Wnt2/2b and β-Catenin Signaling Are Necessary and Sufficient to Specify Lung Progenitors in the Foregut

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SUMMARY

Patterning of the primitive foregut promotes appropriate organ specification along its anterior-posterior axis. However, the molecular pathways specifying foregut endoderm progenitors are poorly understood. We show here 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. The phenotype observed 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 the reprogramming of esophagus and stomach endoderm to a lung endoderm progenitor fate. Together, these data reveal that canonical Wnt2/2b signaling is required for the 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 the 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.

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 the 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 Foregut Development

Previous researchers 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 the regulation of tissue-specific progenitors, we explored whether these ligands are expressed in the appropriate spatial and temporal pattern to regulate the 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 embryonic day (E) 9.0 to E10.5, during the period when the lung is specified (see Figure S1 available online). Wnt2 and Wnt2b are expressed later in the developing lung mesenchyme, with Wnt2 expression persisting into adulthood (Figure S1) (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 the development of the anterior foregut, we generated null alleles of both genes in mice by using homologous recombination in embryonic stem (ES) cells (Figure S2). The majority of Wnt2 / - / null mutants are cyanotic at birth and die within a few minutes, whereas Wnt2b / - / null mutants are viable and have no discernible phenotype (Table S1; data not shown). To explore the reason for perinatal lethality in Wnt2 / - / null mutants, histological analysis was performed on embryos from E10.5 to E18.5. These studies revealed significant lung hypoplasia in Wnt2 / - / null mutants (Figures 1A–1H). Despite the hypoplasia, branching of the terminal airways was relatively normal in Wnt2 / - / null mutants (Figures 1I–1L). Poor development of the lung mesenchyme, leading to a dilated and dysfunctional vascular endothelial plexus by birth, was observed (Figures 1M–1P). Cell proliferation is significantly reduced in both epithelial and mesenchymal cell lineages in Wnt2 / - / - / lungs (Figures 1Q–1S). 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 (Figure 1T) (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 bronchial epithelial cells (Figures 1U–1X).

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 (Figures 2A–2L). Whereas 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 (Figures 2A–2L). 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 (Figures 2M and 2N) (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 (Figures 2N and 2R; Figure S3). Q-PCR confirms loss of Nkx2.1 expression in the anterior foregut (Figure S4). In contrast, Nkx2.1 expression was observed in the thyroid primordium of both wild-type and Wnt2/2b DKO mutants (Figures 2O and 2S). Specification of the 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 (Figure S4). The esophagus was specified normally in Wnt2/2b DKO mutants, as determined by expression of p63, a marker of the esophageal endoderm (Figures 2P and 2T). 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 (Figures 2U and 2V; Figure S4). E-cadherin immunostaining for the foregut endoderm reveals a lack of tracheal budding in Wnt2/2b DKO mutants (Figures 2W and 2X). Development of the liver, stomach, intestine, pancreas, and kidneys was grossly normal in Wnt2/2b DKO mutants (Figure S5). Together, these data reveal that Wnt2/2b are necessary for lung specification, but that 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 the 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 null mice and performed lacZ staining in wild-type BAT-GAL embryos, Wnt2 / - / :BAT-GAL, Wnt2b / - / :BAT-GAL, and Wnt2 / - / :BAT-GAL DKO null mutants. LacZ expression from the BAT-GAL Wnt reporter line was reduced in Wnt2 / - / - / and Wnt2b / - / - / null mutants and was completely lost in the anterior foregut endoderm in Wnt2/2b DKO mutants (Figures 3A–3F). 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 by 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 (Figure S6) (Harfe et al., 2004; Harris et al., 2006). Ctnnb1 / lox / flox / Shh-cre mutants exhibited a phenotype identical to Wnt2/2b DKO mutants and completely lacked lung specification and tracheal budding (Figures 3G–3P). However, specification of other gut-derived tissues, including the esophagus, liver, and thyroid, was unaffected (Figure S6; 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 / lox / flox / 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 that 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 downstream of canonical Wnt signaling in the anterior foregut (Figures 3Q and 3R). 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
Figure 1. Loss of Wnt2 Leads to Lung Hypoplasia and Downregulation of Critical Pathways Required for Lung Development

(A–N) Wnt2−/− null mutants exhibit severe lung hypoplasia, as shown by (A and B) whole-mount comparison and (C, D, F, and G) histological sectioning at E14.5 and E18.5. Dilation of alveolar sacs is observed at (E and H) E18.5 and at (M and N) P0 in Wnt2−/− null mutants. (I–L) Whole-mount staining with E-cadherin shows relatively normal distal branching of airways in Wnt2−/− null mutants.

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Activation of Wnt/β-Catenin Signaling Leads to the 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^{flox/flox}::Shh-cre mutants. These mutants express the stabilized form of β-catenin lacking the phosphorylation sites required for its degradation, which leads to strong activation of Wnt/β-catenin signaling (Harada et al., 1999). Ctnnb1^{flox/flox}; Shh-cre mutants displayed defects in tracheal-esophageal septation compared to wild-type controls (Figures 4A–4C and 4E–4G). Immunostaining revealed expansion of Nkx2.1-positive lung progenitors into the posterior region of the foregut corresponding 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 (Figures 3S and 3T; Figure S4). These data suggest that Wnt2/2b act upstream of Fgf10, but not Gli2/Gli3, in the regulation of lung specification.

Figure 2. Loss of Both Wnt2 and Wnt2b Leads to Specific Loss of Lung Progenitor Specification in the Foregut Endoderm and Complete Lung Agenesis

(A–X) Wnt2/2b DKO mutants exhibit complete lung agenesis, as shown at (A–F) E11.5 and (G–L) E14.5. A clear separation of the esophagus (IB) and [C], arrow) from the trachea (IB) and [C], arrowhead) is observed in wild-type embryos. (M and Q) 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. Whereas 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 (IR, outline). However, Nkx2.1 expression is observed in both (O) wild-type and (S) Wnt2/2b DKO mutants in the thyroid primordium. (P and T) The esophagus epithelial marker p63 is expressed in the single gut tube in Wnt2/2b DKO mutants at E11.5 (arrows). (U and V) Wnt/7b, 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 Wnt/2/2b DKO anterior foregut endoderm. (W–X) E-cadherin whole-mount immunostaining shows a lack of tracheal budding in Wnt2/2b DKO mutants (arrows and brackets).

(O and P) Wnt2^{−/−} null mutants have a dilated endothelial vasculature, indicating significant lung mesenchymal defects (arrows). (Q and R) Wnt2^{−/−} null mutants have a significant decrease in proliferation in both the lung endoderm and mesenchyme. (S) Quantitation of proliferation defects in Wnt2^{−/−} null mutants. Values represent the average of three separate fields of view ± SD. (T) Wnt2^{−/−} null mutants exhibit decreased expression of genes critical for lung development, as assessed by Q-PCR at E14.5. Values represent the average of three samples run in triplicate ± SD. (U–X) Normal proximal-distal patterning in Wnt2^{−/−} null mutants, as assessed by SP-C and CC10 immunostaining. Scale bars in (O), (D), (F), and (G) represent 800 μm; those in (E), (H), (U), and (V) represent 200 μm; those in (W) and (x) represent 400 μm; those in (M) and (N) represent 600 μm; and those in (O)–(R) represent 100 μm.

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Figure 3. Wnt2b Signal through the β-Catenin-Dependent Canonical Pathway to Specify Lung Endoderm Progenitors in the Anterior Foregut

(A and B) Whole-mount staining of Wnt2−/−:BAT-GAL embryos for lacZ expression reveal a significant decrease in canonical Wnt signaling in the anterior foregut at E10.5 (arrows).

(C–F) Histological sections of wild-type BAT-GAL, Wnt2−/−:BAT-GAL, Wnt2b−/−:BAT-GAL, and Wnt2/2b:BAT-GAL DKO mutants show a significant loss of staining in the ventral aspect of the foregut endoderm in the region where the trachea is specified (brackets).
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 both 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 the 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 dkkopf1 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 derm1-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 ~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 the 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 noncanonical 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 the specification of lung endoderm progenitors in the foregut.

In contrast to previous studies in zebrafish that 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 and may have changed 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 Ctnnb1ex3flox;Shh-cre mutants is likely due to the timing and specificity of the Shh-cre line because we do not observe early activity in the liver (Figure S6). It is also important to note that because 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 to be important for both the 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

(G–N) Histological sections from (G–I) E10.5 wild-type and (K–M) Ctnnb1flox/flox;Shh-cre, 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 (J) E10.5, but not in (N) Ctnnb1flox/flox;Shh-cre mutants, indicating loss of lung specification in these mutants.

(O and P) (O) E-cadherin whole-mount immunostaining shows normal tracheal budding in wild-type embryos (arrow) and a lack of tracheal budding in (P) Ctnnb1flox/flox;Shh-cre mutants (arrow). However, the esophagus is still present in the (P) Ctnnb1flox/flox;Shh-cre mutants (bracket).

(Q–T) (Q and R) Fgf10 expression is substantially reduced in the ventral mesoderm surrounding the foregut in Wnt2/2b DKO mutants, whereas (S and T) Gli3 expression is unchanged at E10.0. Es, esophagus; Tr, trachea.

Scale bars in C–F represent 100 μm; those in G–N, Q, and R represent 200 μm; and those in S and T represent 150 μm.
Figure 4. Activation of Wnt/β-Catenin Signaling Leads to Expansion of Lung Endoderm Progenitors into the Stomach

(A–H) H&E-stained sections from (A–C) E10.5 wild-type and (E–G) Ctnnb1\(^{(ex3)flox}:Shh-cre\) mutants show that trachea-esophagus septation is disrupted in these mutants ([F], arrowheads). (D and H) Immunostaining for Nkx2.1 protein expression reveals expansion of Nkx2.1-positive lung endoderm progenitors into the stomach (outlined region).

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Wnt2/2b signaling acting dominantly to specify lung endoderm progenitors in the anterior foregut.

EXPERIMENTAL PROCEDURES

Generation of Wnt2 and Wnt2b Mutant Mice

Wnt2 mutant mice were generated by 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 by using the flip recombinase-expressing mouse (Fiper) from JAX and was confirmed by PCR (Figure S2). Analysis was performed with alleles containing and lacking the neomycin cassette. Wnt2b mutant mice were generated by using recombin-eeing techniques to insert loxP sites flanking exons 2 and 3. Two correctly targeted ES cells were used to generate chimeric mice that 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/6J:129S/J mixed background. Genotyping was performed with the oligonucleotides listed in Table S2. The generation and genotyping of Shh-cre, CMV-cre, Ctnnb1\textsuperscript{flox/flox}, BAT-GAL, and Ctnnb1\textsuperscript{flp/\textsuperscript{flox}} mice has 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 hr and embedded in paraffin for tissue sectioning. In situ hybridization and immunohistochemistry were 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 by 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 by using Trizol reagent, reverse transcribed by using SuperScript First Strand Synthesis System (Invitrogen), and used in quantitative real-time PCR analysis with the oligonucleotides listed in Table S2.

SUPPLEMENTAL DATA

Supplemental Data include six figures and two tables and can be found with this article online at http://www.cell.com/developmental-cell/supplemental/S1534-5807(09)00272-6.

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REFERENCES


I–P) H&E-stained sections from (I and J) E11.5 wild-type and (M and N) Ctnnb\textsuperscript{flox/flox}:Shh-cre mutants. Immunostaining for Nkx2.1 protein expression at E11.5 shows expression of Nkx2.1 in the esophagus of (O) Ctnnb\textsuperscript{flox/flox}:Shh-cre mutants (arrow), but not in (K) wild-type littermates (arrow). (P) Expression of Nkx2.1 is also extended into the stomach of E11.5 Ctnnb\textsuperscript{floxflox}:Shh-cre mutants (arrows). (Q–T) In contrast, expression of p63 is reduced in the esophagus and stomach endoderm of Ctnnb\textsuperscript{flox/flox}:Shh-cre mutants at E11.5. (U–V) Costaining for both Nkx2.1 and p63 show that p63-positive endoderm is lost, whereas Nkx2.1-positive endoderm is present in the esophagus. (W) A model showing the necessity and sufficiency of Wnt2/2b and β-catenin signaling for lung progenitor specification in the anterior foregut endoderm. Lb, lung bud; St, stomach.

Scale bars in A–I, M, K, O, Q, R, and U–V represent 200 μm; those in J, N, L, and P represent 400 μm; and those in S and T represent 30 μm.


