Published in final edited form as:
Dev Cell. 2009 August ; 17(2): 290–298. doi:10.1016/j.devcel.2009.06.005.

Wnt2/2b and β-catenin signaling are necessary and sufficient to specify lung progenitors in the foregut

Ashley M. Goss[1,2], **Ying Tian**[1,3], **Tadasuke Tsukiyama**[5], **Ethan David Cohen**[1,3], **Diane Zhou**[1,3], **Min Min Lu**[1,3], **Terry P. Yamaguchi**[5], and **Edward E. Morrisey**[1,2,3,4,*]

[1] Department of Medicine, University of Pennsylvania, Philadelphia, PA 19104

[2] Department of Cell and Developmental Biology, University of Pennsylvania, Philadelphia, PA 19104

[3] Cardiovascular Institute, University of Pennsylvania, Philadelphia, PA 19104

[4] Institute for Regenerative Medicine, University of Pennsylvania, Philadelphia, PA 19104

[5] Cancer and Developmental Biology Laboratory, National Cancer Institute-National Institutes for Health, Frederick, MD 21702

Abstract

The primitive foregut is patterned in a manner that spatially promotes proper organ specification along the anterior-posterior foregut axis. However, the molecular pathways that specify foregut endoderm progenitors are poorly understood. We show that Wnt2/2b signaling is required to specify lung endoderm progenitors within the anterior foregut. Embryos lacking Wnt2/2b expression exhibit complete lung agenesis and do not express Nkx2.1, the earliest marker of the lung endoderm. In contrast, other foregut endoderm derived organs including the thyroid, liver, and pancreas are correctly specified in Wnt2/2b null animals. We show that this phenotype is recapitulated by an endoderm restricted deletion of β-catenin, demonstrating that Wnt2/2b signaling through the canonical Wnt pathway is required to specify lung endoderm progenitors within the foregut. Moreover, activation of canonical Wnt/β-catenin signaling results in reprogramming of esophagus and stomach endoderm to a lung endoderm progenitor fate. Together, these data reveal that canonical Wnt2/2b signaling is uniquely required for specification of lung endoderm progenitors in the developing foregut.

INTRODUCTION

The vertebrate gut tube is patterned such that organs are specified in a precise spatial location along the anterior-posterior axis of the developing embryo. Signaling molecules expressed in the surrounding lateral plate mesoderm (LPM) are thought to promote development and patterning of these organs including the thyroid, lung, liver, and pancreas in part through proper specification of endoderm progenitors. Although several important signaling pathways have been implicated in the regulation of foregut endoderm development the pathways that uniquely specify the lung within the anterior foregut are unknown.

---

*To whom correspondence should be addressed: Edward E. Morrisey, Ph.D. University of Pennsylvania, 956 BRB II/III, 421 Curie Blvd. Philadelphia, PA 19104, Phone: 215-573-3010, FAX: 215-573-2094, emorrise@mail.med.upenn.edu.

Wnt signaling is one such pathway known to be important for early tissue morphogenesis. Multiple roles for β-catenin in cell proliferation and differentiation have been reported in the endodermal components of multiple tissues including the liver, pancreas, and lung (Apte et al., 2007; Dessimoz et al., 2005; Mucenski et al., 2003; Murtaugh et al., 2005; Shu et al., 2005; Tan et al., 2006). However, whether Wnt signaling plays a role in specification of foregut derived tissues remains unclear. In this report, we show that Wnt2 and Wnt2b play an essential and cooperative role in specifying lung endoderm progenitors within the anterior foregut without affecting the specification of other foregut derived tissues. Moreover, we show that activation of Wnt/β-catenin signaling can reprogram posterior endoderm to a lung progenitor fate indicating the potent role of Wnt signaling in specifying early lung endoderm progenitors. Thus, our studies reveal a unique role for Wnt/β-catenin signaling in promoting lung endoderm specification in the foregut.

RESULTS

Expression of Wnt2 and Wnt2b in forgut development

Previous studiers have reported the expression of several Wnt ligands in the lung including Wnt2, Wnt5a, Wnt7b, and Wnt11 (Li et al., 2002; Weidenfeld et al., 2002). Given the importance of the Wnt pathway in endoderm development, especially in regulation of tissue specific progenitors, we explored whether these ligands are expressed in the appropriate spatial and temporal pattern to regulate specification and development of lung endoderm progenitors in the foregut. These studies revealed that *Wnt2* and *Wnt2b* are expressed in the mesoderm surrounding the ventral aspect of the anterior foregut from E9.0–E10.5 during the period when the lung is specified (Supplemental Figure 1). *Wnt2* and *Wnt2b* are expressed later in the developing lung mesenchyme with *Wnt2* expression persisting into adulthood (Supplemental Figure 1 and (Monkley et al., 1996; Zakin et al., 1998)). These data suggest that *Wnt2/2b* are expressed in the appropriate spatial and temporal manner in the LPM to regulate lung specification and development.

Loss of Wnt2 leads to lung hypoplasia and combined loss of Wnt2 and Wnt2b leads to lung agenesis

To further investigate the role of Wnt2 and Wnt2b in development of the anterior foregut, we generated null alleles in mice of both genes using homologous recombination in embryonic stem (ES) cells (Supplemental Figure 2). The majority of $Wnt2^{-/-}$ null mutants are cyanotic at birth and die within a few minutes whereas $Wnt2b^{-/-}$ null mutants are viable with no discernable phenotype (Supplemental Table 1 and data not shown). To explore the reason for perinatal lethality in $Wnt2^{-/-}$ null mutants, histological analysis was performed on embryos from E10.5–E18.5. These studies revealed significant lung hypoplasia in $Wnt2^{-/-}$ null mutants (Fig. 1A–H). Despite the hypoplasia, branching of the terminal airways was relatively normal in $Wnt2^{-/-}$ null mutants (Fig. 1I–L). Poor development of the lung mesenchyme was observed leading to a dilated and dysfunctional vascular endothelial plexus by birth (Fig. 1M–P). Cell proliferation is significantly reduced in both epithelial and mesenchymal cell lineages in $Wnt2^{-/-}$ lungs (Fig. 1Q–S). Several signaling pathways and transcription factors known to be important for lung growth and differentiation including Fgf10, Nkx2.1, Bmp4, N-myc, and cyclin D1 were significantly reduced in $Wnt2^{-/-}$ null mutants (Fig. 1T and (Eblaghie et al., 2006; Kimura et al., 1996; Okubo et al., 2005; Sekine et al., 1999; Zhang et al., 2008)). In contrast, proximal-distal patterning was unperturbed in $Wnt2^{-/-}$ null mutants as noted by normal expression of SP-C, a marker of distal alveolar epithelial cells, and CC10, a marker of proximal bronchiolar epithelial cells (Fig. 1U–X).

To address the combined role of Wnt2 and Wnt2b in lung development, we generated *Wnt2/2b* double knock-out (DKO) mutants. Examination of *Wnt2/2b* DKO mutants revealed complete lung agenesis (Fig. 2A–L). While the esophagus is readily apparent in wild-type embryos at E11.5 and E14.5, neither lung nor tracheal development could be found in *Wnt2/2b* DKO mutants (Fig. 2A–L). To determine whether the lung endoderm lineage was specified within the anterior foregut, we assessed the expression of Nkx2.1, a homeobox transcription factor that is the earliest marker of the developing lung endoderm (Kimura et al., 1999; Minoo et al., 1995). Nkx2.1 expression is first observed by immunohistochemistry and in situ hybridization at E9.5 and by Q-PCR at E8.5 in the ventral aspect of the foregut demarcating where the trachea will bud off of the anterior foregut (Fig. 2M and N and (Kimura et al., 1996; Serls et al., 2005; Yuan et al., 2000)). Nkx2.1 expression was absent in the anterior foregut region of *Wnt2/2b* DKO mutants confirming the loss of tracheal and lung development (Fig. 2N and R and Supplemental Figure 3). Q-PCR confirms loss of Nkx2.1 expression in anterior foregut (Supplemental Figure 4). In contrast, Nkx2.1 expression was observed in the thyroid primordium of both wild-type and *Wnt2/2b* DKO mutants (Fig. 2O and S). Specification of foregut endoderm was not lost as demonstrated by expression of Foxa2 in *Wnt2/2b* DKO mutants nor was cell proliferation or apoptosis affected in the anterior foregut endoderm of *Wnt2/2b* DKO mutants (Supplemental Figure 4). The esophagus was specified normally in *Wnt2/2b* DKO mutants as determined by expression of p63, a marker of esophageal endoderm (Fig. 2P and T). Expression of Wnt7b, an additional marker of early lung endoderm progenitors in the anterior foregut (Shu et al., 2002), was also lost in *Wnt2/2b* DKO mutants (Fig. 2U and V and Supplemental Figure 4). E-cadherin immunostaining for foregut endoderm reveals lack of tracheal budding in *Wnt2/2b* DKO mutants (Fig. 2W and X). Development of the liver, stomach, intestine, pancreas and kidneys was grossly normal in *Wnt2/2b* DKO mutants (Supplemental Figure 5). Together, these data reveal that Wnt2/2b are necessary for lung specification but specification of other foregut derived tissues including the thyroid, esophagus, liver, pancreas, kidney or stomach is not affected.

Wnt2 and Wnt2b signaling through the canonical Wnt pathway and β-catenin is essential for lung endoderm specification

Wnt ligands can signal through several distinct pathways to regulate cell specification and tissue development. The best understood of these is the β-catenin dependent canonical pathway which has been demonstrated to regulate development and differentiation of several tissues including hair follicles, intestinal epithelium, and the heart (Andl et al., 2002; Cohen et al., 2007; Pinto et al., 2003). To assess whether the canonical Wnt pathway was affected by loss of Wnt2/2b we crossed the *BAT-GAL* (Maretto et al., 2003) canonical Wnt reporter line to *Wnt2/2b* mutants and performed lacZ staining in wild-type *BAT-GAL* embryos, *Wnt2*$^{-/-}$*:BAT-GAL*, *Wnt2b*$^{-/-}$*:BAT-GAL*, and *Wnt2/2b:BAT-GAL* DKO null mutants. LacZ expression from the *BAT-GAL* Wnt reporter line was reduced in *Wnt2*$^{-/-}$ and *Wnt2b*$^{-/-}$ null mutants and completely lost in the anterior foregut endoderm in *Wnt2/2b* DKO mutants (Fig. 3A–F). To further address whether canonical Wnt signaling was necessary in the developing foregut endoderm for lung specification, we genetically deleted the *Ctnnb1* (β-catenin) gene using the *Shh-cre* mouse line which expresses the cre recombinase as early as E8.75 in the anterior foregut endoderm and effectively deletes β-catenin expression by E9.5 (Supplemental Figure 6 and (Harfe et al., 2004; Harris et al., 2006). *Ctnnb1:Shh-cre* mutants exhibited a phenotype identical to *Wnt2/2b* DKO mutants and completely lacked lung specification and tracheal budding (Fig. 3G–P). However, specification of other gut-derived tissues including the esophagus, liver and thyroid was unaffected (Supplemental Figure 6 and data not shown). These data demonstrate that Wnt2/2b act in the β-catenin dependent canonical Wnt pathway, which is required to specify lung endoderm progenitors.

The phenotype of *Wnt2/2b* DKO and *Ctnnb1:Shh-cre* mutants is distinct from other lung hypoplasia phenotypes, including the loss of Fgf10 and loss of Gli2/Gli3 expression, in that the lung is uniquely affected and specification is completely lost. *Fgf10* null mutants form a trachea which does not branch, indicating that the lung endoderm lineage is specified but fails to grow and branch (Min et al., 1998; Sekine et al., 1999). *Gli2/Gli3* double null mutants fail to form a lung but other aspects of foregut development are severely affected, including the loss of the esophagus (Motoyama et al., 1998). To develop a hierarchical model of lung specification, we assessed expression of Fgf10, Gli2, and Gli3 to determine whether their expression was affected by loss of Wnt2/2b expression. Expression of Fgf10 was reduced in *Wnt2/2b* DKO mutants, suggesting that it acts down-stream of canonical Wnt signaling in the anterior foregut (Fig. 3Qand R). Although we have demonstrated that Fgf10 is a direct target of Wnt/β-catenin signaling (Cohen et al., 2007), it remains possible that loss of Fgf10 expression is secondary to a loss of lung specification. In contrast to Fgf10 expression, Gli2 and Gli3 expression were unchanged in the anterior foregut region of *Wnt2/2b* DKO mutants (Fig. 3S and T and Supplemental Figure 4). These data suggest that Wnt2/2b act upstream of Fgf10 but not Gli2/Gli3 in the regulation of lung specification.

Activation of Wnt/β-catenin signaling leads to reprogramming of posterior foregut endoderm to a lung endoderm fate

The potent role for Wnt/β-catenin signaling in specifying lung endoderm progenitors suggested that ectopic activation of this pathway might dominantly expand lung endoderm progenitor identity outside the normal region in the foregut. To test this hypothesis, we generated *Ctnnb1:Shh-cre* mutants. These mutants express the stabilized form of β-catenin lacking the phophorylation sites required for its degradation which leads to strong activation of Wnt/β-catenin signaling (Harada et al., 1999). *Ctnnb1:Shh-cre* mutants displayed defects in tracheal-esophageal septation compared to wild-type controls (Fig. 4A–C, E–G). Immunostaining revealed expansion of Nkx2.1 positive lung progenitors into the hindgut region corresponding to stomach endoderm in *Ctnnb1:Shh-cre* E10.5 mutants (Fig. 4D and H). This expansion is also evident at E11.5 in *Ctnnb1:Shh-cre* mutants where the esophagus as well as proximal stomach are populated with Nkx2.1 positive lung progenitors (Fig. 4I–P). To determine whether this expansion of Nkx2.1 lung progenitors represented a reprogramming of foregut endoderm or an increase in Nkx2.1 expression in esophagus and stomach endoderm, E11.5 wild-type and *Ctnnb1:Shh-cre* mutants where immunostained to detect expression of the esophagus and stomach marker p63. These data reveal that p63 expression is lost in the esophagus and proximal stomach of *Ctnnb1:Shh-cre* mutants suggesting that activation of Wnt/β-catenin signaling reprograms esophagus and stomach endoderm to a lung endoderm progenitor fate (Fig. 4Q–V). Together, these data indicate that activation of Wnt/β-catenin signaling reprograms posterior regions of the foregut endoderm to a lung endoderm progenitor fate, suggesting that activation of this pathway drives lung endoderm specification in a dominant manner. Thus, our work identifies Wnt2/β-catenin dependent activity as the required signal for specification of lung endoderm progenitors within the foregut (Fig. 4W).

DISCUSSION

Mutations in other genes have resulted in either a severe truncation in lung development (i.e. *Fgf10* null mutants) or defects in LPM leading to severe foregut agenesis including the lung and esophagus (i.e. *Gli2/Gli3* double mutants) (Motoyama et al., 1998; Sekine et al., 1999). Here, we show the Wnt2/2b are distinct in their ability to specify lung progenitors within the developing foregut while sparing other organs including the thyroid, esophagus, liver, and pancreas. Moreover, we show that activation of Wnt signaling can reprogram esophagus and stomach endoderm to a lung progenitor fate. Our data support the importance of mesoderm to endoderm signaling that promotes development of foregut derived tissues and extends these findings to provide a molecular hierarchy of foregut endoderm specification.

Previous reports have elucidated additional roles for Wnt signaling in the developing lung. Loss of β-catenin or expression of the Wnt inhibitor dikkopf1 in lung epithelium after lung specification leads to decreased distal airway epithelial development and an overall proximalization of the lung (Mucenski et al., 2003; Shu et al., 2005). A dermo1-cre mesenchymal specific loss of β-catenin in the lung leads to defective lung mesenchymal proliferation and development (De Langhe et al., 2008; Yin et al., 2008). A previous report on a different Wnt2 allele did not report a lung phenotype although approximately 50% of null animals died by birth (Monkley et al., 1996). This could be explained by the presence of significant levels of a truncated Wnt2 mRNA species observed in this previous allele (Monkley et al., 1996). Expression of several other Wnt ligands besides Wnt2 and Wnt2b has been reported in the lung including Wnt7b and Wnt5a (Li et al., 2002; Weidenfeld et al., 2002). Wnt7b has been shown to regulate mesenchymal proliferation as well as epithelial proliferation and maturation (Rajagopal et al., 2008; Shu et al., 2002). Loss of Wnt7b also disrupts lung smooth muscle development leading to a loss of vascular integrity (Shu et al., 2002). The decreased proliferation observed in Wnt7b mutant lungs is similar to that observed in the Wnt2 null lungs suggesting that one of the major roles for Wnt signaling in the lung post-specification is regulation of organ growth and size. Wnt5a is expressed initially in both the mesenchyme and distal epithelium of the developing lung (Li et al., 2002). After E12.5, however, expression of Wnt5a becomes restricted to the distal epithelium (Li et al., 2002). Loss of Wnt5a leads to increased mesenchymal proliferation and a loss in late airway maturation (Li et al., 2002). Since Wnt5a has been reported to act in the non-canonical Wnt pathway (Topol et al., 2003), which can antagonize β-catenin dependent canonical signaling, the increased proliferation observed in the lung mesenchyme of Wnt5a mutants could be due to increased canonical Wnt signaling in this tissue. The present study shows that in addition to regulation of lung development and growth, Wnt signaling through Wnt2/2b is essential for specification of lung endoderm progenitors in the foregut.

In contrast to previous studies in zebrafish which demonstrated an important role for *wnt2b* in liver development and specification as well as fin development in zebrafish, our data show that Wnt2/2b are not required for mammalian liver specification (Ng et al., 2002; Ober et al., 2006). The studies described here suggest that the role for Wnt/β-catenin signaling along the anterior-posterior axis of the foregut varies between species which may have occurred as Wnt2/2b and the canonical Wnt pathway were co-opted during evolution to specify the lung during the vertebrate expansion into the terrestrial environment. The specificity for Wnt signaling, in particular Wnt2 and Wnt2b, in regulating specification of the lung is interesting in light of previous reports showing an important role for this pathway in pancreas and liver development (Apte et al., 2007; Dessimoz et al., 2005; Murtaugh et al., 2005; Tan et al., 2006). This may be due to the precise expression pattern of these two Wnt ligands or to an important sensitivity of lung endoderm progenitors to canonical Wnt signaling. Moreover, the phenotype in *Ctnnb1:Shh-cre* mutants is likely due to the timing and specificity of the Shh-cre line since we do not observe early activity in the liver (Supplemental Figure 6). It is also important to note that since Fgf10 is a direct target of Wnt/β-catenin signaling (Cohen et al., 2007), the ability of Wnt2/2b to regulate its expression in the mesoderm surrounding the anterior foregut in a cell autonomous manner could affect other pathways important for mesoderm-endoderm signaling during lung development. Given the critical importance of Wnt2/2b signaling in lung endoderm specification, it will be interesting in future studies to determine whether simple activation of Wnt signaling can rescue the Wnt2/2b phenotype in foregut endoderm. Previous reports have shown that Wnt/β-catenin signaling is also important in adult lung progenitor expansion after injury (Reynolds et al., 2008; Zhang et al., 2008). Thus, Wnt signaling plays a key role in both embryonic as well as adult lung endoderm progenitor development, which reinforces the importance of critical developmental pathways that are recapitulated upon injury and repair.

Wnt/β-catenin signaling is one of the critical developmental pathways that are considered important for both self-renewal and differentiation of stem/progenitor cells. With vigorous efforts underway to determine whether agonists or antagonists can be used to manipulate this pathway for therapeutic purposes, our findings that Wnt signaling is central to the specification and ability to reprogram foregut endoderm to a lung endoderm fate provides important information for investigating lung regeneration. In summary, our data provide a molecular hierarchy of foregut endoderm progenitor specification with Wnt2/2b signaling acting dominantly to specify lung endoderm progenitors in the anterior foregut.

MATERIALS AND METHODS

Generation of Wnt2 and Wnt2b mutant mice

Wnt2 mutant mice were generated using recombineering techniques to replace a portion of the coding region of the first exon with the reverse tet-activator cDNA. Three correctly targeted ES clones were used to generate chimeric mice and all three clones transmitted the mutant allele through the germline. The neomycin selection cassette was removed using the flp recombinase expressing mice (Flper) from JAX and confirmed by PCR (Supplemental Figure 2). Analysis was performed with alleles containing and lacking the neomycin cassette. *Wnt2b* mutant mice were generated using recombineering techniques to insert loxP sites flanking exons 2 and 3. Two correctly targeted ES cells were used to generate chimeric mice which were bred to transmit these mutant alleles through the germline. *Wnt2b* mutants were crossed to *CMV-cre* mice to delete exons 2 and 3 and generate a null allele. Both lines were maintained on a C57BL/6:129SVJ mixed background. Genotyping was performed using the oligonucleotides listed in Supplemental Table 2. The generation and genotyping of *Shh-cre*, *CMV-cre*, *Ctnnb1*, *BAT-GAL*, and *Ctnnb1* mice have been previously described (Brault et al., 2001; Harada et al., 1999; Harfe et al., 2004; Maretto et al., 2003).

Histology

Embryos were fixed in 4% paraformaldehyde for 24 hours and embedded in paraffin for tissue sectioning. In situ hybridization and immunohistochemistry was performed as previously described (Shu et al., 2001). Antibodies and dilutions used are as follows: Ki67 (Vector Laboratories, 1:50), Nkx2.1 (Santa Cruz, 1:50), p63 (Santa Cruz, 1:50) β-catenin (BD Biosciences, 1:50). Quantitation of positive cell populations was performed using at least three different tissue sections from at least three different embryos of the same genotype. LacZ histochemical staining of embryos was performed as previously described (Shu et al., 2002). TUNEL staining was performed as previously described (Shu et al., 2007).

Quantitative RT-PCR

Total RNA was isolated from lung tissue at the indicated time points using Trizol reagent, reverse transcribed using SuperScript First Strand Synthesis System (Invitrogen), and used in quantitative real time PCR analysis using the oligonucleotides listed in Supplemental Table 2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

A.M.G. was supported by an American Heart Association Predoctoral fellowship and Y.T. and E.D.C. were supported by American Heart Association Postdoctoral fellowships. This work was support by funding from the NIH to E.E.M. (HL075215 and HL087825). The authors would like to thank the animal care personnel in the Animal Services Unit of the John Morgan Animal facility at the University of Pennsylvania for help in animal husbandry.

References

Andl T, Reddy ST, Gaddapara T, Millar SE. WNT signals are required for the initiation of hair follicle development. Dev Cell 2002;2:643–653. [PubMed: 12015971]

Apte U, Zeng G, Thompson MD, Muller P, Micsenyi A, Cieply B, Kaestner KH, Monga SP. beta-Catenin is critical for early postnatal liver growth. Am J Physiol Gastrointest Liver Physiol 2007;292:G1578–1585. [PubMed: 17332475]

Brault V, Moore R, Kutsch S, Ishibashi M, Rowitch DH, McMahon AP, Sommer L, Boussadia O, Kemler R. Inactivation of the beta-catenin gene by Wnt1-Cre-mediated deletion results in dramatic brain malformation and failure of craniofacial development. Development 2001;128:1253–1264. [PubMed: 11262227]

Cohen ED, Wang Z, Lepore JJ, Lu MM, Taketo MM, Epstein DJ, Morrisey EE. Wnt/beta-catenin signaling promotes expansion of Isl-1-positive cardiac progenitor cells through regulation of FGF signaling. J Clin Invest 2007;117:1794–1804. [PubMed: 17607356]

De Langhe SP, Carraro G, Tefft D, Li C, Xu X, Chai Y, Minoo P, Hajihosseini MK, Drouin J, Kaartinen V, Bellusci S. Formation and differentiation of multiple mesenchymal lineages during lung development is regulated by beta-catenin signaling. PLoS ONE 2008;3:e1516. [PubMed: 18231602]

Dessimoz J, Bonnard C, Huelsken J, Grapin-Botton A. Pancreas-specific deletion of beta-catenin reveals Wnt-dependent and Wnt-independent functions during development. Curr Biol 2005;15:1677–1683. [PubMed: 16169491]

Eblaghie MC, Reedy M, Oliver T, Mishina Y, Hogan BL. Evidence that autocrine signaling through Bmpr1a regulates the proliferation, survival and morphogenetic behavior of distal lung epithelial cells. Dev Biol 2006;291:67–82. [PubMed: 16414041]

Harada N, Tamai Y, Ishikawa T, Sauer B, Takaku K, Oshima M, Taketo MM. Intestinal polyposis in mice with a dominant stable mutation of the beta-catenin gene. Embo J 1999;18:5931–5942. [PubMed: 10545105]

Harfe BD, Scherz PJ, Nissim S, Tian H, McMahon AP, Tabin CJ. Evidence for an expansion-based temporal Shh gradient in specifying vertebrate digit identities. Cell 2004;118:517–528. [PubMed: 15315763]

Harris KS, Zhang Z, McManus MT, Harfe BD, Sun X. Dicer function is essential for lung epithelium morphogenesis. Proc Natl Acad Sci U S A 2006;103:2208–2213. [PubMed: 16452165]

Kimura S, Hara Y, Pineau T, Fernandez-Salguero P, Fox CH, Ward JM, Gonzalez FJ. The T/ebp null mouse: thyroid-specific enhancer-binding protein is essential for the organogenesis of the thyroid, lung, ventral forebrain, and pituitary. Genes Dev 1996;10:60–69. [PubMed: 8557195]

Kimura S, Ward JM, Minoo P. Thyroid-specific enhancer-binding protein/thyroid transcription factor 1 is not required for the initial specification of the thyroid and lung primordia. Biochimie 1999;81:321–327. [PubMed: 10401665]

Li C, Xiao J, Hormi K, Borok Z, Minoo P. Wnt5a participates in distal lung morphogenesis. Dev Biol 2002;248:68–81. [PubMed: 12142021]

Maretto S, Cordenonsi M, Dupont S, Braghetta P, Broccoli V, Hassan AB, Volpin D, Bressan GM, Piccolo S. Mapping Wnt/beta-catenin signaling during mouse development and in colorectal tumors. Proc Natl Acad Sci U S A 2003;100:3299–3304. [PubMed: 12626757]

Min H, Danilenko DM, Scully SA, Bolon B, Ring BD, Tarpley JE, DeRose M, Simonet WS. Fgf-10 is required for both limb and lung development and exhibits striking functional similarity to Drosophila branchless. Genes Dev 1998;12:3156–3161. [PubMed: 9784490]

Minoo P, Hamdan H, Bu D, Warburton D, Stepanik P, deLemos R. TTF-1 regulates lung epithelial morphogenesis. Dev Biol 1995;172:694–698. [PubMed: 8612983]

Monkley SJ, Delaney SJ, Pennisi DJ, Christiansen JH, Wainwright BJ. Targeted disruption of the Wnt2 gene results in placentation defects. Development 1996;122:3343–3353. [PubMed: 8951051]

Motoyama J, Liu J, Mo R, Ding Q, Post M, Hui CC. Essential function of Gli2 and Gli3 in the formation of lung, trachea and oesophagus. Nat Genet 1998;20:54–57. [PubMed: 9731531]

Mucenski ML, Wert SE, Nation JM, Loudy DE, Huelsken J, Birchmeier W, Morrisey EE, Whitsett JA. beta-Catenin is required for specification of proximal/distal cell fate during lung morphogenesis. J Biol Chem 2003;278:40231–40238. [PubMed: 12885771]

Murtaugh LC, Law AC, Dor Y, Melton DA. Beta-catenin is essential for pancreatic acinar but not islet development. Development 2005;132:4663–4674. [PubMed: 16192304]

Ng JK, Kawakami Y, Buscher D, Raya A, Itoh T, Koth CM, Rodriguez Esteban C, Rodriguez-Leon J, Garrity DM, Fishman MC, Izpisua Belmonte JC. The limb identity gene Tbx5 promotes limb initiation by interacting with Wnt2b and Fgf10. Development 2002;129:5161–5170. [PubMed: 12399308]

Ober EA, Verkade H, Field HA, Stainier DY. Mesodermal Wnt2b signalling positively regulates liver specification. Nature 2006;442:688–691. [PubMed: 16799568]

Okubo T, Knoepfler PS, Eisenman RN, Hogan BL. Nmyc plays an essential role during lung development as a dosage-sensitive regulator of progenitor cell proliferation and differentiation. Development 2005;132:1363–1374. [PubMed: 15716345]

Pinto D, Gregorieff A, Begthel H, Clevers H. Canonical Wnt signals are essential for homeostasis of the intestinal epithelium. Genes Dev 2003;17:1709–1713. [PubMed: 12865297]

Rajagopal J, Carroll TJ, Guseh JS, Bores SA, Blank LJ, Anderson WJ, Yu J, Zhou Q, McMahon AP, Melton DA. Wnt7b stimulates embryonic lung growth by coordinately increasing the replication of epithelium and mesenchyme. Development 2008;135:1625–1634. [PubMed: 18367557]

Reynolds SD, Zemke AC, Giangreco A, Brockway BL, Teisanu RM, Drake JA, Mariani T, Di PY, Taketo MM, Stripp BR. Conditional stabilization of beta-catenin expands the pool of lung stem cells. Stem Cells 2008;26:1337–1346. [PubMed: 18356571]

Sekine K, Ohuchi H, Fujiwara M, Yamasaki M, Yoshizawa T, Sato T, Yagishita N, Matsui D, Koga Y, Itoh N, Kato S. Fgf10 is essential for limb and lung formation. Nat Genet 1999;21:138–141. [PubMed: 9916808]

Serls AE, Doherty S, Parvatiyar P, Wells JM, Deutsch GH. Different thresholds of fibroblast growth factors pattern the ventral foregut into liver and lung. Development 2005;132:35–47. [PubMed: 15576401]

Shu W, Guttentag S, Wang Z, Andl T, Ballard P, Lu MM, Piccolo S, Birchmeier W, Whitsett JA, Millar SE, Morrisey EE. Wnt/beta-catenin signaling acts upstream of N-myc, BMP4, and FGF signaling to regulate proximal-distal patterning in the lung. Dev Biol 2005;283:226–239. [PubMed: 15907834]

Shu W, Jiang YQ, Lu MM, Morrisey EE. Wnt7b regulates mesenchymal proliferation and vascular development in the lung. Development 2002;129:4831–4842. [PubMed: 12361974]

Shu W, Lu MM, Zhang Y, Tucker PW, Zhou D, Morrisey EE. Foxp2 and Foxp1 cooperatively regulate lung and esophagus development. Development 2007;134:1991–2000. [PubMed: 17428829]

Shu W, Yang H, Zhang L, Lu MM, Morrisey EE. Characterization of a new subfamily of winged-helix/forkhead (Fox) genes that are expressed in the lung and act as transcriptional repressors. J Biol Chem 2001;276:27488–27497. [PubMed: 11358962]

Tan X, Behari J, Cieply B, Michalopoulos GK, Monga SP. Conditional deletion of beta-catenin reveals its role in liver growth and regeneration. Gastroenterology 2006;131:1561–1572. [PubMed: 17101329]

Topol L, Jiang X, Choi H, Garrett-Beal L, Carolan PJ, Yang Y. Wnt-5a inhibits the canonical Wnt pathway by promoting GSK-3-independent beta-catenin degradation. J Cell Biol 2003;162:899–908. [PubMed: 12952940]

Weidenfeld J, Shu W, Zhang L, Millar SE, Morrisey EE. The WNT7b promoter is regulated by TTF-1, GATA6, and Foxa2 in lung epithelium. J Biol Chem 2002;277:21061–21070. [PubMed: 11914369]

Yin Y, White AC, Huh SH, Hilton MJ, Kanazawa H, Long F, Ornitz DM. An FGF-WNT gene regulatory network controls lung mesenchyme development. Dev Biol 2008;319:426–436. [PubMed: 18533146]

Yuan B, Li C, Kimura S, Engelhardt RT, Smith BR, Minoo P. Inhibition of distal lung morphogenesis in Nkx2.1(−/−) embryos. Dev Dyn 2000;217:180–190. [PubMed: 10706142]

Zakin LD, Mazan S, Maury M, Martin N, Guenet JL, Brulet P. Structure and expression of Wnt13, a novel mouse Wnt2 related gene. Mech Dev 1998;73:107–116. [PubMed: 9545553]

Zhang Y, Goss AM, Cohen ED, Kadzik R, Lepore JJ, Muthukumaraswamy K, Yang J, Demayo FJ, Whitsett JA, Parmacek MS, Morrisey EE. A Gata6-Wnt pathway required for epithelial stem cell development and airway regeneration. Nat Genet. 2008

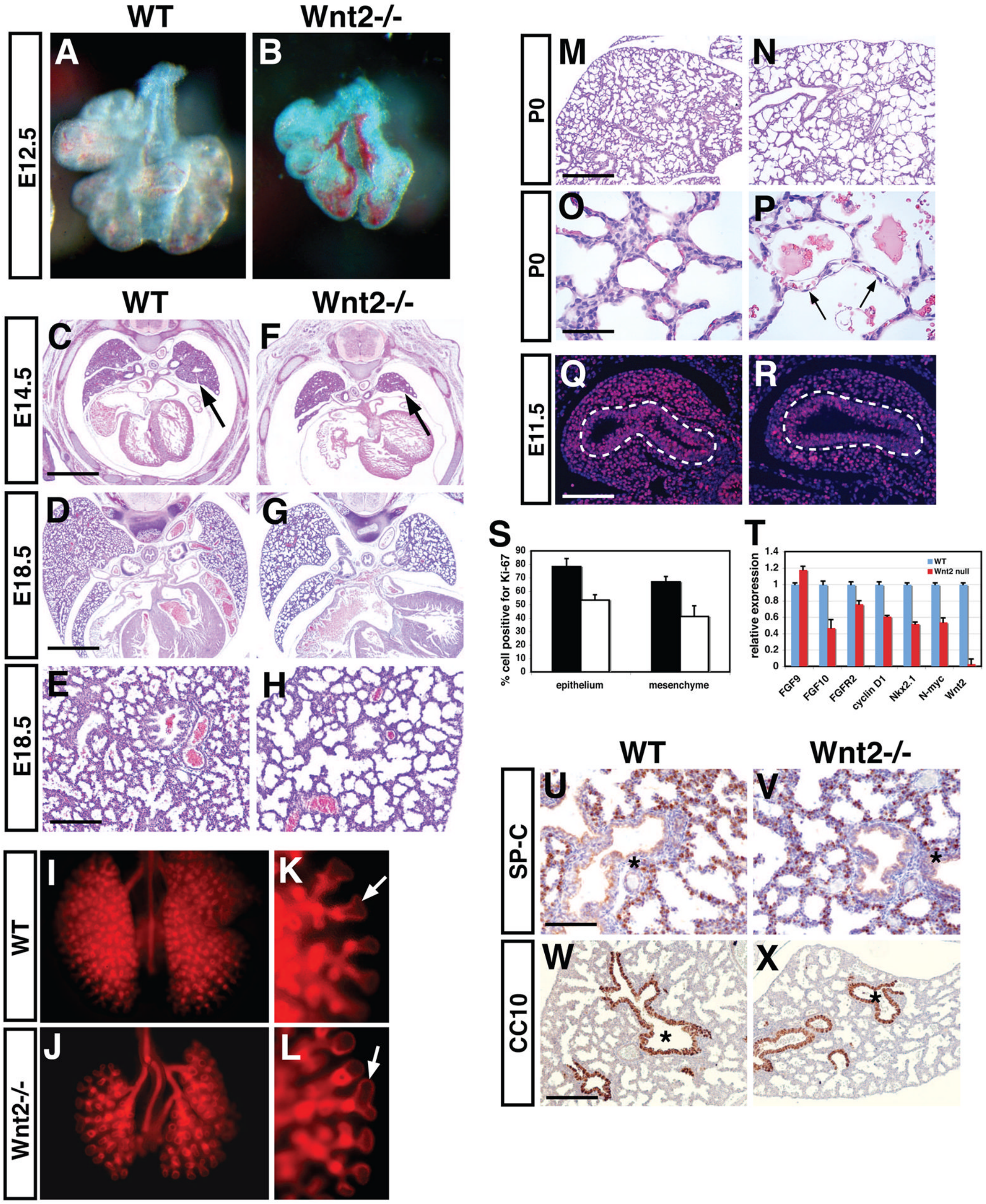

Figure 1. Loss of Wnt2 leads to lung hypoplasia and down-regulation of critical pathways required for lung development

Wnt2$^{-/-}$ null mutants exhibit severe lung hypoplasia as shown by whole mount comparison (A and B) and histological sectioning at E14.5 and E18.5 (C, D, F, G). Dilation of alveolar sacs is observed at E18.5 (E and H) and at P0 (M and N) in *Wnt2*$^{-/-}$ null mutants. Whole mount staining with E-cadherin shows relatively normal distal branching of airways in *Wnt2*$^{-/-}$ null mutants (I–L). *Wnt2*$^{-/-}$ null mutants have a dilated endothelial vasculature indicating significant lung mesenchymal defects (O and P, arrows). *Wnt2*$^{-/-}$ null mutants have a significant decrease in proliferation in both the lung endoderm and mesenchyme (Q and R). Quantitation of proliferation defects in Wnt2$^{-/-}$ null mutants (S). *Wnt2*$^{-/-}$ null mutants exhibit decreased expression of genes critical for lung development as assessed by Q-PCR at E14.5 (T). Normal proximal-distal patterning in *Wnt2*$^{-/-}$ null mutants as assessed by SP-C and CC10 immunostainng (U–X). Scale bars: C, D, F, G=800 μm, E, H, U and V=200 μm, W and X=400 μm, M and N=600 μm, O–R=100 μm.

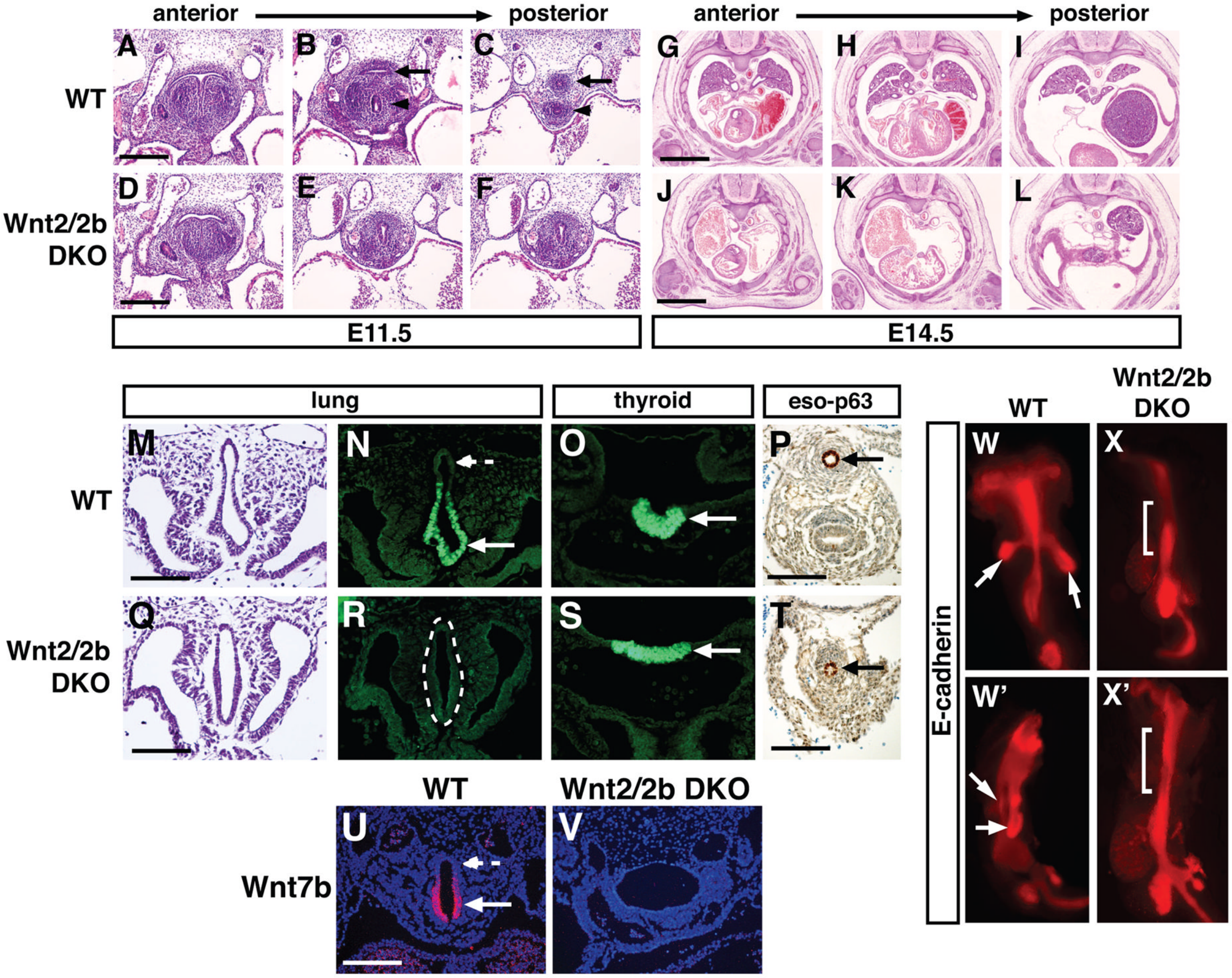

Figure 2. Loss of both Wnt2 and Wnt2b leads to specific loss of lung progenitor specification in the foregut endoderm and complete lung agenesis

Wnt2/2b DKO mutants exhibit complete lung agenesis as shown at E11.5 (A–F) and E14.5 (G–L). A clear separation of the esophagus (B and C, arrow) from the trachea (B and C, arrowhead) is observed in wild-type embryos. At E9.5, when the lung is initially specified, there is no detectable budding of the trachea from the anterior foregut in *Wnt2/2b* DKO mutants (M and Q). While wild-type embryos express Nkx2.1 in the region where the trachea will bud from the foregut (N), expression is not observed in *Wnt2/2b* DKO mutants (R, outline). However, Nkx2.1 expression is observed in both wild-type (O) and *Wnt2/2b* DKO mutants (S) in the thyroid primordium. The esophagus epithelial marker p63 is expressed in the single gut tube in *Wnt2/2b* DKO mutants at E11.5 (P and T, arrows). Wnt7b, which also marks early lung endoderm progenitors in the ventral aspect of the anterior foregut (arrow) versus the dorsal aspect (dashed arrow), is lost in *Wnt2/2b* DKO anterior foregut endoderm (U and V). E-cadherin whole mount immunostaining shows lack of tracheal budding in Wnt2/2b DKO mutants (W and X, arrows and brackets). Scale bars: A–F=200 μm, G–L=800 μm, M–V=100 μm.

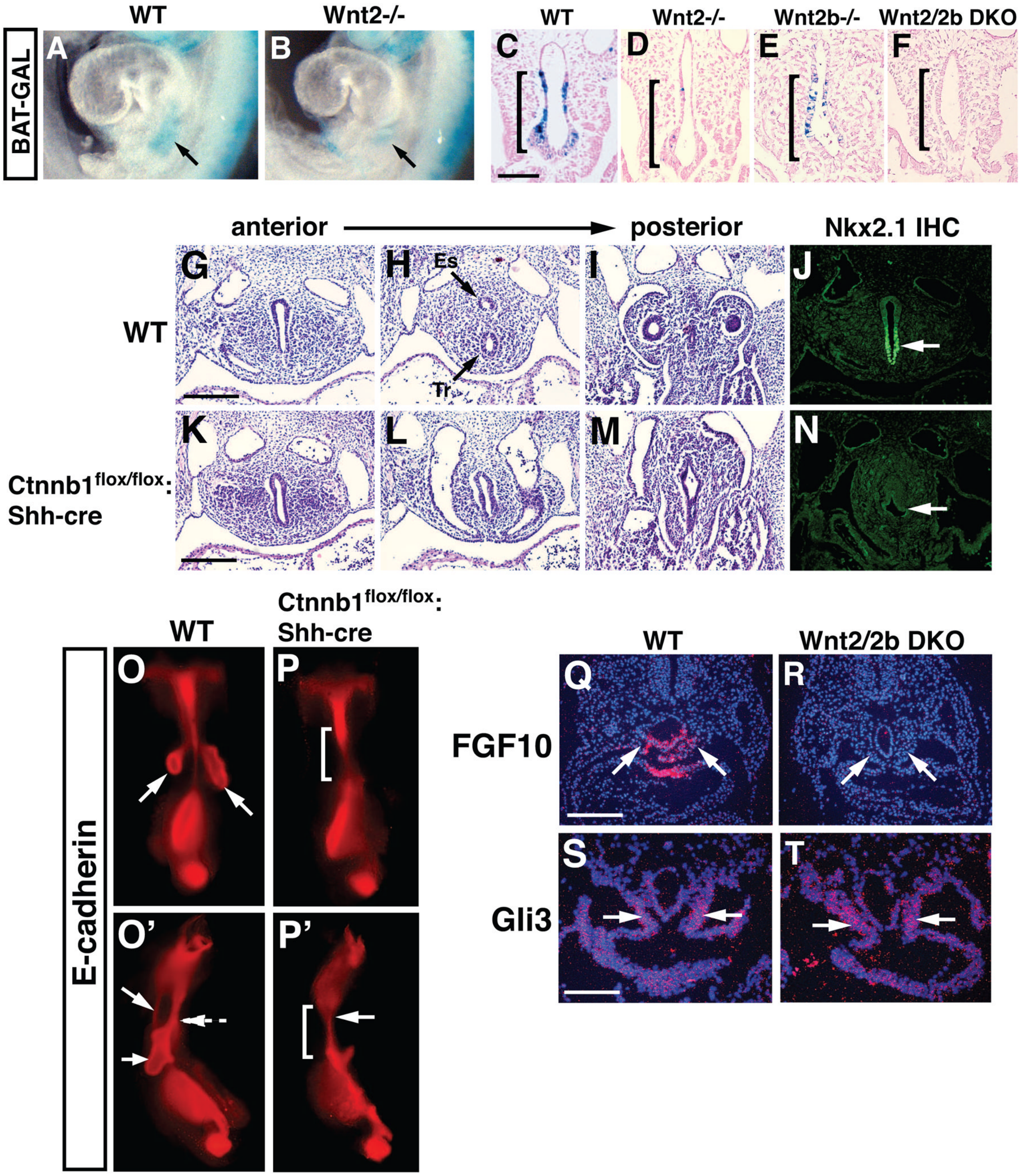

Figure 3. Wnt2/2b signal through the β-catenin dependent canonical pathway to specify lung endoderm progenitors in the anterior foregut

Whole mount staining of *Wnt2*$^{-/-}$:*BAT-GAL* embryos for lacZ expression revealing a significant decrease in canonical Wnt signaling in the anterior foregut at E10.5 (A and B, arrows). Histological sections of wild-type *BAT-GAL*, *Wnt2*$^{-/-}$:*BAT-GAL*, *Wnt2b*$^{-/-}$:*BAT-GAL*, and *Wnt2/2b:BAT-GAL* DKO mutants shows a significant loss of staining in the ventral aspect of the foregut endoderm in the region where the trachea is specified (C–F, brackets). Histological sections from E10.5 wild-type (G–I) and *Ctnnb1:Shh-cre* (K–M) demonstrating lung agenesis upon deletion of foregut endoderm β-catenin expression. Nkx2.1 expression is observed in the ventral foregut endoderm of wild-type embryos at E10.5 (J) but not in

Ctnnb1:Shh-cre mutants (N) indicating loss of lung specification in these mutants. E-cadherin whole mount immunostaining shows normal tracheal budding in wild-type embryos (O, arrow) and lack of tracheal budding in *Ctnnb1:Shh-cre* mutants (P, arrow). However, the esophagus is still present in the *Ctnnb1:Shh-cre* mutants (P′, bracket). Fgf10 expression is substantially reduced in the ventral mesoderm surrounding the foregut in *Wnt2/2b* DKO mutants (Q and R) while Gli3 expression is unchanged at E10.0 (S and T). Es=esophagus, Tr=trachea. Scale bars: C–F=100 μm, G–N, Q, R=200 μm, and S and T=150 μm.

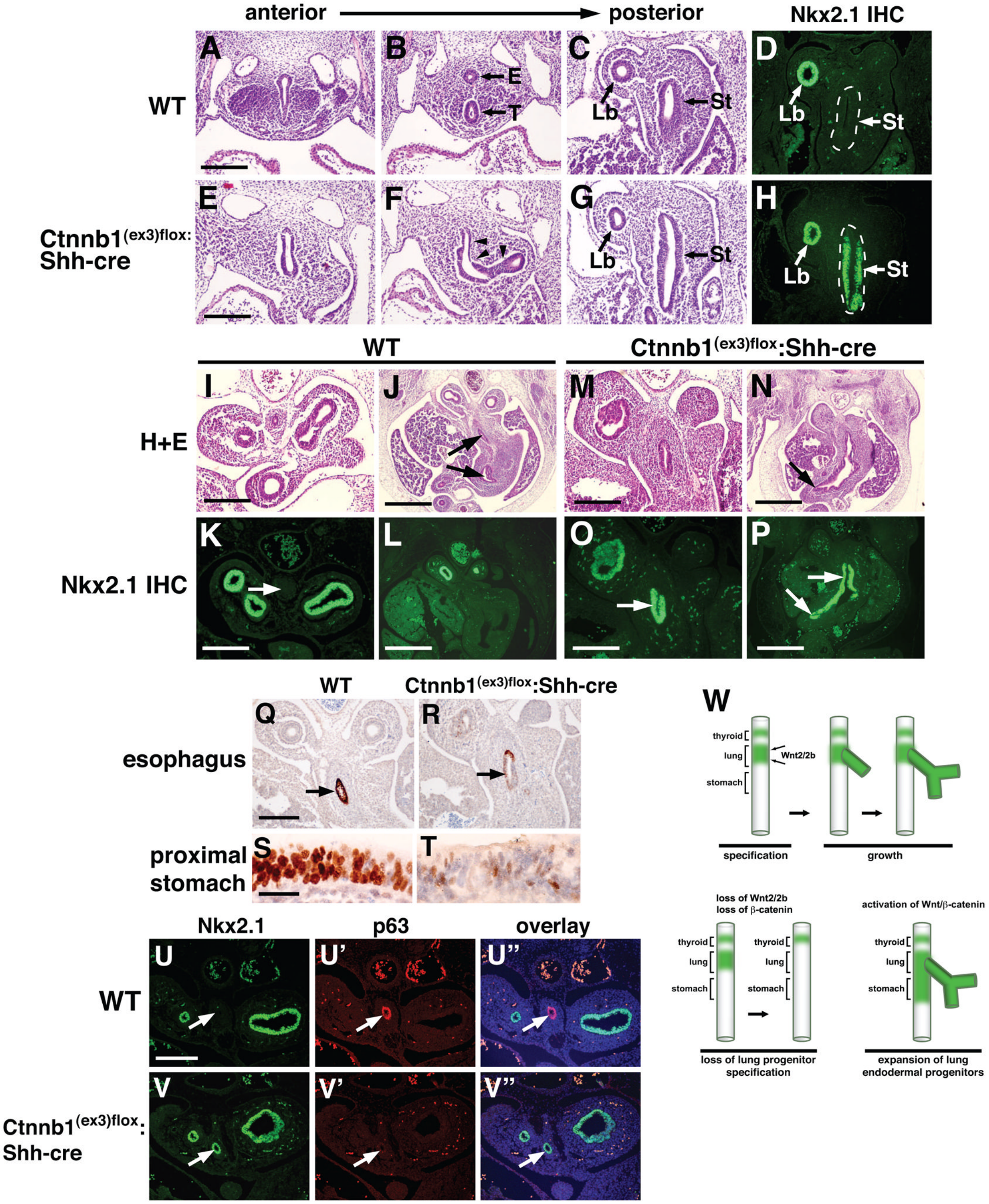

Figure 4. Activation of Wnt/β-catenin signaling leads to expansion of lung endoderm progenitors into the stomach

H+E stained sections from E10.5 wild-type (A–C) and *Ctnnb1:Shh-cre* (E–G) mutants show that trachea-esophagus septation is disrupted in these mutants (F, arrowheads). Immunostaining for Nkx2.1 protein expression reveals expansion of Nkx2.1 positive lung endoderm progenitors into the stomach (D and H, outlined region). H+E stained sections from E11.5 wild-type (I and J) and *Ctnnb1:Shh-cre* (M and N) mutants. Immunostaining for Nkx2.1 protein expression at E11.5 shows expression of Nkx2.1 in esophagus of *Ctnnb1:Shh-cre* mutants (O, arrow) and not in wild-type littermates (K, arrow). Expression of Nkx2.1 is also extended into the stomach of E11.5 *Ctnnb1:Shh-cre* mutants (P, arrows). In contrast, expression of p63 is reduced in the esophagus and stomach endoderm of *Ctnnb1:Shh-cre* mutants at E11.5 (Q–T). Co-staining for both Nkx2.1 and p63 show that p63 positive endoderm is lost while Nkx2.1 positive endoderm is present in the esophagus (U–V). Model showing necessity and sufficiency of Wnt2/2b and β-catenin signaling for lung progenitor specification in the anterior foregut endoderm (W).