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Research Article

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Wnt signaling regulates smooth muscle precursor development in the mouse lung via a tenascin C/PDGFR pathway

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Paracrine signaling from lung epithelium to the surrounding mesenchyme is important for lung SMC development and function and is a contributing factor in an array of pulmonary diseases such as bronchopulmonary dysplasia, pulmonary hypertension, and asthma. Wnt7b, which is exclusively expressed in the lung epithelium, is important for lung vascular smooth muscle integrity, but the underlying mechanism by which Wnt signaling regulates lung SMC development is unclear. In this report, we have demonstrated that Wnt7b regulates a program of mesenchymal differentiation in the mouse lung that is essential for SMC development. Genetic loss-of-function studies showed that Wnt7b and β -catenin were required for expression of Pdgfr α and Pdgfr β and proliferation in pulmonary SMC precursors. In contrast, gain-of-function studies showed that activation of Wnt signaling increased the expression of both Pdgfr α and Pdgfr β as well as the proliferation of SMC precursors. We further showed that the effect on Pdgfr expression was, in part, mediated by direct transcriptional regulation of the ECM protein tenascin C (Tnc), which was necessary and sufficient for Pdgfr α / β expression in lung explants. Moreover, this pathway was highly upregulated in a mouse model of asthma and in lung tissue from patients with pulmonary hypertension. Together, these data define a Wnt/Tnc/Pdgfr signaling axis that is critical for smooth muscle development and disease progression in the lung.

Introduction

The mammalian lung is a highly complex organ that develops from the anterior foregut through interactions between the anterior foregut endoderm and the surrounding mesenchyme. Early in development epithelial-mesenchymal interactions pattern the lung endoderm and mesenchymal components leading to distinct cell types along the proximal-distal axis of the lung. These patterning events are essential for proper development of both the airway epithelial cells required for gas exchange and surfactant expression and development of the pulmonary vasculature and airway smooth muscle. Some of the key pathways that regulate these epithelial-mesenchymal interactions include Vegf, Bmp, Shh, and Wnt, but the mechanisms by which they regulate lung mesenchymal development, in particular smooth muscle development, are poorly understood (reviewed in ref. 1). Defects in these developmental processes lead to several important lung diseases, including pulmonary hypertension and asthma. For example, mutations in the bone morphogenetic protein receptor 2 gene are a leading cause of familial pulmonary hypertension, underscoring the link between important developmental pathways and adult lung disease (2, 3).

Wnt signaling is a key regulator of multiple aspects of tissue development. The canonical Wnt pathway involves the stabilization and nuclear translocation of β -catenin through the binding of Wnt ligands to the frizzled/LRP coreceptor complex. β -Catenin acts as a powerful trans-activator of LEF/TCF transcription factors, regulating important downstream target genes that promote

cell proliferation, differentiation, and tissue development. Despite a growing list of general target genes, less is known about the tissue-specific targets for this pathway. In the lung, Wnt signaling has been shown to regulate airway epithelial differentiation, proliferation, and vascular smooth muscle development (4–8). In particular, loss of Wnt7b leads to decreased mesenchymal differentiation and proliferation and later in development decreased VSMC integrity and pulmonary hemorrhage (8). VSMCs in *Wnt7b^{lacZ}* mutants exhibit either a hypertrophic and apoptotic appearance or significant thinning, which suggests that defective VSMC development leads to hypertrophy, cell death, and subsequent thinning of the vessel wall and hemorrhage (8). Since Wnt7b is expressed exclusively in the developing airway epithelium, these data demonstrate important epithelial-mesenchymal signaling by Wnt7b that regulates lung VSMC development and function.

Given the importance of Wnt signaling in lung epithelial-mesenchymal interactions and lung development in general, we sought to define the molecular program acting downstream of Wnt7b and β -catenin signaling in the development of smooth muscle precursors in the mesenchyme. Using *in vivo* gain- and loss-of-function analyses combined with differential gene expression assays, we define a molecular program regulated by Wnt7b and β -catenin through tenascin C (Tnc), Pdgfr α , and Pdgfr β , which are important for smooth muscle precursor development. Importantly, we find that this Wnt/Tnc/Pdgfr pathway is upregulated in mouse and human models of asthma and pulmonary hypertension. These data provide important insight into the role for Wnt signaling in lung SMC development, providing evidence that Wnt signaling regulates early smooth muscle precursor differentiation in the lung as well as influences SMC development in the adult lung.

Conflict of interest: The authors have declared that no conflict of interest exists.

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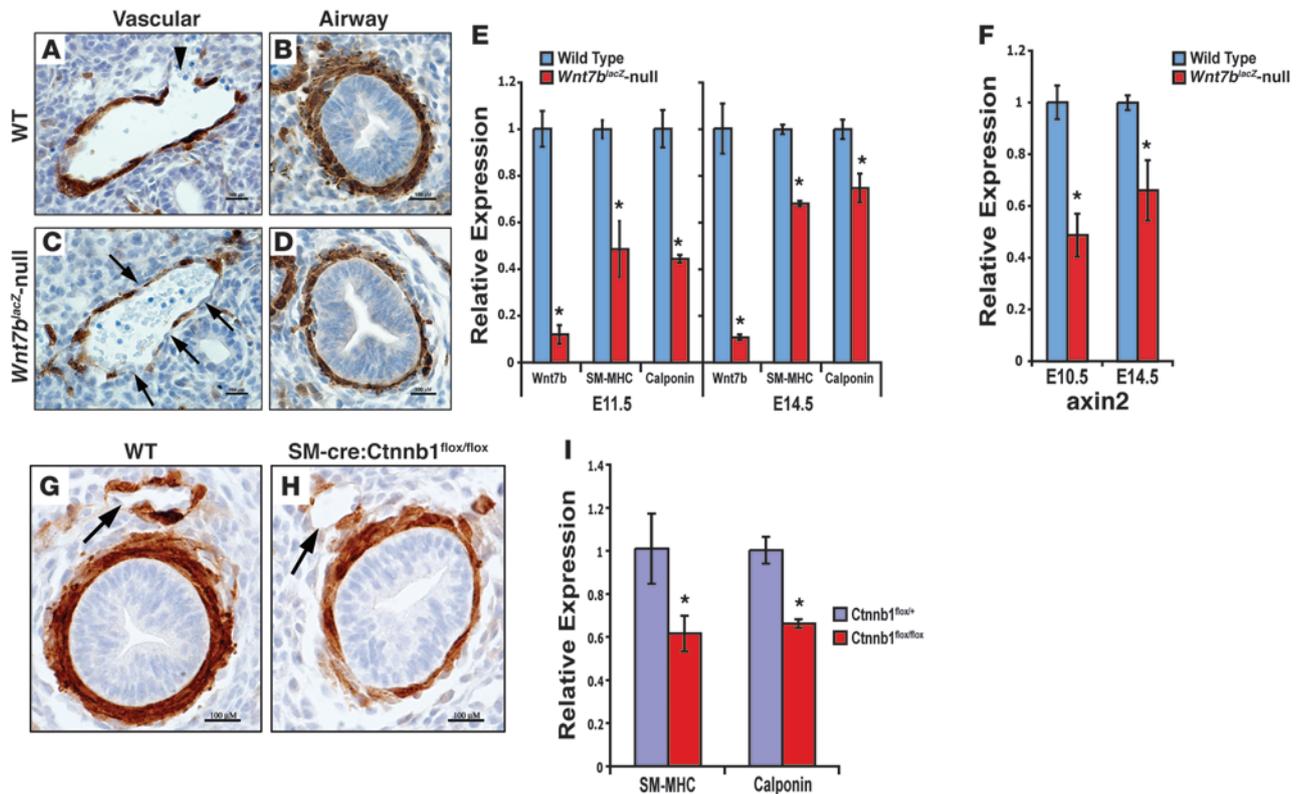


Figure 1

Loss of *Wnt7b* expression leads to early defects in SMC development in the lung. (A–D) SM22 α immunostaining showed vascular (A and C) and airway (B and D) smooth muscle development in wild-type (A and B) and *Wnt7b^{lacZ}-null* (C and D) mutants. Arrowhead in A indicates a smaller branching vessel in wild-type embryos, and arrows in C point to gaps in SM22 α expression in surrounding larger blood vessels of *Wnt7b^{lacZ}-null* mutants. (E) Loss of *Wnt7b* expression in *Wnt7b^{lacZ}-null* mutants led to decreased expression of other SMC markers, including SM-MHC and calponin, as shown by Q-PCR. (F) *Wnt7b^{lacZ}-null* mutants exhibited decreased *axin2* expression. (G and H) SM22 α -cre:*Ctnnb1^{flox/flox}* mutants at E12.5 exhibited decreased SM22 α expression surrounding both airways and blood vessels (arrows) compared with wild-type littermates. (I) SM22 α -cre:*Ctnnb1^{flox/flox}* mutants had decreased SM-MHC and calponin expression as shown by Q-PCR. **P* < 0.01. Scale bars: 100 μ m.

Results

Wnt7b and β -catenin are required for vascular and airway smooth muscle development in the developing lung. To address the underlying molecular mechanism of *Wnt7b* function in SMC development in the lung, immunohistochemistry for the smooth muscle-specific protein SM22 α was performed on sections through the lungs of control and *Wnt7b^{lacZ}* mutant embryos at E14.5. In wild-type lungs, a contiguous layer of SM22 α -positive cells was observed surrounding large blood vessels, with gaps in SM22 α expression restricted to the openings of smaller vessels while a thick layer of SMCs encircled the developing proximal airways (Figure 1, A and B). In contrast, the layer of SM22 α -positive cells surrounding similarly sized vessels and airways in *Wnt7b^{lacZ}* mutant lungs was consistently thinner than those found in control lungs with large gaps in SM22 α expression, and the layer of SMCs surrounding the proximal airways was also thinner (Figure 1, C and D). These data suggest that early defects in SMC development leads to the later loss of these cells in *Wnt7b^{lacZ}* mutants (8).

To quantify the effect of loss of *Wnt7b* expression on pulmonary SMC development, expression of the Ca²⁺ binding protein calponin-h1 and smooth muscle myosin heavy chain (SM-MHC) was examined by quantitative real-time PCR (Q-PCR). At E11.5 and E14.5, calponin-h1 and SM-MHC were significantly reduced in the lung buds of *Wnt7b* mutants (Figure 1E). The decreasing severity

of this loss at E14.5 may be due to compensatory upregulation of the related ligand *Wnt7a* after loss of *Wnt7b* expression (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI38079DS1) (8, 9). We also examined the expression of *axin2*, a well-characterized target of *Wnt*/ β -catenin signaling that provides an accurate readout of signaling activity, in wild-type and *Wnt7b^{lacZ}* mutant lungs (10). *Axin2* expression was reduced at both E10.5 and E14.5 in *Wnt7b^{lacZ}* mutants, indicating that loss of *Wnt7b* signaling leads to decreased β -catenin-dependent transcription (Figure 1F).

To determine whether β -catenin (*Ctnnb1*) was required for lung smooth muscle development, we examined expression of SM22 α in SM22 α -cre:*Ctnnb1^{flox/flox}* mutants. Of note, the SM22 α -cre line expresses the cre recombinase in smooth muscle precursors as well as definitive SMCs, and the *Ctnnb1^{flox/flox}* allele contains loxP sites flanking exons 2–6 and upon cre recombination results in a null allele (11, 12). As predicted, control embryos at E12.5 had a thick ring of SM22 α expression surrounding the large airways and developing blood vessels (Figure 1G). In contrast, the smooth muscle layer was thinner and SM22 α staining less intense surrounding the large airways and developing blood vessels of SM22 α -cre:*Ctnnb1^{flox/flox}* mutants (Figure 1H). Consistent with the apparent reduction in SM22 α staining seen in the SM22 α -cre:*Ctnnb1^{flox/flox}* mutant lungs, expression of the smooth muscle marker genes calponin-h1 and

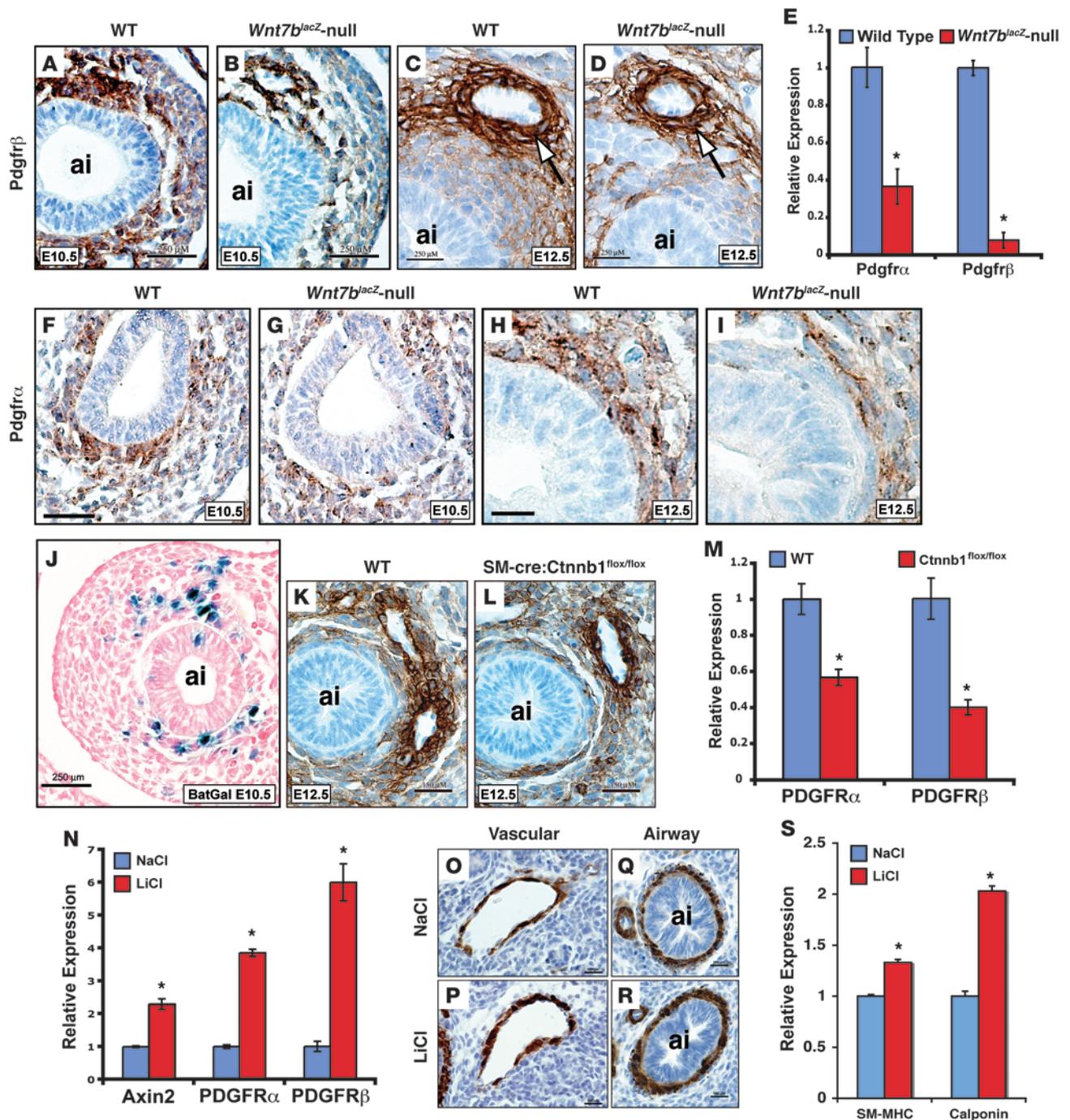
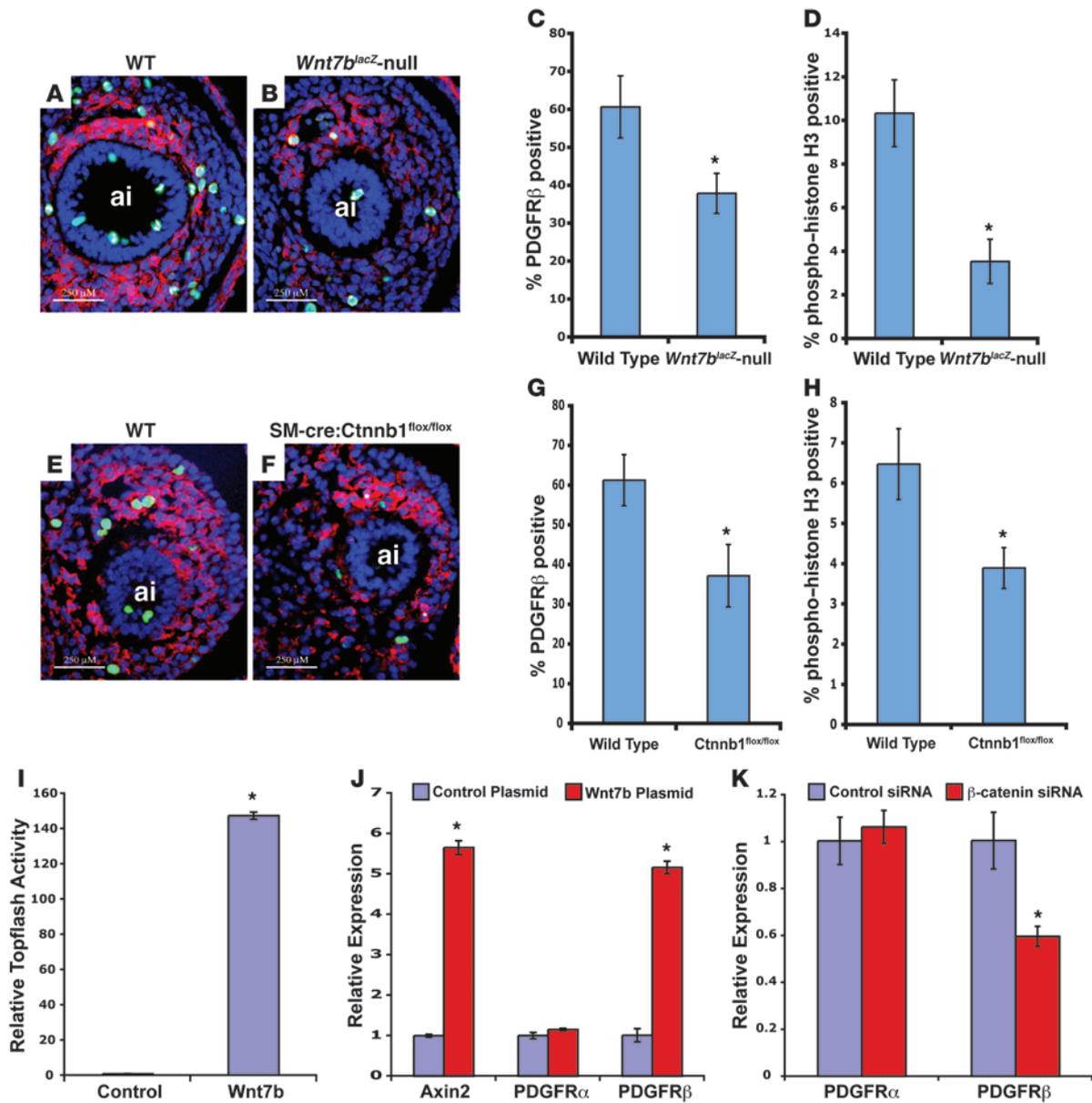


Figure 2

Wnt7b and β -catenin signaling regulates *Pdgfra* and *Pdgfrb* expression in the lung. (A–D) Immunostaining for *Pdgfrb* expression in the developing lungs of wild-type (A and C) and *Wnt7b^{lacZ}-null* mutants (B and D) at E10.5 (A and B) and in developing blood vessels (arrows) at E12.5 (C and D). (E) Q-PCR for *Pdgfra* and *Pdgfrb* expression at E10.5. (F–I) Immunostaining for *Pdgfra* expression in the developing lungs of wild-type (F and H) and *Wnt7b^{lacZ}-null* mutants (G and I) at E10.5 (F and G) and E12.5 (H and I). (J) BAT-GAL Wnt reporter activity at E10.5 in the mesenchyme of the lung in an overlapping region where *Pdgfrb* expression was observed. (K and L) Immunostaining for *Pdgfrb* expression in wild-type (K) and *SM22 α -cre:Ctnnb1^{flox/flox}* mutants (L) at E12.5. (M) Q-PCR for *Pdgfra* and *Pdgfrb* expression in wild-type and *SM22 α -cre:Ctnnb1^{flox/flox}* mutants at E10.5. (N) Q-PCR for *axin2*, *Pdgfra*, and *Pdgfrb* expression from the lung buds of NaCl- and LiCl-treated embryos at E10.5. (O–R) *SM22 α* immunostaining in lungs of NaCl- and LiCl-treated E15.5 embryos. (S) Q-PCR for SM-MHC and calponin expression from the lung buds of NaCl- and LiCl-treated embryos at E14.5. ai, airway. * $P < 0.01$. Scale bars: 250 μ m (A–D, F, G, and J), 150 μ m (H, I, K, and L), 100 μ m (O–R).

**Figure 3**

Wnt7b regulates proliferation of Pdgfr β -expressing SMC precursors, and β -catenin specifically regulates Pdgfr β versus Pdgfr α expression in VSMCs. (A and B) Immunostaining for Pdgfr β (red) and PO₄-H3 (green) expression in wild-type (A) and *Wnt7b^{lacZ}-null* mutants (B) at E10.5. (C and D) The percentage of Pdgfr β -expressing cells (C) as well as proliferating lung mesenchyme was significantly reduced in *Wnt7b^{lacZ}-null* mutants (D). (E and F) Immunostaining for Pdgfr β (red) and PO₄-H3 (green) expression in wild-type (E) and *SM22 α -cre:Ctnnb1^{flx/flx}* mutants (F). (G and H) The percentage of Pdgfr β -expressing cells (G) as well as proliferating lung mesenchyme was significantly reduced in *SM22 α -cre:Ctnnb1^{flx/flx}* mutants (H) at E10.5. DAPI was used as a counterstain to detect cell nuclei. (I) Wnt7b activated the SuperTOPFLASH Wnt reporter in Pac1 cells. (J) Wnt7b activated expression of Pdgfr β and axin2 but not Pdgfr α in Pac1 cells. (K) siRNA-mediated knockdown of β -catenin in Pac1 cells led to decreased Pdgfr β but not Pdgfr α expression. **P* < 0.001. Scale bars: 250 μ m.

SM-MHC was downregulated relative to those of heterozygous controls (Figure 11). These data demonstrate that smooth muscle development in the lung requires Wnt7b and β -catenin signaling and suggests that the affects of Wnt7b on smooth muscle development are mediated by canonical Wnt signaling.

Smooth muscle precursor development requires Wnt7b and β -catenin signaling in the lung. Given the significant affect that loss of Wnt7b has on smooth muscle development early in lung development,

we sought to determine whether smooth muscle precursors are affected in Wnt7b mutant lungs. Control and *Wnt7b^{lacZ}* mutant lung buds isolated at E10.5 were immunostained to detect Pdgfr β , which is highly expressed in vascular smooth muscle precursors and was required for their proliferation and migration (13). These analyses show that the numbers of Pdgfr β - and Pdgfr α -positive cells surrounding the epithelium of the early lung bud were significantly reduced in Wnt7b mutant lung buds compared with con-



Table 1
Genes regulated by Wnt7b expression in Pac1 cells

Gene symbol	Gene title	Fold change ^A	Public ID	Q-PCR verified
Genes implicated in canonical Wnt signaling				
<i>Axin2</i>	Axin 2	12.56	NM_024355	Yes
<i>Nkd2</i>	Naked homolog 2 (<i>Drosophila</i>)	3.01	AI072336	Yes
<i>Tcf7</i>	T cell-specific transcription factor 7	2.80	AW251860	No
<i>Sfrp4</i>	Secreted frizzled-related protein 4	2.78	AF140346	Yes
Genes implicated in vascular remodeling and smooth muscle development				
<i>Irx3</i>	Iroquois related homeobox 3 (<i>Drosophila</i>)	3.72	AI030203	No
<i>Tnc</i>	Tenascin C	3.02	AI176034	Yes
<i>Alcam</i>	Activated leukocyte cell adhesion molecule	2.58	NM_031753	Yes
<i>Prrx1</i>	Paired related homeobox 1	2.48	AA957064	Yes
<i>Timp3</i>	Tissue inhibitor of metalloproteinase 3	2.18	AA893169	Yes
<i>Vegfd</i>	Vascular endothelial growth factor D	2.14	AI170324	Yes
<i>Edn1</i>	Endothelin 1	2.04	NM_012548	Yes
<i>Twist1</i>	Twist homolog 1 (<i>Drosophila</i>)	2.04	BF420349	No
<i>Tgfb2</i>	Transforming growth factor _2	2.04	NM_031131	No

^ARelative to mock-transfected Pac1 cells.

trols (Figure 2, A–D and F–I). There was no significant difference in apoptosis in wild-type or *Wnt7b^{lacZ}* mutant lung buds, indicating that the decrease in Pdgfrβ-positive cells was not due to cell death (Supplemental Figure 2). Moreover, Q-PCR analysis showed that expression of both Pdgfrα and Pdgfrβ was reduced in *Wnt7b^{lacZ}* mutant lung buds, indicating that expression of both receptors was reduced by loss of Wnt7b expression (Figure 2E).

BAT-GAL Wnt reporter mice revealed canonical Wnt activity in mesenchyme adjacent to airway epithelium at E10.5 in a pattern overlapping with Pdgfrα and Pdgfrβ expression (Figure 2J). To determine whether β-catenin was required for the expression of Pdgfrα and Pdgfrβ in the early lung, E12.5 *SM22α-cre: Ctnnb1^{lox/lox}* mutant and control lungs were immunostained for Pdgfrβ expression. In control lungs, Pdgfrβ protein was broadly expressed throughout the mesenchyme, with the highest levels of staining found close to the airway epithelium, coalescing around developing blood vessels (Figure 2K). In contrast, both the intensity and breadth of Pdgfrβ staining was drastically reduced in *SM22α-cre: Ctnnb1^{lox/lox}*

mutant lungs (Figure 2L). Q-PCR analysis showed that expression of both Pdgfrα and Pdgfrβ was significantly downregulated in *SM22α-cre: Ctnnb1^{lox/lox}* mutants (Figure 2M).

To determine whether Wnt/β-catenin signaling was sufficient to promote smooth muscle precursor development in the lung, mouse embryos were treated with either NaCl or LiCl during embryogenesis. LiCl inhibits the kinase activity of Gsk3β, and previous studies have shown that systemic treatment of developing embryos in utero with LiCl activates Wnt/β-catenin signaling in vivo (14–16). A 2.5-fold increase in axin2 expression was observed in the lung of LiCl-treated embryos relative to NaCl-treated embryos, indicating activation of canonical Wnt signaling (Figure 2N). Remarkably, Pdgfrβ and Pdgfrα expression in the lung was dramatically increased in LiCl-treated embryos relative to NaCl-treated embryos, indicating that Wnt/β-catenin signaling activates expression of these receptors (Figure 2N). In addition, airway and vascular smooth muscle layers were thicker and expression levels of SM-MHC and calponin-h1 were increased in LiCl-treated embryos,

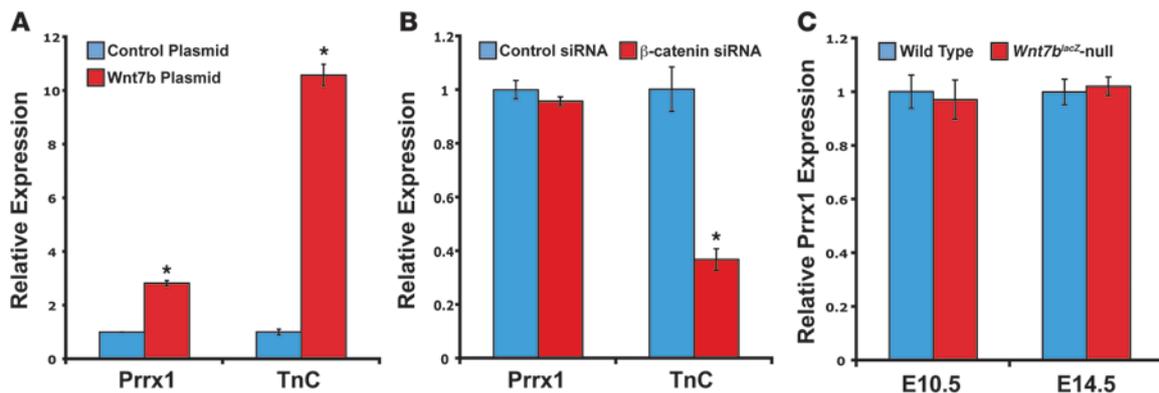
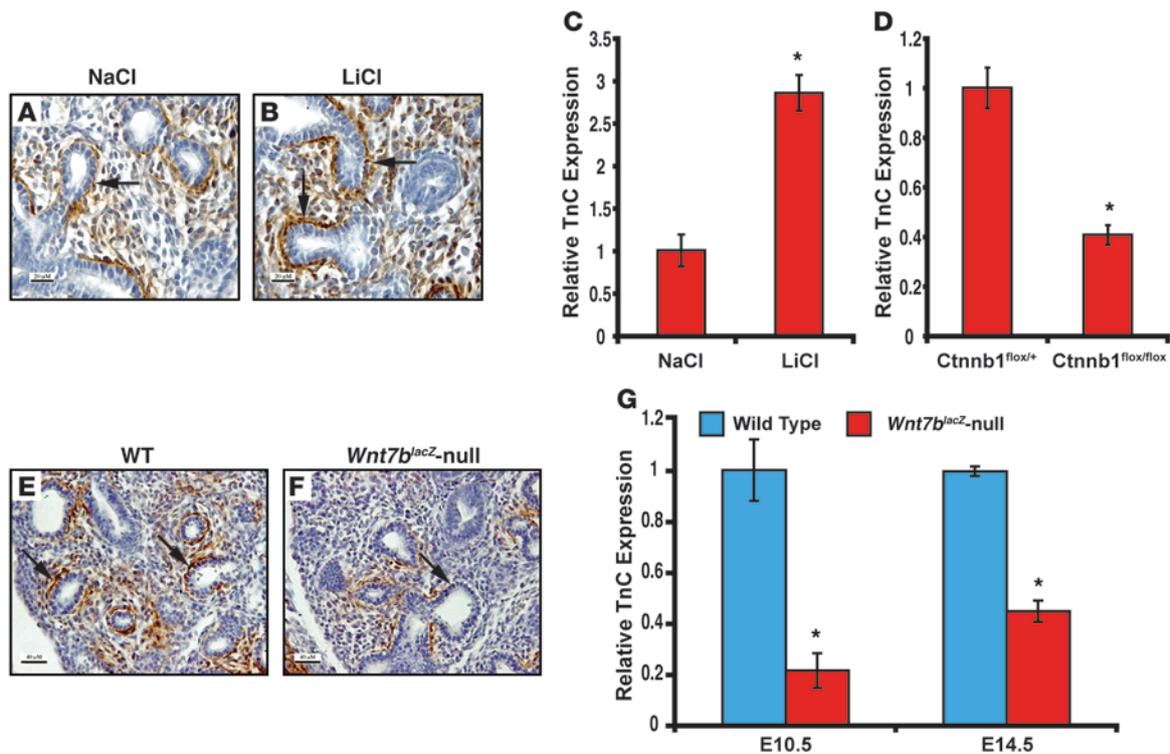


Figure 4
Wnt/β-catenin is necessary and sufficient for Tnc expression. (A) Wnt7b expression activated Prrx1 and Tnc expression in Pac1 cells. (B) siRNA-mediated knockdown of β-catenin expression led to decreased Tnc but not Prrx1 expression. (C) Prrx1 expression was unchanged in *Wnt7b^{lacZ}-null* mutants at both E10.5 and E14.5. **P* < 0.01.

**Figure 5**

β -Catenin and Wnt7b signaling regulate Tnc expression in vivo. (A and B) Immunostaining for Tnc expression in NaCl- and LiCl-treated wild-type embryos at E15.5 showed increased Tnc expression (arrows) surrounding the airways of LiCl-treated embryos. (C) Expression of Tnc was induced approximately 3-fold by LiCl treatment. (D) Loss of β -catenin expression in SMC precursors in *SM22 α -cre:Ctnnb1^{flox/flox}* mutants resulted in a quantitative loss of Tnc expression in vivo at E10.5. (E–G) Tnc expression was significantly reduced in *Wnt7b^{lacZ}-null* mutants as assessed by immunohistochemistry at E14.5 (E and F) and quantitatively by Q-PCR (G). * $P < 0.001$. Scale bars: 20 μ m (A and B), 40 μ m (E and F).

indicating that Wnt/ β -catenin signaling is sufficient to promote smooth muscle development in the lung (Figure 2, O–S).

Wnt7b and β -catenin signaling regulate Pdgfr β expression in VSMC. We and others have previously shown that Wnt7b is required for mesenchymal proliferation during lung development (8, 17). The loss of Pdgfr β in Wnt7b mutants may therefore reflect either a requirement for Wnt7b signaling in the expansion of Pdgfr β -expressing smooth muscle precursors in the surrounding mesenchyme or a role in their specification and differentiation. Costaining for phospho-histone H3 (PO₄-H3) and Pdgfr β protein expression revealed that loss of either Wnt7b or β -catenin leads to dramatic reductions in the proliferation of Pdgfr β -expressing cells (Figure 3, A–H), suggesting that reduced proliferation is at least partially responsible for the loss of SMC precursors in Wnt7b mutant lungs.

In addition to the reduced proliferation of Pdgfr β -expressing cells, the overall level of Pdgfr β expression in each cell also appeared to be lower in *Wnt7b^{lacZ}* and *SM22 α -cre:Ctnnb1^{flox/flox}* mutants, suggesting that Wnt signaling may control Pdgfr β expression in SMC precursors. To assess this possibility, Wnt7b was overexpressed in the pulmonary VSMC line Pac1 and levels of Pdgfr β expression were determined by Q-PCR. Of note, expression of Wnt7b was sufficient to activate canonical Wnt signaling in Pac1 cells, as demonstrated by increased activation of a Wnt/ β -catenin reporter and increased axin2 expression relative to control transfected cells (Figure 3, I and J). Expression of Wnt7b was sufficient to induce Pdgfr β expression in Pac1 cells (Figure 3J).

Furthermore, siRNA-mediated inhibition of β -catenin expression led to a significant reduction in Pdgfr β expression (Figure 3K). Although Pdgfr β expression responded specifically to Wnt7b and β -catenin signaling, CHIP did not reveal a direct association of β -catenin with conserved TCF binding elements (TBEs) in the mouse Pdgfr β promoter (data not shown) (18). Moreover, expression of neither Wnt7b nor β -catenin siRNA affected Pdgfr α expression in Pac1 cells, which may correlate with the vascular lineage of this cell line (Figure 3, J and K). These data suggest that Wnt7b regulates overall lung mesenchymal proliferation, including Pdgfr β positive smooth muscle precursors, but this effect may be indirect and transmitted through additional pathways.

Wnt7b is necessary and sufficient for Tnc expression in VSMCs and in lung development. The regulation of Pdgfr β expression by Wnt7b signaling raised the possibility that Wnt7b might regulate a broad transcriptional program critical for smooth muscle development. We therefore compared the gene expression profiles of Pac1 cells expressing Wnt7b and non-expressing cells using microarray differential gene expression analysis. Several known targets of Wnt signaling including axin2 and naked2 were upregulated in Pac1 cells expressing Wnt7b (Table 1). Interestingly, multiple genes implicated in smooth muscle development were also upregulated in Wnt7b-expressing Pac1 cells, including the paired-homeobox transcription factor Prrx1 and the ECM protein Tnc, both of which have been shown to promote smooth muscle growth and differentiation and are required for proper lung development (Table 1) (19–22). To con-

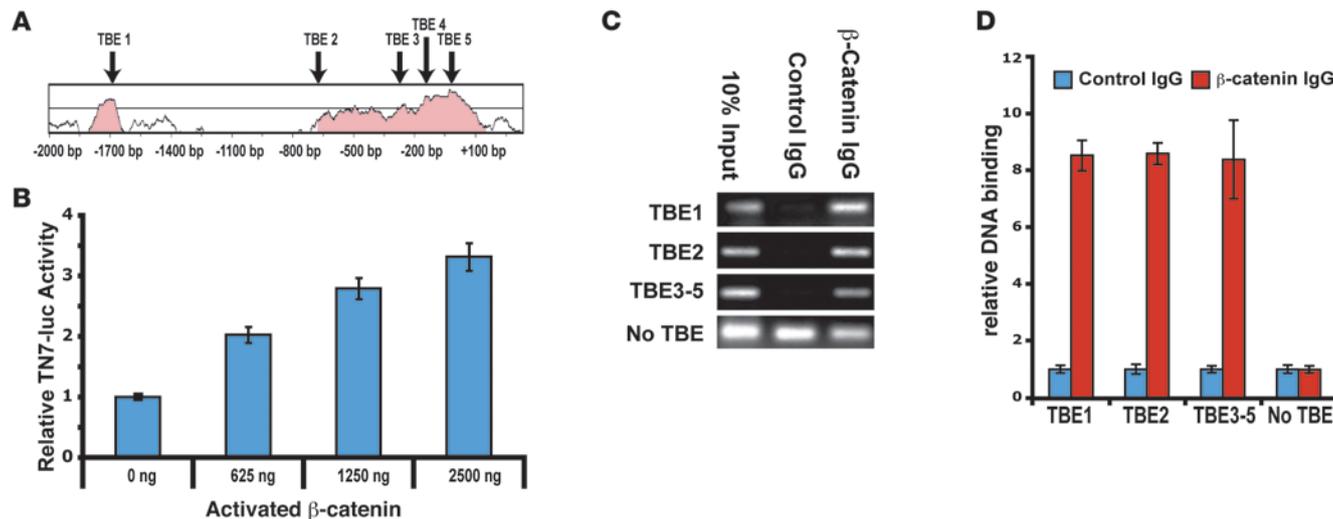


Figure 6 *Tnc* is a direct target of Wnt signaling in the lung. (A) The proximal *Tnc* mouse promoter region contains 5 TBEs. (B) The p*Tnc*.luc reporter was activated in a dose-dependent manner by expression of an activated form of β -catenin in Pac1 cells. (C and D) ChIP assays using agarose gel electrophoresis (C) and Q-PCR (D) showed that β -catenin was associated with the TBE1, TBE2, and TBE3–5 regions in the mouse *Tnc* promoter in the lung.

firm that Wnt7b signaling was sufficient to activate these genes in Pac1 cells, we examined the mRNA levels for *Prrx1* and *Tnc* in control and Wnt7b-transfected cells by Q-PCR. *Prrx1* and *Tnc* were both significantly upregulated in Wnt7b-transfected cells relative to control (Figure 4A). Previous studies have shown that *Prrx1* can directly activate *Tnc* (19). Thus, Wnt signaling could directly activate *Tnc* or activate *Tnc* through direct activation of *Prrx1*. To discern between these two possibilities, we examined mRNA levels for *Prrx1* and *Tnc* in Pac1 cells transfected with either control or β -catenin siRNA. While *Tnc* mRNA levels were significantly reduced in β -catenin siRNA-treated Pac1 cells, *Prrx1* expression was unaffected (Figure 4B). In agreement with these data, *Prrx1* levels were unaffected by the loss of Wnt7b in vivo (Figure 4C).

In order to determine whether activating canonical Wnt signaling induces *Tnc* expression in vivo, immunostaining for *Tnc* expression was performed on the lungs of NaCl- and LiCl-treated embryos. *Tnc* staining was significantly increased in the lungs of LiCl-treated embryos using both immunostaining and Q-PCR analysis (Figure 5, A–C). To determine whether β -catenin signaling was required for *Tnc* expression during lung development, Q-PCR was used to compare *Tnc* expression in *SM22 α :Ctnnb1^{lox/flox}* and control embryos. Again, consistent with the results obtained above, the levels of *Tnc* mRNA were significantly reduced in the lungs of *SM22 α :cre:Ctnnb1^{lox/flox}* mutant embryos relative to those of control embryos (Figure 5D). Finally, to determine whether Wnt7b was required for *Tnc* expression during lung development, immunostaining and Q-PCR analysis for *Tnc* expression was performed on control and *Wnt7b^{lacZ}* mutants. *Tnc* expression was significantly reduced in *Wnt7b^{lacZ}* mutants lungs relative control (Figure 5, E–G). Together, these data indicate that Wnt7b signaling via the canonical Wnt/ β -catenin pathway is necessary and sufficient for *Tnc* expression during lung development.

Tnc is a direct target of Wnt/ β -catenin signaling and regulates *Pdgfr α* and *Pdgfr β* expression in the lung. Comparative genomic analysis between the mouse and human *TNC* genes identified 5 conserved

TBEs located within a 2-kb region upstream of the transcriptional start site (Figure 6A). One of these sites, TBE5, is located within a previously described *Tnc* promoter construct, p*Tnc*.luc (23). To determine whether this site was responsive to β -catenin trans-activation, Pac1 cells were transfected with the p*Tnc*.luc reporter along with an expression plasmid encoding an activated form of β -catenin. These studies showed that expression of activated β -catenin trans-activates the p*Tnc*.luc reporter in a dose-dependent manner (Figure 6B). ChIP assays were performed on chromatin obtained from E12.5 lung tissue using an anti- β -catenin antibody to determine whether β -catenin associated with any of the 5 TBE sites in the 2-kb mouse *Tnc* promoter. ChIP analysis showed that β -catenin associates with all 5 TBEs in the proximal 2-kb *Tnc* mouse promoter (Figure 6, C and D). In contrast, β -catenin binding was not enriched in an unrelated region of the genome upstream of the *VegfD* locus (Figure 6, C and D).

Tnc has previously been shown to be required for *Pdgfr α* expression in glomerular mesangial cells (13), raising the possibility that the regulation of *Pdgfr α* and *Pdgfr β* expression by Wnt7b and β -catenin signaling during lung development shown in Figure 2 may be mediated by its ability to regulate *Tnc*. This hypothesis is concordant with the lack of evidence supporting a direct regulation of the *Pdgfr β* promoter by Wnt/ β -catenin signaling (data not shown). To test this hypothesis, E11.5 lung buds were cultured for 48 hours in the presence of either an antibody that blocks *Tnc* function (*Tnc* IgG) or a nonspecific control antibody (control IgG). In control IgG-treated explanted lung buds, high levels of *Pdgfr β* expression were found in mesenchymal cells adjacent to airway epithelium at the periphery of the explant, which corresponds to the distal region of the developing lung in vivo (Figure 7A). In contrast, both the number of cells expressing *Pdgfr β* and the intensity of their expression were reduced in *Tnc* IgG-treated explants (Figure 7B). Q-PCR analysis revealed that the levels of both *Pdgfr α* and *Pdgfr β* mRNA were significantly reduced in *Tnc* IgG-treated explants relative to controls (Figure 7C). Moreover,

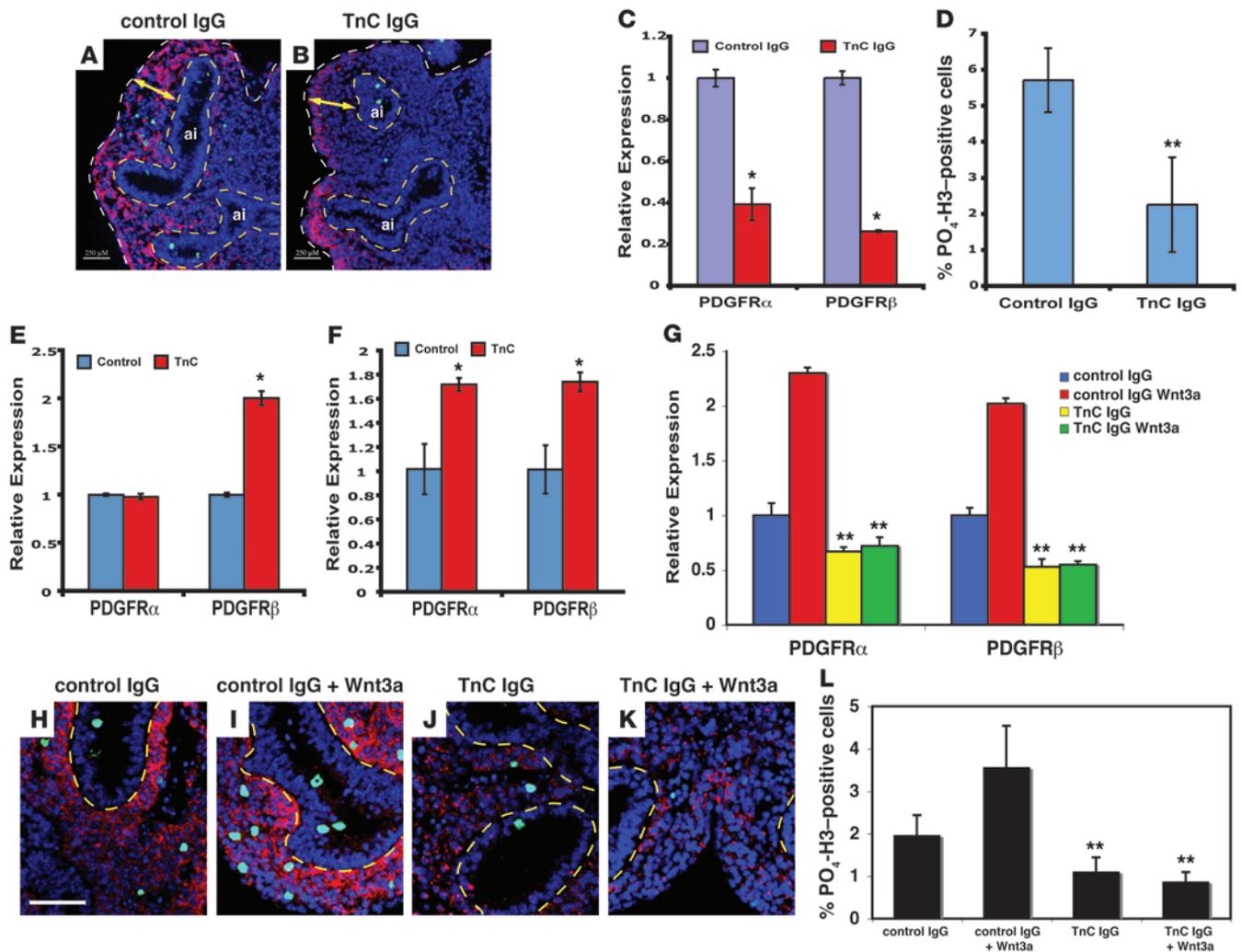


Figure 7

Tnc expression directly modulates Pdgfr β expression. (A–C) Treatment of lung explants from E11.5 embryos with a Tnc-blocking antibody reduced Pdgfr β expression (arrows) by immunohistochemistry (A and B) and reduced both Pdgfr α and Pdgfr β expression by Q-PCR (C). The white dotted lines in A and B denote the external outline of lung explant; yellow dotted lines denote developing airways. (D) Antibody-mediated blocking of Tnc function reduced lung mesenchymal proliferation. (E) Tnc protein treatment of Pac-1 cells led to increased Pdgfr β but not Pdgfr α expression by Q-PCR. (F) Tnc protein treatment of E11.5 mouse lung explants resulted in increased expression of both Pdgfr α and Pdgfr β as detected by Q-PCR. (G–K) Treatment of lung explants with Wnt3a and either non-immune IgG (control IgG) or Tnc-blocking antibody (Tnc IgG) showed that blocking Tnc decreased Wnt3a-induced Pdgfr α and Pdgfr β expression, as assessed by Q-PCR (G) and immunohistochemistry (H–K). (H–L) Blocking Tnc also inhibited the Wnt3a-induced increase in proliferation observed in lung explants as noticed by PO₄-H3 immunostaining (green nuclei). **P* < 0.001; ***P* < 0.03. Scale bars: 250 μ m.

proliferation of Pdgfr β -expressing cells was significantly reduced in Tnc IgG-treated explants relative to those treated with control antibody (Figure 7D).

To determine whether Tnc was sufficient to induce Pdgfr expression, Pac1 cells were treated with purified Tnc protein and expression of Pdgfr α and Pdgfr β was assessed. Expression of Pdgfr β was significantly higher in Tnc-treated Pac1 cells relative to control cells (Figure 7E). In contrast, Tnc did not increase Pdgfr α expression in Pac1 cells, which is consistent with their vascular origin (Figure 7E). To determine whether Tnc was sufficient to increase Pdgfr α and Pdgfr β signaling in developing lung tissue, E11.5 lung buds were cultured for 3 days in either control media or media containing purified Tnc protein. Expression of

both Pdgfr α and Pdgfr β was significantly higher in Tnc-treated explants than in control explants (Figure 7F).

Finally, to determine whether the regulation of Pdgfr α and Pdgfr β by Wnt signaling was dependent upon Tnc activity, we treated lung explants with recombinant Wnt3a and either a control antibody or the Tnc-blocking antibody and assessed Pdgfr α and Pdgfr β expression by immunostaining and Q-PCR and proliferation by PO₄-H3 immunostaining. As expected, treatment of lung explants with Wnt3a increased Pdgfr α and Pdgfr β expression in control IgG-treated explants (Figure 7, G–I). In contrast, Pdgfr α and Pdgfr β expression was significantly reduced in Wnt3a/Tnc blocking antibody-treated explants (Figure 7, G–K). Moreover, proliferation in Tnc-treated explants was reduced compared with

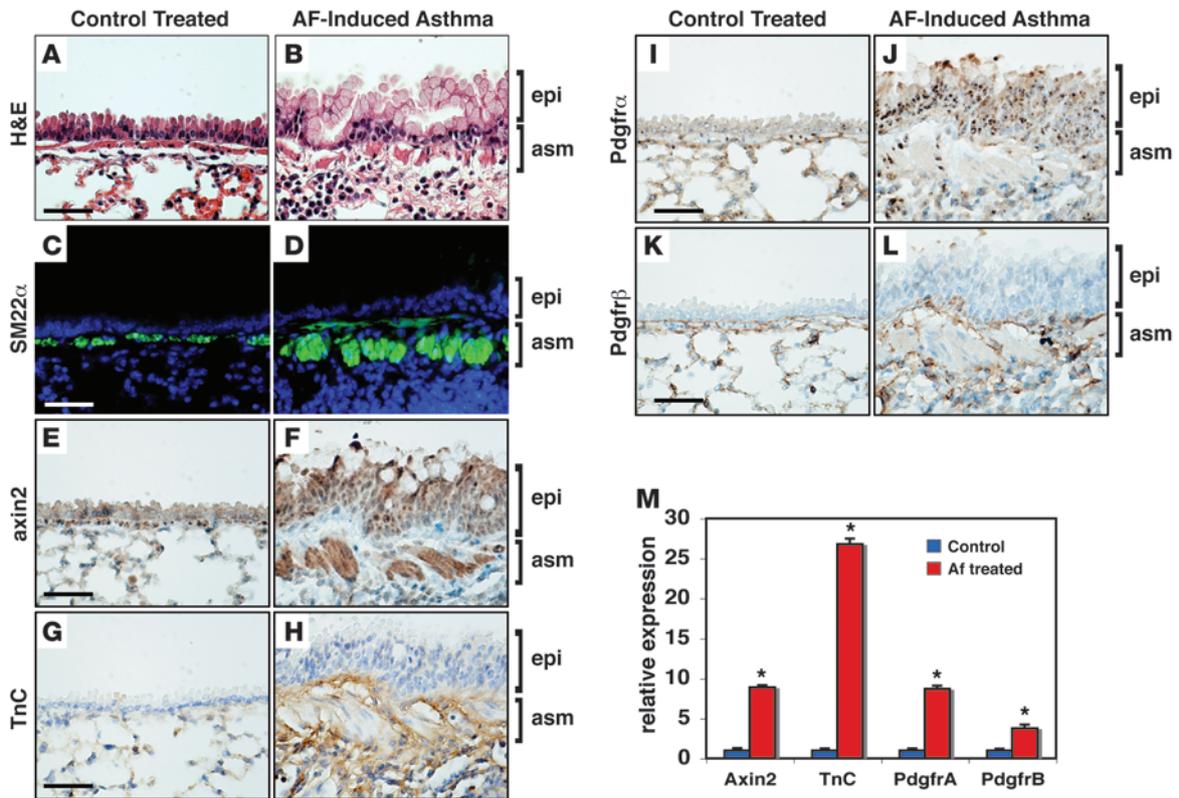


Figure 8 Increased expression of the Wnt/Tnc/Pdgr pathway in *A. fumigatus*-induced asthma in mice. (A–L) Mice treated with *A. fumigatus* to induce airway hyperresponsiveness and inflammation showed increased smooth muscle mass as assessed by H&E staining (A and B) and SM22 α immunohistochemistry (C and D). Coincident with this reaction, increased expression of axin2 (E and F), Tnc (G and H), Pdgr α (I and J), and Pdgr β (K and L) were observed. (M) Q-PCR was used to quantitate the increase in expression of axin2, Tnc, Pdgr α , and Pdgr β . * $P < 0.001$. Epi, epithelium; asm, airway smooth muscle. Scale bars: 100 μ m.

controls (Figure 7, H–L). These data show that the increase in Pdgr α and Pdgr β as well as the increase in proliferation by Wnt in the lung is dependent on Tnc activity.

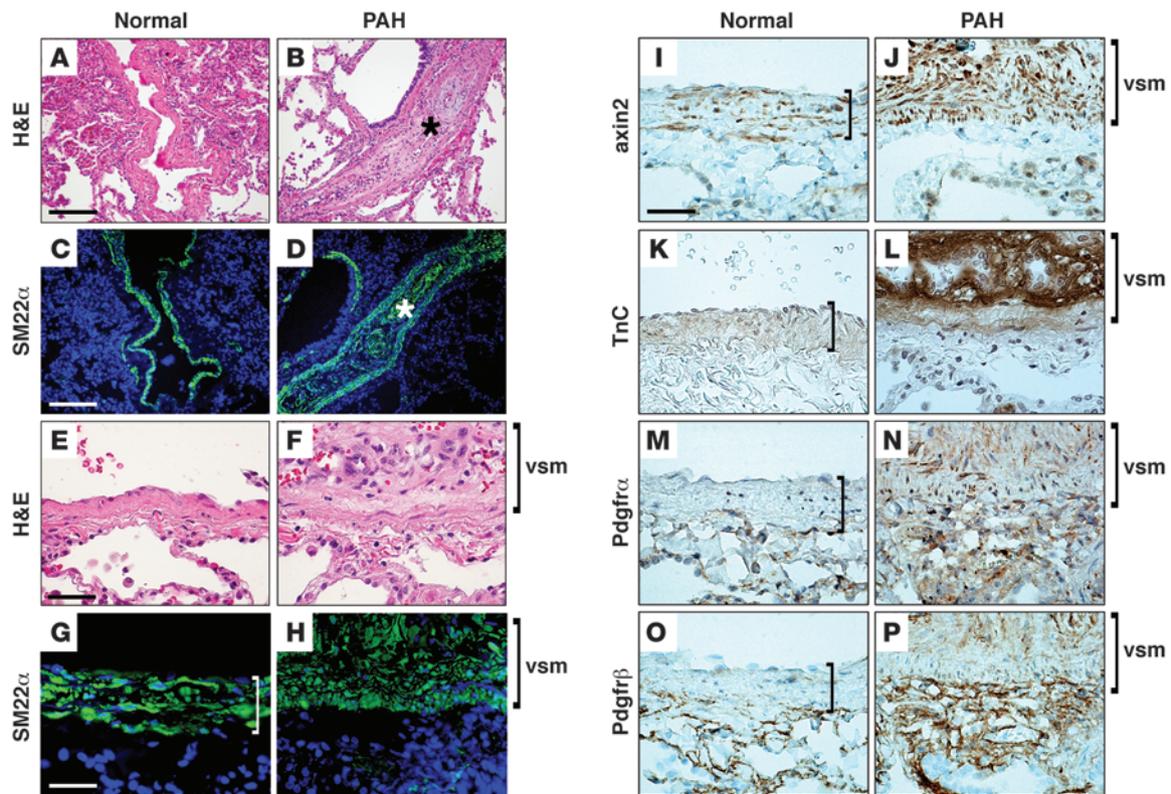
Increased Wnt/Tnc/Pdgr expression in a mouse model of asthma and in human pulmonary hypertension patients. To determine whether expression of this pathway was altered in adult smooth muscle-associated lung diseases, we examined expression and activity of the Wnt, Tnc, and Pdgr components in a mouse model of allergen-induced asthma and samples from human patients with pulmonary arterial hypertension (PAH). The *Aspergillus fumigatus*-induced airway asthma model mimics human asthma by inducing airway smooth muscle proliferation, leading to increased airway smooth muscle (ASM) mass (24). As shown in Figure 8, increased ASM mass was observed in *A. fumigatus*-treated animals (Figure 8, A–D). Moreover, increased expression of axin2, Tnc, Pdgr α , and Pdgr β was observed in the airway smooth muscle of *A. fumigatus*-treated animals (Figure 8, E–L). Expression of axin2 and Pdgr α was also upregulated in airway epithelium of *A. fumigatus*-treated animals (Figure 8, E, F, I, and J). Q-PCR showed that these increases were highly significant (Figure 8M).

Human PAH is characterized by a dramatic increase in pulmonary vascular smooth muscle proliferation and eventual narrowing of these vessels, leading to right ventricular overload in the heart and eventual heart failure (25). Histological analysis of human PAH samples showed increased levels of smooth muscle α -actin and Tnc staining (Figure 9, A–H, K, and L), as has been previously described (23).

Remarkably, increased expression of axin2, Pdgr α , and Pdgr β was also observed in PAH samples (Figure 9, I, J, and M–P). Taken together, these data support a model in which Wnt signaling regulates both the expression and the number of Pdgr α - and Pdgr β -positive SMC precursors, in part through direct regulation of Tnc transcription, to promote SMC precursor proliferation and development in embryonic lung development as well as in adult lung disease (Figure 10).

Discussion

Defects in pulmonary smooth muscle development and function are leading contributors to numerous lung diseases, including pulmonary hypertension and asthma. Although signaling between airway epithelium and airway smooth muscle is critical for development of each of these cell lineages, little is understood about the relevance of these interactions in development and human disease. Expression of Tnc and Pdgr β is linked with several adult lung diseases, including asthma, pulmonary fibrosis, and pulmonary hypertension (23, 26–28). We show that paracrine Wnt signaling from the developing airway epithelium is required for expansion and development of smooth muscle precursors in the lung through a Wnt/Tnc/Pdgr signaling axis. Moreover, we show that expression and activity of this pathway is increased in a mouse asthma model and in human PAH patients. Thus, our data establish the critical importance of a Wnt/Tnc/Pdgr pathway in both lung development and adult lung disease.

**Figure 9**

Increased expression of the Wnt/Tnc/Pdgfr pathway in pulmonary hypertension in humans. (A–H) Histological sections from human patients with PAH were stained with H&E (A, B, E, and F) or SM22 α (C, D, G, and H) to detect vascular smooth muscle overgrowth in pulmonary arteries. (I–P) Expression of axin2 (I and J), Tnc (K and L), Pdgfr α (M and N), and Pdgfr β (O and P) was increased in patients with PAH compared with normal controls. Vsm, vascular smooth muscle. Scale bars: 400 μ m (A–D), 100 μ m (E–P).

Many airway diseases in the lung are the result of environmental insults and the subsequent inflammatory response from these chronic injuries. Diseases such as pulmonary fibrosis, pulmonary hypertension, and asthma involve damage by prolonged exposure to environmental agents followed by successive rounds of inflammation and wound healing. During these processes, SMCs and myofibroblasts rapidly proliferate and expand to replace damaged tissue. However, expansion of these mesenchymal cell types and subsequent deposition of abnormal amounts of ECM proteins can become disruptive to airway and vascular function. Interestingly, Wnt7b and Tnc expression are both upregulated in mouse models of pulmonary fibrosis, and Wnt7b and Pdgfr β are upregulated in some forms of human idiopathic pulmonary fibrosis and hypertension, respectively (23, 26–30). Moreover, polymorphisms in Tnc and Pdgfr β have been linked to asthma, while Tnc mutations attenuate ovalbumin-induced asthma in mice including the associated increase in the numbers of bronchiolar goblet cells, a phenotype that can be mimicked by activating canonical Wnt signaling in the airway epithelium (31–34). Taken together, our results suggest that the Wnt/Tnc/Pdgfr signaling axis described in this paper may be a common link in human airway disease.

Our findings demonstrate that Tnc is a direct target of Wnt signaling in the lung and that loss of Tnc, and in turn Pdgfr β expression, is in part responsible for the SMC defects observed in *Wnt7b^{lacZ}*-null mutants. A previous report showed that decreased Tnc expression leads to SMC apoptosis (22). Thus, loss of Tnc might be a

primary cause of the increased apoptosis observed in the VSMCs of Wnt7b-deficient animals (8). Other ECM proteins, including fibronectin (Fn), have been shown to be targets of Wnt signaling (35). ECM proteins are critical regulators of stem/progenitor cell differentiation and act primarily through regulation of cell-ECM interactions, which helps create specific microenvironments or niches within tissues that are critical for stem/progenitor cell self-renewal and differentiation. Given the role of Wnt signaling in the regulation of precursor and progenitor populations, governing the expression of ECM proteins is likely an important mechanism underlying the ability of Wnt signaling to regulate proliferation and differentiation of SMC precursors in the lung. Tnc likely acts cooperatively with other ECM proteins to promote proper SMC development in part through regulation of smooth muscle precursor expansion and differentiation. Interestingly, Tnc and Fn have been shown to cooperatively regulate SMC proliferation and migration, respectively, in models of pulmonary hypertension (20). Thus, the ability of Wnt signaling to regulate both Tnc and Fn reveals a critical role for this pathway not only in lung SMC development and diseases but also in SMC populations in other tissues.

Although our data demonstrate that Wnt7b plays an important role in the paracrine regulation of lung smooth muscle development, other Wnt ligands likely act redundantly with Wnt7b in this process. One such ligand, Wnt7a, is expressed in airway epithelium, but lung defects have not been reported (36). Importantly, we show that Wnt7a expression is significantly upregulated in the lungs of

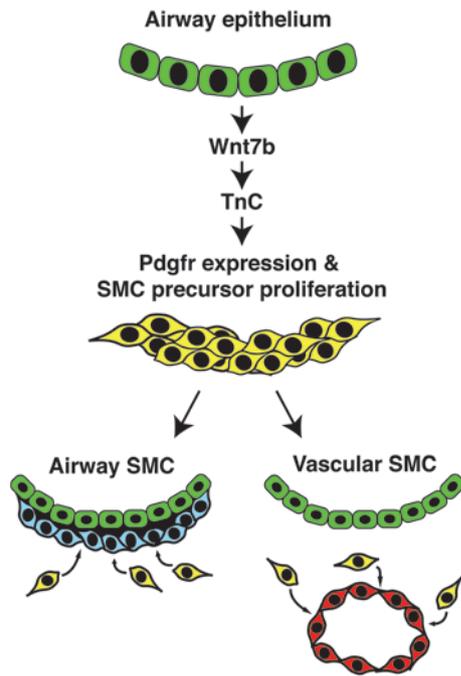


Figure 10
Model of a Wnt/Tnc/Pdgfr signaling axis regulating lung SMC development. Airway epithelium (green) expresses Wnt7b, which acts on mesenchymal SMC precursors (yellow). Wnt7b and β -catenin signaling directly activates Tnc expression and in turn promotes airway SMC (blue) and VSMC (red) precursor proliferation and development.

Wnt7b^{lacZ}-null mutants. Mouse *Wnt5a* mutants exhibit defects in lung mesenchymal proliferation, although specific smooth muscle defects were not reported (37). Conversely, overexpression of *Wnt5a* leads to defects in lung vascular development through repression of *Shh*, which in turn leads to increased *Fn* expression (38). Since *Wnt5a* is a non-canonical Wnt and non-canonical signaling often antagonizes canonical signaling, these findings suggest that multiple Wnt ligands expressed in lung epithelium might act cooperatively to balance SMC differentiation in the lung. In addition to Wnt ligand redundancy, the source of SMC precursors has recently expanded to include mesothelial cell migration followed by epithelial to mesenchymal transformation to generate VSMCs in the lung (39). Thus, Wnt signaling may be important in orchestrating the complex series of developmental processes required for proper SMC development injury response in the lung.

Our findings showing that Wnt7b and canonical Wnt/ β -catenin signaling affects both smooth muscle development and survival provides a new and important pathway to target for therapeutic intervention in smooth muscle-dependent lung diseases including asthma and pulmonary hypertension. Wnt signaling is under intensive investigation as a target for anticancer drugs, and therapies derived from these studies may be useful in treating pulmonary diseases, especially ones characterized by excessive smooth muscle proliferation.

Methods

Animals. Generation and genotyping of the *Wnt7b^{lacZ}*, *Ctmb1^{lox/lox}*, *BAT-GAL*, and *SM22 α -cre* mouse lines have been previously described (8, 11, 12, 40). All animals were kept on a mixed C57BL/6:129/SvJ background. Genera-

tion of lithium-treated embryos was performed as described (14). Pregnant females were given 2 intraperitoneal injections of 600 mM LiCl or NaCl (10 μ l/g body weight) between E8.5 and E10.5 of gestation. Embryos were collected either at E10.5 and lung buds isolated for Q-PCR analysis or at E14.5 and processed for immunohistochemical analysis.

Histology. Mouse tissue samples were harvested and fixed overnight in 4% paraformaldehyde, dehydrated in a series of ethanol washes, and embedded in paraffin. All results shown in the figures and table are representative of at least 4 samples of each genotype. Tissue sections were stained with the following antibodies: anti-SM22 α (Abcam; catalog AB10135), phosphohistone H3 (Cell Signaling Technology Inc.; catalog 9706L), anti-Tnc (Millipore; catalog AB1951), and anti-Pdgfr β (Cell Signaling Technology Inc.; catalog 3169), using protocols previously described (4). β -Galactosidase histochemical staining was performed as previously described (8).

Cell culture. Pac1 cells were cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin, as previously described (5). Cells were transfected with the indicated expression and reporter plasmids using FuGENE 6 (Roche Applied Science) and luciferase assays performed with the Dual-Glo assay system (Promega) according to the manufacturer's protocols. For knockdown experiments, cells were transfected with siRNA pools targeting β -catenin and Tnc (Thermo Fisher Scientific) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Reported values are the mean \pm SEM of 3 assays performed in duplicate.

Q-PCR and microarray analysis. Total RNA was isolated from cells and tissue with Trizol (Invitrogen) and reverse transcribed using Superscript II (Invitrogen) according to the manufacturer's protocols. Q-PCR was performed using the oligonucleotides listed in Supplemental Table 1 on an Applied Biosystems 7900HT system with SYBR Green reaction mixture (Applied Biosystems) according to the manufacturer's protocol. Reported values are the mean \pm SEM of 3 assays performed in duplicate. For microarray analysis, total RNA was isolated as previously described (4) from control and *Wnt7b*-expressing Pac-1 cells (2 samples each) and biotinylated cRNA probes generated for use as probes for Affymetrix rat 230A GeneChips (4). Two chips were hybridized for each condition. Array data were normalized using Microarray Suite 5.0 (MAS5; Affymetrix) and Significance Analysis of Microarrays (<http://stat.stanford.edu/~tibs/SAM/>). Changes in gene expression of 2.0-fold or greater, with *P* values below 0.05, were considered significant.

Lung explant cultures. Lung buds were explanted from E11.5 embryos using tungsten needles and cultured in BGJb media (Invitrogen) supplemented with 0.1 mg/ml ascorbic acid and penicillin / streptomycin for 48 hours, as previously described (4). At least 4 lung buds were used in each experiment. For antibody blocking experiments, explants were placed directly into media containing 25 μ g/ml of either Tnc IgG (catalog AB1951; Millipore) or nonspecific control IgG. For Tnc protein treatment, explants were placed directly in either control media or media containing 10 μ g/ml purified human Tnc protein (GeneWay Biotech). All explant experiments were repeated at least 3 times.

ChIP assays. Lung buds were isolated from wild-type embryos at E12.5, minced, fixed with 1% formaldehyde, and the chromatin sheared to an average length of 500–600 bp with a Branson Model 450 sonicator. Immunoprecipitation was performed with a commercially available kit (Upstate Biotechnology) and an antibody recognizing β -catenin (BD Biosciences; catalog 610154). After reverse cross-linking, precipitated chromatin was subjected to PCR and Q-PCR using the primers listed in Supplemental Table 1.

A. fumigatus-induced allergic asthma model. The method of sensitization and challenge with *A. fumigatus* has been previously described (24). Briefly, female BALB/c mice were injected intraperitoneally with 20 μ g *A. fumigatus* combined with 2 mg alum (Imject Alum; Pierce Biotechnology) in 100 μ l PBS on days 1 and 14, followed by a single intranasal challenge on day 27 with 25 μ g of *A. fumigatus* extract (Hollister-Stier) in PBS (12.5 mg in 21% glycerol, PBS).



Human lung tissue preparation. Lung sections from human pulmonary hypertension subjects were obtained through the Cardiovascular Medical Research Education Network and were processed by in accordance with institutional and NIH guidelines. Adult normal lung sections were obtained from ProSci. All protocols were reviewed and approved by the University of Alabama Institutional Review Board.

Statistics. A 2-tailed Student's *t* test was used in these analyses. *P* values less than 0.05 were considered statistically significant.

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