signaling in developing conducting airways.

Deletion of *Notum* causes abnormal tracheal patterning and perinatal death

To study the role of Notum in embryonic development in vivo, we used Crispr-Cas9 to mutate *Notum*. We targeted a region between exon 3 and 4 to produce a truncated protein lacking the C-terminal region required for deacylase activity (Fig. 4A). We obtained four founder mice, two of them containing non-functional alleles (Fig. 4B). The offspring of mouse 9103, bore mutations containing a 158 bp deletion resulting in a frameshift and premature stop codon in exon 4 (Fig. 4C). *Notum 1*-123 is a non-functional allele as assessed in vitro. The truncated protein did not inhibit the Wnt3a induced Top-Flash activity. While a full-length construct inhibited the Top Flash reporter, Notum1-123 lacked the repressive activity. Likewise, a ΔN mutant lacking the region required for deacylase activity failed to inhibit Top-Flash activity (Fig. 4D).

Homozygous mice for the *Notum* 1-123 allele, hereafter $Notum^{150/150}$, died in the perinatal period due to abnormal kidney development, confirming published data obtained with a different *Notum* allele wherein defects in tooth development and perinatal lethality were also observed (Vogel et al., 2016). Examination of the respiratory tract in our *Notum KO* mice revealed that chondroblasts and myoblasts were specified; however, tracheas were stenotic accompanied with mispatterned cartilage extending into the dorsal side of the trachea and reduced trachealis muscle, indicating a ventralization of the tracheal mesenchyme (Fig. 5A, C). These defects extended into the bronchi that also have smaller luminal area (Fig. 5B). Number of cartilaginous rings were decreased, and gaps in chondrogenesis were seen on the ventral aspect of the trachea (Fig. 5D,E). These data demonstrate that Notum is critical for postnatal survival and that it influences tracheal mesenchyme patterning.

Differential cell proliferation in tracheal epithelium of $Notum^{150/150}$ embryos

Since the trachea in $Notum^{150/150}$ embryos were stenotic we sought to investigate whether changes in cell proliferation caused the reduction in luminal circumference of the tracheal tube. Pregnant mice were injected with BrdU at E11.5 and sacrificed at E13.5. αSMA and Sox9 stainings were performed to differentiate smooth muscle cells and chondroblasts. Cell proliferation was reduced in the tracheal epithelium but not in the mesenchyme (Fig. 6).

Abnormal mesenchymal condensations in *Notum*$^{150/150}$ *developing trachea*

Given the abnormalities in cartilage observed in conducting airways of *Notum* pups, we studied whether mesenchymal condensation, a prerequisite for the formation of cartilage, was impaired. At E13.5, PNA-Lectin staining, which labels condensing mesenchymal cells, was diffused in Notum tracheas as compared to the well-define staining observed in control tracheas. Similarly, Sox9 staining of E14.5 tracheal tissue demonstrated loss of condensations, compared to the well-condense masses of cells observed in the control trachea (Fig. 7A).

Since morphogenetic processes during tracheal formation require cell migration, we performed scratch assays using primary mesenchymal cells isolated from E10.5 tracheal mesenchyme. Cells were incubated with Wnt3a or Wnt5a conditioned media, ligands shown to influence migration of mesenchymal cells (Snowball et al., 2015a). Wnt5a promoted, while Wnt3a did not alter cell migration. Addition of Notum conditioned media (Supplementary Fig. 4) prevented the Wnt5a or serum induced migration of the cells (Fig. 7B). Furthermore, addition of Notum conditioned media decreased the speed of the cell migration (Fig. 7C). Cells incubated in Notum conditioned media tended to remain attached to the plate surface with a rounded morphology compared to a more elliptical morphology seen in control or Wnt5a treated cells (Fig. 7D). These data support the concept that Notum influences mesenchymal cell migration and likely influences cytoskeletal cell rearrangements necessary for cell migration.

Deletion of *Notum* increases Wnt/β-catenin activity in tracheal mesenchyme

Since Notum is a modulator of Wnt/β-catenin signaling we tested Wnt activity using the *Axin2Lacz* reporter mouse crossed with the *Notum*$^{150/150}$ mice. In the resulting *Notum*$^{150/150}$;*Axin2*$^{LacZ/Wt}$ mice, we observed increased in Wnt/β-catenin signaling. Reporter activity was expanded throughout the entire mesenchyme of the Notum mutant compared to the more localized subepithelial activity observed in control mice (Fig. 8A,B). *Axin2* transcripts were increased while levels of *Col2a1* and *Myh11* were similar in control and *Notum*$^{150/150}$ samples at E13.5 as determined by RNA in situ hybridization (Fig. 8E,F). β-catenin was increased in mesenchyme of the *Notum*$^{150/150}$ trachea (Fig. 8G,H) compared to the control trachea. Taken together these data support the concept that Notum is capable of regulating Wnt/β-catenin signaling in the trachea and mesenchyme of the developing respiratory tract.

Notum RNA overlaps with *Axin2* in ventral tracheal mesenchyme where both molecules would inhibit Wnt/β-catenin activity. Since Wnt β-catenin is increased after mutation of *Notum* (Fig. 8) or deletion of *Axin2*, we sought to test whether increased β-catenin activity causes abnormal tracheal development ex-vivo. We utilized a *Sox9-GFP* mouse model to monitor mesenchymal condensations using PNA lectin or Alcian blue staining of the tracheal tissue after incubation in LiCl, a known activator of Wnt/β-catenin. Addition of 20mM and 40 mM LiCl to E13.5 tracheal lung tissue blocked formation of mesenchymal condensations and cartilage (Fig. 9A,B). To test whether Wntβ-catenin affects tracheal cartilage formation in vivo, we generated *Ctnnb*$^{\Delta 3}$;*Dermo1*$^{Cre/wt}$ mutants that overexpressed β-catenin in tracheal mesenchyme. Immunofluorescence analysis was performed in sections

of E12.5 embryos to detect Sox9 and αSMA in tracheal mesenchyme, two critical molecules for cartilage and muscle development. Increased expression of β-catenin decreased Sox9 and aSMA (Fig. 9C). Taken together these data indicate that Wnt/β-catenin activity must be tightly regulated for normal tracheal mesenchyme differentiation, in particular the cartilage that requires attennuation of Wnt/β-catennin.

Since deletion of *Notum* causes increased β-catenin in tracheal mesenchyme, we tested whether the increased of Wntβ-catenin signaling could affect Wntβ-catenin independent signaling mediated by Wnt5a-Ror2. Wnt5a and Ror2 participate in tracheal development, but how deletion of these genes affect tracheal cartilage and muscle is unclear (Li et al., 2002; Oishi et al., 2003). We generated *Ror2* $^{f/f}$*;Dermo1*$^{Cre/wt}$ embryos, to delete *Ror2* in tracheal mesenchyme. In these embryos mesenchymal deletion of *Ror2* causes tracheal stenosis as determined by decreased lumen area at E14.5 as seen in *Notum*$^{150/150}$ (Fig. 9D). The similarities observed in *Notum*$^{150/150}$ and *Ror2*$^{f/f}$*;Dermo1*$^{Cre/wt}$ tracheas, lead us to test whether increased Wntβ-catenin signaling antagonized Wntβ-catenin independent signaling. We tested this possibility in vitro using mouse mesenchymal NIH3T3 cells. An ATF2: luciferase reporter was used to monitor Wnt/β-catenin independent signaling (Ohkawara and Niehrs, 2011). Co-transfection of ATF2 reporter and Wnt5a stimulated luciferase activity, while co-transfection of ATF2 and Wnt3a failed to activate the reporter. Co-transfection of ATF2, Wnt5a and Wnt3a dramatically decreased the luciferase activity induced by Wnt5a, suggesting that Wnt3a impairs Wnt5a induced Wnt/β-catenin independent signaling (Fig. 9E). Taken together, these data demonstrate that *Notum* attenuates Wntβ-catenin signaling in tracheal mesenchyme and could mediate a switch between Wnt/β-catenin dependent and independent signaling, thus enabling normal progression of cartilage and muscle development.

Discussion and Conclusions

Using a mouse model of tracheobronchomalacia, we identified *Notum*, a modulator of Wnt/β-catenin activity, as a *Wls* target in the developing respiratory tract (Giraldez et al., 2002). Deletion of *Notum* caused tracheal stenosis, ectopic Sox9 expression and reduced trachealis muscle. We determined that mesenchymal condensations were impaired partially because of the increased Wnt/β-catenin activity detected in developing *Notum*$^{150/150}$ trachea. Further, Notum partially recapitulates the tracheal stenosis observed in Ror2$^{f/f}$;Dermo1$^{Cre/wt}$ embryos, a model in which Wnt/β-catenin independent signaling is impaired. We identified Notum as a critical modulator of Wnt/β-catenin signaling in ventral mesenchyme of developing trachea that is required for formation and patterning of tracheal cartilage and muscle.

Epithelial Wls modulates expression of Notum to promote tracheal morphogenesis

Our previous studies demonstrated that conditional deletion of *Wls* in the epithelium of mouse developing trachea virtually abolished expression of *Sox9 as well as* cartilage development. These studies also demonstrated the critical role of Wnt ligands produced and secreted by the tracheal epithelium in regulation of genes driving cartilage formation in the ventral aspect of the trachea (Fig. 1) (Snowball et al., 2015c).

Performing unbiased RNA sequence analysis of the developing *Wls*$^{f/f}$;*Shh*$^{Cre/wt}$ and control tracheal tissue allowed us to identify *genes differentially regulated by Wls including the Wnt/β-catenin modulators Notum (*Giraldez et al., 2002; Kakugawa et al., 2015; Zhang et al., 2015*) and Lgi3 (*Gu et al., 2005; Lee et al., 2006) as well as the synapse modulator and podocyte slit diaphragm gene *Lrrc7*.

Notum encodes an extracellular deacylase that removes the lipid moieties from Wnt ligands rendering them inactive. We identified Notum as a target of Wls and Wntβ-catenin signaling expressed in early stages of trachea development, near the time after separation of trachea and esophagus. Notum RNA was found in a very defined pattern restricted to the ventral aspect of the trachea in the subepithelial mesenchyme, where Wnt/β-catenin activity is high. Present and previous findings demonstrated that both *Notum* and *Axin2* are direct targets of Wnt/β-catenin (Kakugawa et al., 2015; Zhang et al., 2015). We found that *Axin2* RNA overlapped with Notum RNA in the developing trachea. In invertebrates, fish and frog Notum plays a critical role in cephalic development by repressing Wnt/β-catenin (Ayers et al., 2012; Flowers et al., 2012; Roberts-Galbraith and Newmark, 2013; Zhang et al., 2015). Recent studies in chick embryo demonstrated that Notum participates in patterning of developing neural tube, wherein increased expression of *Notum* caused a dorsalization in developing neural tube (Saad et al., 2017). These findings support the concept that Notum participates in embryonic patterning processes.

We identified a role for *Notum* in formation of condensations of mesenchymal cells, an essential step for patterning of tracheal cartilage and smooth muscle. Deletion of *Notum* caused a ventralization, wherein chondrocytes were found further dorsally than in control tracheas (Fig. 5A). While trachealis muscle was diminished in the *Notum*$^{150/150}$ tracheas, it is presently unclear if this is a direct effect of Notum in development of trachealis muscle or a secondary effect caused by abnormalities in patterning of tracheal cartilage. Taken together, our findings support a critical role for Notum, mediating the dorsal-ventral patterning of the tracheal mesenchyme.

How is Wnt/β-catenin modulated in the respiratory tract?

Organogenesis requires precise regulation of Wnt signaling, since both increased and decreased activity leads to abnormal development and disease (Clevers and Nusse, 2012; Freese et al., 2010). Studies in invertebrates, fish and frog showed that Notum attenuates Wnt/β-catenin signaling (Flowers et al., 2012; Kakugawa et al., 2015; Zhang et al., 2015); however, it is unknown how anomalous levels of Notum affect the strength of Wnt/β-catenin signaling during respiratory tract development. We showed that during organogenesis, Notum is highly expressed in upper airways and its deletion causes increases mesenchymal Wnt/β-catenin activity (Fig. 8 and 9).

Published studies determined that deletion of the β-catenin inhibitor APC in mesenchyme of the respiratory tract, reduced tracheal length. Shortening tracheal phenotype was partially due to increased β-catenin signaling (Luo, Y et al 2015). Our studies using β-catenin gain of function determined that increased levels of mesenchymal Wntβ-catenin signaling impaired differentiation of the tracheal mesenchyme, inhibiting formation of mesenchymal condensations, and decreased Sox9 and αSMA (Fig. 9C). In the present study, we observed

poor differentiation of mesenchymal condensations and increased β-catenin in the *Notum*$^{150/150}$ trachea, supporting a role of *Notum* in attenuating Wnt/β-catenin signaling to influence patterning of the tracheal mesenchyme.

We found that *Notum and Axin2* co-localized in the ventrolateral aspect of the trachea. In developing frog embryos, *Notum* synergizes with *Tiki2,* another Wntβ-catenin inhibitor also identified by our RNAseq analysis, to pattern anterior axial body by inhibiting Wnt/β-catenin signaling (Zhang et al., 2016). We suggest that Notum, may also synergize with Axin2 to promote tracheal development by further inhibiting Wnt/β-catenin signaling. Genetic studies are required to formaly test whether *Notum and Axin2* interact to promote tracheal cartilage and muscle.

A role for Notum in balancing Wnt/β-catenin dependent and independent signaling?

Notum is enriched in the subepithelial mesenchyme of the trachea in a layer of cells between domains of expression of epithelial Wnt7b and mesenchymal Wnt5a (Fig. 2). This spatial localization suggests that Notum could create a physical and functional barrier attenuating the signaling elicited by each of the Wnt ligands. In developing respiratory tract, several studies demonstrated that Wnt7b primarily acts through Wnt/β-catenin signaling to promote growth and differentiation of the pulmonary epithelium and muscle layer of peripheral lung vasculature (Cohen et al., 2009; Shu et al., 2002; Wang et al., 2005). Deletion of Wnt7b has been linked to a mild tracheal cartilage malformation (Rajagopal et al., 2008).

Wnt5a, expressed in the mesenchyme that gives rise to tracheal cartilage, participates in tracheal development and its deletion causes stenotic and truncated tracheas (Li et al., 2002). Similarly, *Ror2*, which serves as a receptor for Wnt5a via Wnt/β-catenin independent signaling (Ho et al., 2012b), is also required for tracheal patterning and its germline deletion leads to tracheal stenosis, recapitulating the Wnt5a phenotype (Oishi et al., 2003). We found that conditional deletion of Ror2 in the tracheal mesenchyme caused tracheal stenosis (Fig. 9). Deletion of Notum caused tracheal stenosis (Fig. 5), partially recapitulating the tracheal phenotype observed after mesenchymal deletion of *Ror2*.

Wnt5a and Wnt3a can compete for binding to receptors in the cell membrane, shifting the output of Wnt signaling between Wnt/β-catenin dependent and independent signaling (Bryja et al., 2009; Grumolato et al., 2010). Present in vitro studies demonstrated that Wnt3a represses the Wnt5a induced activity in a reporter assay in vitro (Fig. 9). The presence of Notum RNA in cells located between Wnt7b and Wnt5a expressing cells in developing trachea (Fig. 2), supports the concept that Notum may balance Wnt/β-catenin dependent and independent signaling.

Increased levels of Notum prevented the Wnt5a-induced migration of the tracheal mesenchymal cells in vitro, while in vivo, deficiency of Notum impaired the formation of mesenchymal condensations (Fig. 7). Modulation of Wnt ligands activity mediated by Notum may be required for chemotaxis of mesenchymal cells, similarly to its role in neural tissue of the zebrafish, wherein Notum 2 provides guidance for axonal cone growth (Cantu et al., 2013). Wnt5a promotes both directional cell migration, critical for morphogenesis, and cytoskeletal rearrangements, required for cell condensations (He et al., 2008; Nishita et

al., 2006; Nomachi et al., 2008). Impaired Wnt5a induced signaling, resulting from increased of Wnt/β-catenin activity, will disrupt cells migration and condensation. Failure to form mesenchymal condensations at the right time may prevent exposure of mesenchymal cells to critical signals necessary for proper formation of cartilaginous rings, causing the abnormal cartilage patterning as observed in the *Notum*$^{150/150}$ tracheas.

Finally, it is possible that Notum may also affect Wnt5a activity independently of its role in balancing Wnt/β-catenin dependent and independent signaling. While there is not in vivo data on the effects of Notum on Wnt/β-catenin independent signaling, in vitro studies suggest that Notum impairs Wnt5a-induced activity (Supplementary Fig. 5) (Zhang et al., 2015). Wnt5a is critical for cartilage formation; however, abnormally increased levels of Wnt5a may also affect cartilage formation (Yang et al., 2003). Thus, Notum could modulate Wnt5a signaling activity to promote cartilage formation of developing trachea.

In summary, we have identified Notum as a novel regulator of the Wnt signaling in developing trachea (Fig. 10). Our data indicate that *Notum* plays a fundamental role in modulating the strength of Wnt signaling, which is required for cell differentiation and patterning of the respiratory tract.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Notum is a target of epithelial-induced Wnt/β-catenin signaling in developing trachea.
- Notum is a critical regulator of Wnt/β-catenin in developing tracheal mesenchyme.
- Notum is required for proper patterning of tracheal mesenchyme.

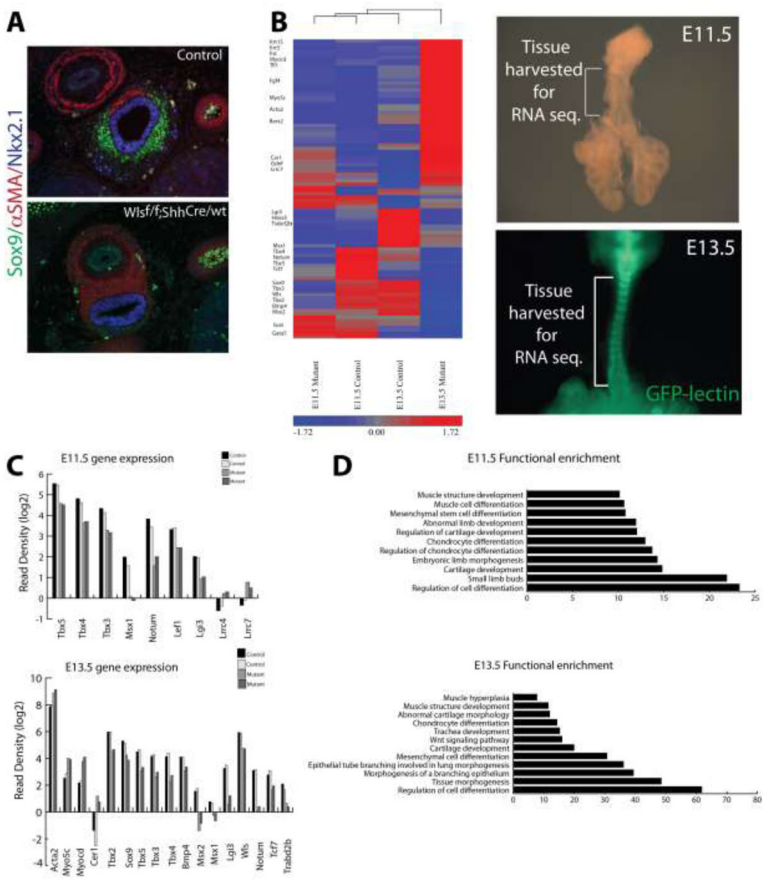

Figure 1.

A) Epithelial deletion of *Wls* results in loss of Sox9 positive cells within the tracheal mesenchyme. Irregular cartilage and muscle development is shown. Sections of tracheal tissue depict the lack of ventral mesenchymal Sox9 staining within $Wls^{f/f};Shh^{Cre/wt}$ samples and increased extent of αSMA staining. **B)** RNA sequencing studies of $Wls^{f/f};Shh^{Cre/wt}$ trachea at E11.5 and E13.5 demonstrates differential gene regulation, Z-score normalized heat map of all differentially expressed genes is shown. **C)** Log_2 transformed expression of select genes from E11.5 and E13.5 RNA seq analysis show differences in RNA levels. **D)** Representative, Toppfun functional enrichment hits. P-values were $-Log_2$ transformed. Functional enrichment analysis was performed using all differentially expressed RNA from both E11.5 or E13.5 RNA-Seq. Specific characteristics resulting from changes in gene expression were predicted for each developmental stage.

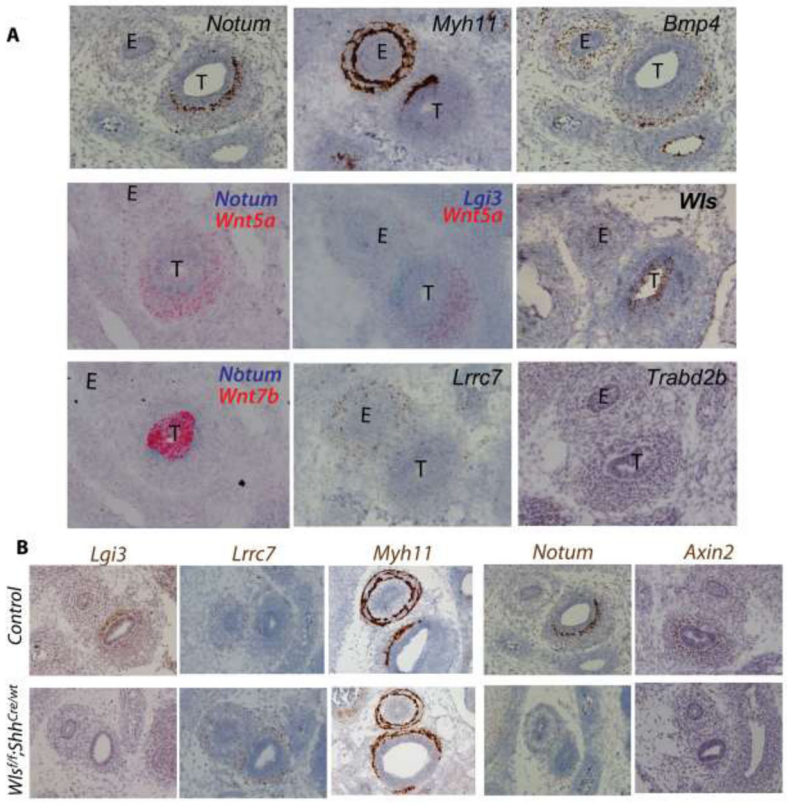

Figure 2.

A) In-situ hybridization analysis of E13.5 transverse sections using RNA scope demonstrates differential gene regulation. *Myh11* is expressed in muscle cells within the developing trachea, *Wls* is an epithelial marker of the developing trachea. Notum expression was complementary to *Wnt7b, Wnt5a, and Myh11*. **B)** Deletion of Wls from tracheal epithelium results in decreased *Lgi3, Notum* and *Axin2* and increased *Myh11* and *Lrrc7* within the complete tracheal mesenchyme. Notum RNA was enriched in the ventro-lateral aspect of the trachea, where cartilage forms. E: Esophagus, T: Trachea.

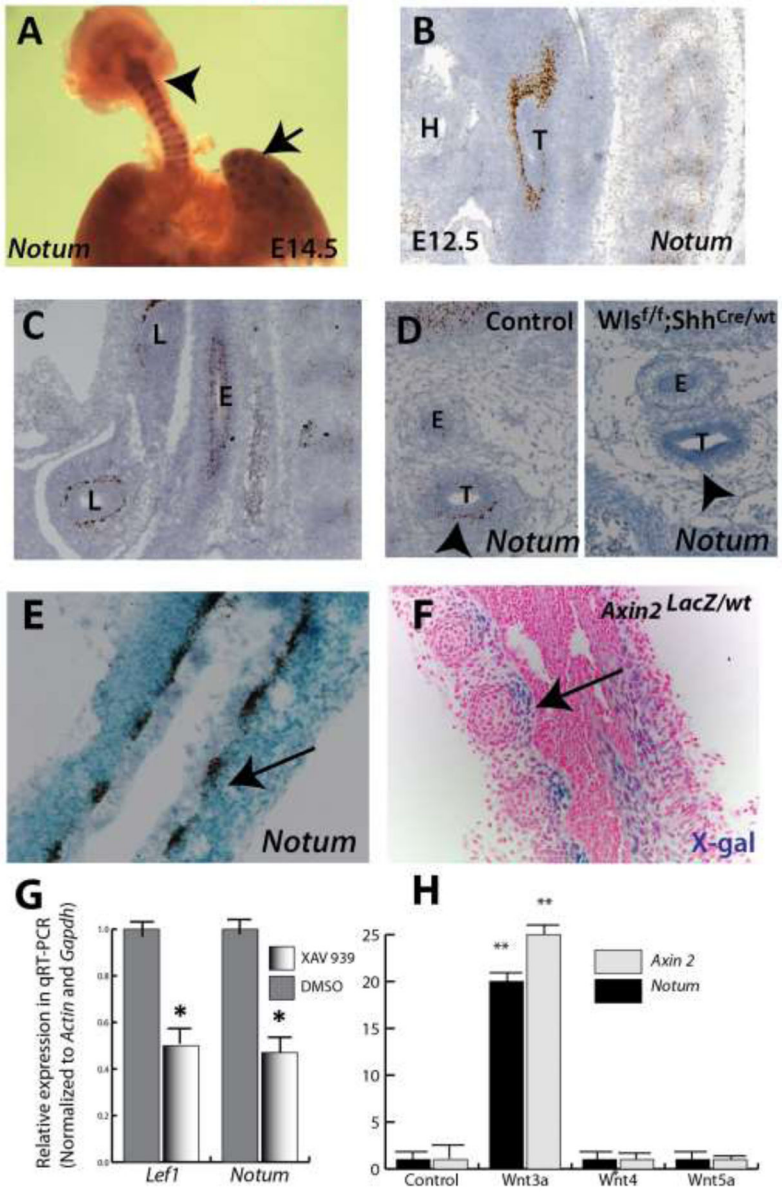

Figure 3.

A) Notum is expressed in developing lung and trachea (arrowheads) as detected by digoxygenin labeled RNA probe. Specificity was confirmed by sense probe that yielded no staining. **B and C)** In-situ hybridization on E12.5 longitudinal sections of trachea (B) and lung (C) using RNAscope are shown. Notum RNA is enriched in the ventral side of the trachea and the subepithelial mesenchyme of the lung. Note expression in esophagus. **D)** Notum was decreased in tracheal tissue after deletion of Wls in respiratory tract epithelium as determined by in situ hybridization. **E**) Notum RNA is restricted to the periphery of mesenchymal condensations. **F**) Wnt/β-catenin activity is detected in periphery of mesenchymal condensations. **G)** qRT-PCR of Lef1 and Notum performed in W11.5 tracheal tissue incubated in presence of Wnt/β-catenin inhibitor XAV939 is shown (N=4). **H)** qRT-PCR was performed to test the ability of different Wnt ligands to induce Notum and Axin 2

RNA in mesenchymal primary cells isolated from E10.5 tracheal tissue. Wnt ligands were added at concentration 400ng/ml (N=5). T: Trachea, H: Heart, E: Esophagus. L=Lung * $p<0.05$, ** $p<0.01$

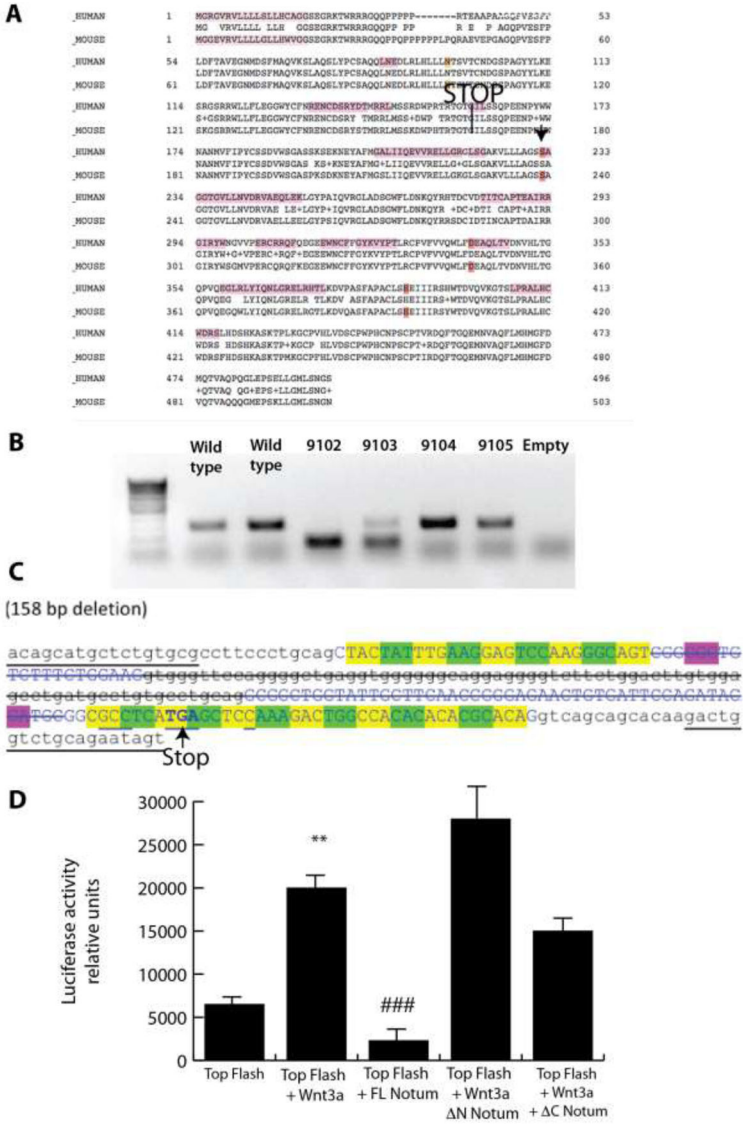

Figure 4.

A) Notum KO mouse was generated using Crispr-Cas9. The gRNAs targeted a region between exon 3 and 4 to generate a truncated protein (Stop) without active deacylase site (arrow in A). **B)** The wild type Notum band is detected at 250bp; mice 9103 and 9105 carry non-functional Notum alleles. **C**) Offspring of 9103 carry a mutation resulting in a premature stop codon. Sequence in C corresponds to exon 3,4 and corresponding intronic regions. Underlined sequences correspond to gRNAs targeted DNA regions. **D)** A top flash reporter assay was performed to test Notum activity. Notum conditioned media was tested as well (Supplementary Fig. 3). Notum represses Wnt/β-catenin activity as determined by the reporter, while truncated forms of Notum, that cannot be secreted ΔN Notum or do not contain the active site of the enzyme ΔC Notum did not repress the Wnt3a induced Wnt/β-catenin activity. N=4 ANOVA, Tukey's multiple comparisons test ** $p < 0.006$ Top flash vs Top Flash + Wnt3a; ### $p < 0.0002$ Top Flash + Wnt3a vs Top Flash + Wnt3a FL Notum.

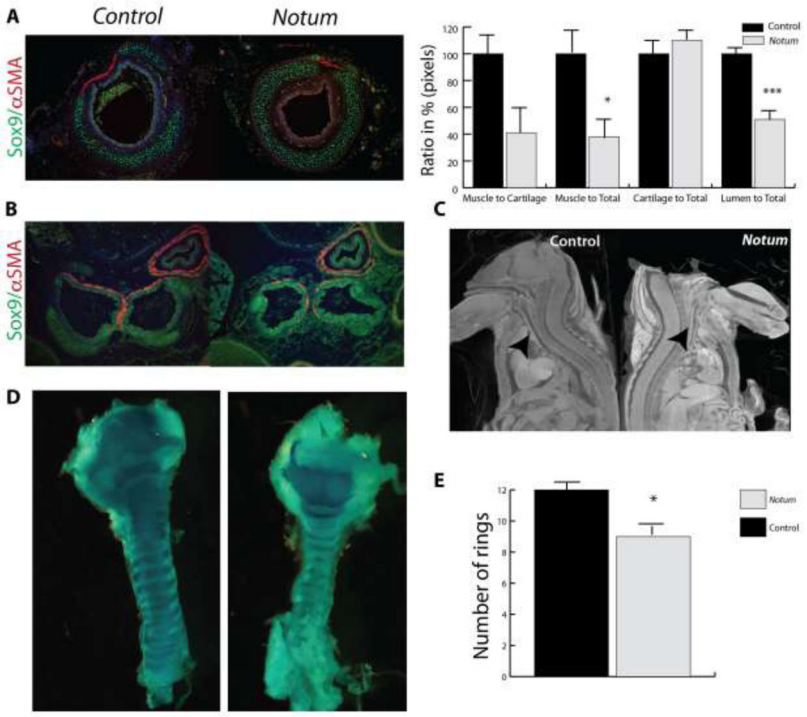

Figure 5. A–C) Stenosis is observed in Notum$^{150/150}$ bronchi and trachea

Immunofluorescence staining of transverse sections of E16.5 trachea and bronchi and micro CT scan of E16.5 embryos show stenosis and abnormal mesenchymal patterning. Smooth muscle is reduced on the dorsal side and the luminal area of the trachea is decreased. Areas of cartilage, muscle, lumen and total area of the trachea were quantified. **D**,**E**) Alcian blue staining of E16.5 tracheal explants was performed to determine the sites of chondrogenesis. In *Notum*$^{150/150}$ tracheas, the number of cartilaginous rings is reduced when compared to the control trachea. N=6 *p< 0.05; ***p< 0.001.

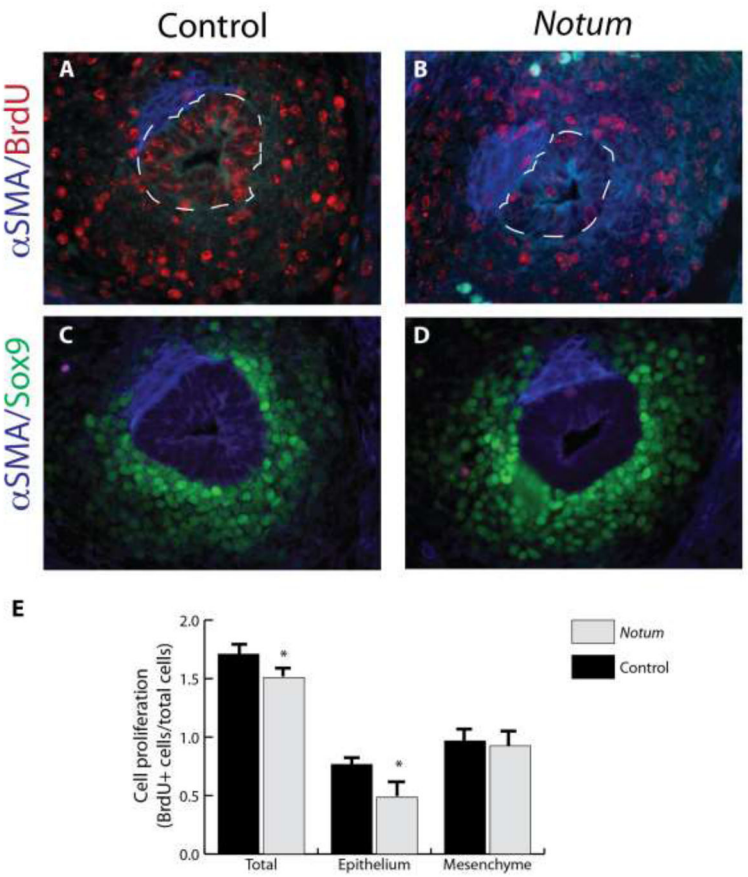

Figure 6. Deletion of Notum alters cell proliferation and patterning in developing trachea

A) *Notum*$^{150/Wt}$ females were injected with BrdU at E11.5. Sections of E13.5 embryos were stained with αSMA, Sox9 and anti-BrdU antibodies. For convenience in counting the number of BrdU positive cells, the images were separated as αSMA and anti BrdU (A and B) and αSMA and Sox9 (C and D). Dotted lines in A and B represent the limits of tracheal epithelium. **E)** Cell proliferation was decreased in the epithelium compared to mesenchyme of *Notum*$^{150/150}$ embryos. N=8 *p<0.05

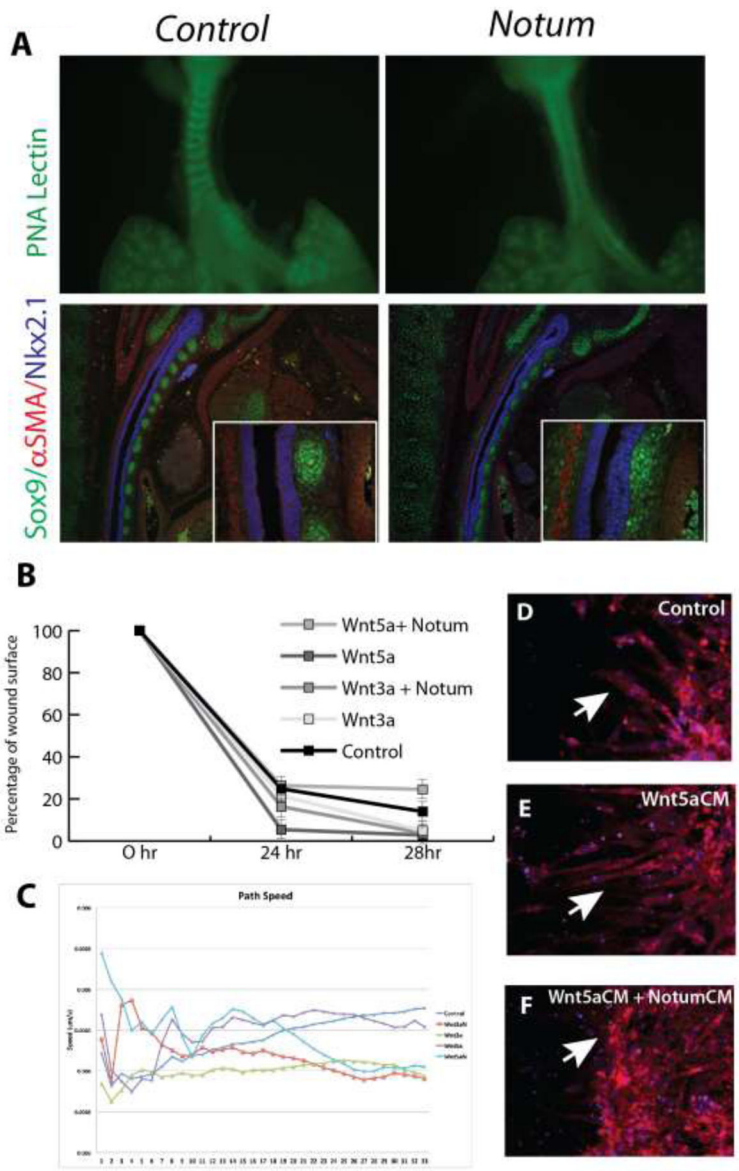

Figure 7. Notum$^{150/150}$ embryos lack well defined mesenchymal condensations

A: Tracheal tissue of E14.*5* embryos was stained with PNA Lectin. Staining was restricted to the periphery of the trachea, demonstrating the lack of well-defined mesenchymal condensations in *Notum*$^{150/150}$ embryos. Longitudinal sections of E14.5 sections were stained for αSMA, Sox9 and Nkx2.1. Sox9 positive cells in the trachea of *Notum*$^{150/150}$ embryos demonstrated poorly developed mesenchymal condensations. Trachealis smooth muscle stained for αSMA on the dorsal side while tracheal epithelium stained for Nkx2.1. **B:** Scratch assays performed on MEF cells determined migration rates when treated with Wnt3a, Wnt5 and or Notum conditioned media. Notum inhibited Wnt5a induced cell migration. **C:** Cells were tracked and analyzed using Path speed within NIS Elements. Path speed was measured by calculating the total path traveled by the cells divided by time elapsed. Representative images of the morphological changes observed in migrating cells

incubated with control (**D)**, Wnt5a **(E),** or Wnt5a + Notum **(F)** conditioned media are shown.

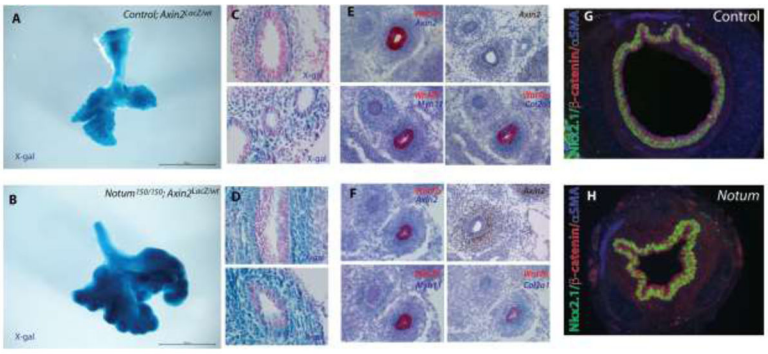

Figure 8.

A) X-gal staining was performed on trachea-lung tissue of E13.5 *Notum 150/Wt; Axin* $^{LacZ/Wt}$ and *Notum* ^{150150t}; *Axin* $^{LacZ/Wt}$ embryos. X-gal staining was observed in trachea and lung of *Notum* $^{150/Wt}$; *Axin* $^{LacZ/Wt.}$ **B)** Intense X-gal staining was observed throughout the lungs and trachea of *Notum* $^{150/150}$; *Axin* $^{LacZ/Wt}$. **C)** X-gal staining was primarily detected in the subepithelial mesenchyme of the airways. **D)** Prominent X-gal staining was observed in the mesenchyme of the lung of *Notum* $^{150/150}$; *Axin* $^{LacZ/Wt}$ embryos. **E, F)** *Myh11*, *Col2a1* and *Wnt7b* RNA were unchanged while expression of *Axin2* was increased after deletion of *Notum*. Transverse sections of E16.5 tracheas show increased β-catenin expression in mesenchyme of *Notum* ^{150150} tissue. Note the collapsed appearance of the epithelium and the reduced luminal area in the *Notum* $^{150/150}$ trachea **(H)**, compared to control **(G).**

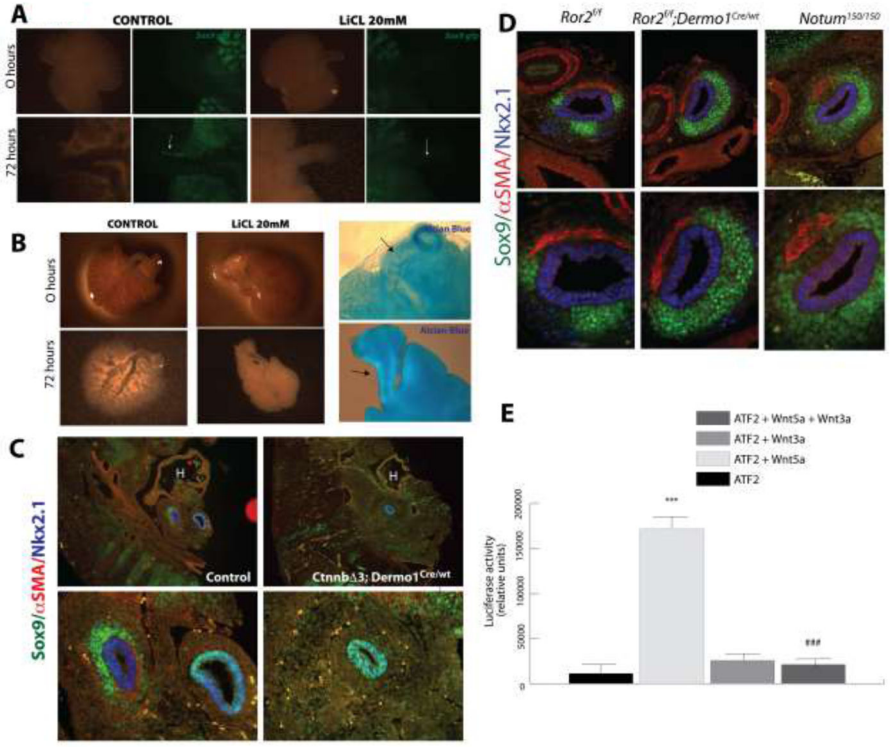

Figure 9.

A) Lung and trachea tissue was isolated from Sox9GFP mice at E13.5 and incubated in air-liquid interphase for 72 hour in presence of 20 mM LiCl. Mesenchymal condensations are well formed in control samples, but are lacking in samples treated with LiCl (white arrow). **B)** Alcian blue staining depicted sites of cartilage in control samples. **C:** Note the lack of Alcian blue staining (black arrow) as well as the reduced branching of the peripheral lung of LiCl treated samples **D)** Increased mesenchymal expression of β-catenin in *Ctnnb*$^{\Delta 3}$*; Dermo1*$^{Cre/wt}$ embryos results in absence of Sox9 and αSMA staining in E12.5 respiratory tract mesenchyme, while epithelial expression of Nkx2.1 is preserved. Low and high-power magnifications of respiratory tract longitudinal sections are shown. **E**) E14.5 Mesenchymal deletion of Ror2 causes tracheal stenosis partially recapitulating the phenotype observed after deletion of Notum. **F**) ATF2 luciferase reporter assay shows that Wnt5a promotes activation of the reporter, while co-transfection of Wnt5a and Wnt3a impaired the Wnt5a induced luciferase activity. H:Heart. N=4 ANOVA, Tukey's multiple comparisons test *** $p<0.001$ ATF2 vs ATF2 + Wnt5a; ###$p<0.001$ ATF2 + Wnt5a vs ATF2 +Wnt5a + Wnt3a.

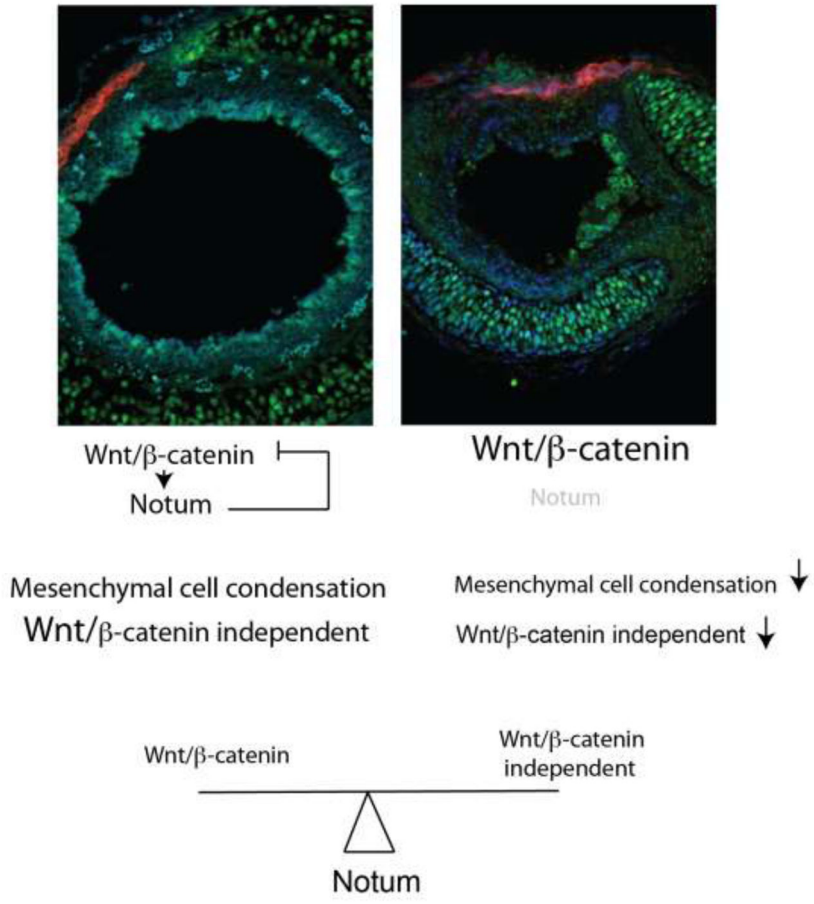

Figure 10.

Model: Notum is a target of Wnt/β-catenin signaling in developing trachea. Wnt/β-catenin signaling is attenuated by Notum during formation of mesenchymal condensations. Notum is required for normal patterning of tracheal cartilage and muscle. In absence of Notum, Wnt/β-catenin dependent signaling is increased, leading to abnormal mesenchymal condensation formation affecting the normal tracheal development.