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Apc Deficiency Alters Pulmonary Epithelial Cell Fate and Inhibits Nkx2.1 via Triggering TGF-beta Signaling

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Abstract

Wnt signaling is critical for cell fate specification and cell differentiation in many organs, but its function in pulmonary neuroendocrine cell (PNEC) differentiation has not been fully addressed. In this study, we examined the role of canonical Wnt signaling by targeting the gene for Adenomatous Polyposis Coli (Apc), which controls Wnt signaling activity via mediating phosphorylation of beta-catenin (Ctnnb). Targeting the *Apc* gene in lung epithelial progenitors by *Nkx2.1-cre* stabilized Ctnnb and activated canonical Wnt signaling. Apc deficiency altered lung epithelial cell fate by inhibiting Clara and ciliated cell differentiation and activating Uchl1, a marker of neuroendocrine cells. Similar to PNEC in normal lung, Uchl1^{positive} cells were innervated. In mice with targeted inactivation of Ctnnb by Nkx2.1-cre, PNEC differentiation was not interrupted. These indicate that, after lung primordium formation, Wnt signaling is not essential for PNEC differentiation; however, its over-activation promotes PNEC features. Interestingly, Nkx2.1 was extinguished in Apc deficient epithelial progenitors before activation of Uchl1. Examination of Nkx2.1 null lungs suggested that early deletion of Nkx2.1 inhibits PNEC differentiation, while late repression does not. Nkx2.1 was specifically inhibited in Apc deficient lungs but not in Ctnnb gain-of-function lungs indicating a functional difference between Apc deletion and Ctnnb stabilization, both of which activate Wnt signaling. Further analysis revealed that Apc deficiency led to increased TGF-beta signaling, which inhibited Nkx2.1 in cultured lung endodermal explants. In contrast, TGF-beta activity was not increased in Ctnnb gain-of-function lungs. Therefore, our studies revealed an important mechanism involving Apc and TGF-beta signaling in regulating the key transcriptional factor, Nkx2.1, for lung epithelial progenitor cell fate determination.

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Introduction

Development of the mammalian lung represents a tractable model for analysis of signaling pathways crucial to cell fate determination & differentiation. In the mouse lung development commences around embryonic day 9.5 via outgrowth of anterior foregut endoderm into the surrounding mesenchyme. The enodermally-derived epithelial structure then undergoes repeated branching, controlled by epithelial-mesenchymal interaction, to build the architecture of the lung. During this process, both epithelial cells and mesenchymal cells undergo cell fate determination and differentiation to give rise to what is thought to be more than 40 distinct differentiated pulmonary cell types, specialized in carrying out the function of the mature lung (Morrisey and Hogan, 2010). The earliest epithelial progenitors in the lung express Nkx2.1, a homeodomain transcriptional factor (Minoo et al., 1999). Expression of Nkx2.1 is intimately linked to lung epithelial cell identity. The epithelial progenitors differentiate into multiple types of functionally specialized cells, among which the most abundant in the conducting airways are Clara and ciliated cells, and the most abundant in alveoli are alveolar type 1 and type 2 (AT1 and AT2) cells. The epithelial airways also contain neuroendocrine cells, identified by markers such as Uchl1, Cgrp, & Syp.

Pulmonary neuroendocrine cells (PNECs) are essential for lung function. Disruption of PNEC differentiation results in neonatal death due to respiratory failure (Borges et al., 1997). PNEC hyperplasia is observed in several pediatric lung diseases including BronchoPulmonary Dysplasia (BPD), Sudden Infant Death Syndrome (SIDS) and Congenital Central Hypoventilation Syndrome (CCHS) (Cutz et al., 2007a; Cutz et al., 2007b). To date, it is unclear whether PNECs originate from neural crest or from the same progenitor as other cells that comprise the airway epithelium (Ito et al., 1997). Lineage-tracing studies have shown that some PNECs are differentiated from ID2 expressing epithelial cells, whereas none of the PNECs seem to originate from epithelial cells expressing a 3.7kb human Spc promoter (Perl et al., 2002; Rawlins et al., 2009). It has been reported that Mash1 is required for PNEC differentiation (Ito et al., 2000).

Wnt signaling, mediated by beta-catenin (Ctnnb), is critical for normal organogenesis, stem cell renewal and tumorigenesis (Ling et al., 2009; Logan and Nusse, 2004; van Amerongen and Berns, 2006). The activity of Ctnnb is regulated by a protein complex, a major component of which is Adenomatous Polyposis Coli or Apc, a 310-kDa multifunctional protein (Polakis, 1997). In the absence of Wnt ligands, Ctnnb is phosphorylated at the destruction complex composed of Apc, Axin, and Gsk3beta, and is subsequently ubiquitinated and degraded. Deletion of Apc disrupts the destruction complex, which results in Ctnnb stabilization and activation of canonical Wnt signaling. Mutations of Apc or Ctnnb are frequently observed in patients with colorectal cancer. Experimentally, two approaches have been developed to hyper-activate canonical Wnt signaling in mice. These are deletion of Ctnnb exon 3 (Ctnnb gain-of-function) and deletion of Apc. Ctnnb exon 3 is the site of phosphorylation targets (S33, S37, T41, S45) that lead to its degradation. Both models have been used extensively and it is generally thought that the two approaches have similar functional consequences. (Harada et al., 1999; Kuraguchi et al., 2006; Oshima et al., 1995).

Functions of Ctnnb in the lung have been studied and found to be closely linked to cell fate specification (De Langhe and Reynolds, 2008). In gain-of-function studies, deletion of Ctnnb exon 3 by Ccsp-cre which targets mostly Clara cells leads to expansion of a lung stem cell pool. Using Nkx2.1-cre which targets epithelial progenitors early in lung morphogenesis, deletion of Ctnnb exon 3 was shown to result in cell fate changes that favored neuroendocrine features (Li et al., 2009). Deletion of Ctnnb exon 3 by Spc-cre which targets distal lung endoderm led to loss of bronchiolar epithelium and distal airway ectasis, a phenotype similar to human Congenital Cystic Adenomatous Malformation or

CCAM (Hashimoto et al.). Deletion of Ctnnb exon 3 by *Shh-cre* which targets foregut endoderm before the specification of the lung primordium resulted in expansion of ectopic Nkx2.1 expression into domains that include the esophagus and stomach endoderm, while Ctnnb loss-of-function by the same cre inhibited commitment of foregut endoderm to Nkx2.1^{positive} lung epithelial progenitors (Goss et al., 2009; Harris-Johnson et al., 2009). In addition, deletion of Ctnnb (loss-of-function) by an SpC-rtTA;tetO-cre strategy proximalized the lung (Mucenski et al., 2003). PNECs labeled by Cgrp, although reduced, were present in the mutant lungs. Since in lineage tracing studies, the epithelial cells expressing the latter Spc promoter (lung epithelial-specific) do not give rise to any PNECs (Perl et al., 2002), it remains to be clarified whether deleting Ctnnb in PNEC progenitors will affect PNEC differentiation.

In the current study, two major observations were made. First, epithelial Apc deficiency disrupted lung morphology and altered lung epithelial cell fate toward a neuroendocrine lineage, confirming a role of WNT signaling in epithelial cell fate specification. Second, Apc deficiency, but not Ctnnb exon 3 deletion, activated TGF-beta signaling, which in turn inhibited Nkx2.1 expression. These findings have a practical cautionary implication for studies in which Apc inactivation has been thought to functionally equate exon3-deletion-induced Ctnnb stabilization.

Results

Apc deficiency disrupts lung morphogenesis

To determine the function of *Apc* in lung development, we characterized the lungs of *Nkx2.1-cre; Apc$^{f/f}$* (*Apc$^{Nkx2.1}$* for abbreviation) mice. These mice were generated by crossing *Apc$^{f/f}$* mice in which exon 14 of the *Apc* gene is sandwiched by two loxP sequences to *Nkx2.1-cre* mice in which cre-recombinase is expressed in airway epithelial progenitors from early stages of lung development (Tiozzo et al., 2009). Levels of Apc protein were decreased in the mutant lungs as determined by western blot analysis (Figure 1). The *Apc$^{Nkx2.1}$* pups died perinatally with two obvious morphological defects in the lung and trachea. First, the *Apc$^{Nkx2.1}$* lungs showed severe dilation in lung parenchyma resembling the human neonatal condition known as CCAM (Morotti et al., 1999). In addition, polyp-like structures were formed in trachea, main-stem bronchi and intralobular proximal airways. Both defects were first observed in E14 embryos.

Hematoxylin and eosin (H&E) staining of E18 control and *Apc$^{Nkx2.1}$* lungs showed that dilation of the airways occurred uniformly in lung parenchyma with variations in size (Figure 1). Histological analysis of the polyps revealed that they were composed of a group of mesenchymal cells covered by a layer of epithelial cells. This structure was similar to that in *Nkx2.1-cre;Ctnnb$^{+/lox(ex3)}$* [*Ctnnb(ex3)$^{Nkx2.1}$* for abbreviation] lungs, that express a truncated/stabilized Ctnnb (exon 3 deletion) in embryonic lung epithelial progenitors (Li et al., 2009).

To determine the impact of *Apc* deficiency on canonical Wnt signaling, we first examined levels of Ctnnb, the target of Apc/Gsk3beta/Axin complex. As shown in Figure 1, Ctnnb was markedly increased in clusters of epithelial cells along the main-stem-bronchi (MSB) and intralobular airways. We also examined levels of Lef1, a transcriptional factor that is activated by and is involved in canonical Wnt signaling (Planutiene et al., 2011). As expected, Lef1 levels were increased in the epithelial cells with accumulated Ctnnb (Figure 1), indicating functionally increased canonical Wnt signaling activity.

Apc deficiency alters lung epithelial cell fate by inhibiting Clara and Ciliated cell differentiation and activating neuroendocrine markers

Canonical Wnt signaling is critical for cell fate specification in the lung (Goss et al., 2009; Harris-Johnson et al., 2009; Li et al., 2009). We therefore examined the impact of *Apc* deficiency on lung epithelial cell fate. During normal lung development, *Nkx2.1*^{positive} epithelial progenitors differentiate into Clara and ciliated cells, which account for the majority of epithelial cells along proximal airways (Figure 2A). As shown in Figure 2, in *Apc*$^{Nkx2.1}$ lungs, differentiation toward Clara and ciliated cells was inhibited in epithelial cells with accumulated Ctnnb (*Apc* deficiency). Instead, cell populations expressing high levels of Uchl1, a neuroendocrine cell marker were significantly expanded (Figure 2E vs. 2D) (Schofield et al., 1995).

To determine whether the expansion of Uchl1^{positive} cells in *Apc*$^{Nkx2.1}$ lungs was caused by increased cell proliferation, we examined phospho-H3 (p-H3). Figure 2 shows that the ratio of p-H3^{positive} cells in epithelial cells with Ctnnb accumulation was much lower than the ratio in epithelial cells of wild-type, control lungs. This finding was further validated by immunostaining with Ki67 which also revealed reduced cell proliferation in the epithelial cells with Ctnnb accumulation (supplemental data, Figure S1). Thus, increased population of Uchl1^{positive} cells in the mutant lungs was likely caused by cell fate changes, rather than over-proliferation of Uchl1^{positive} cells.

The Uchl1^{positive} cells are innervated

Within the airway epithelium, PNECs are innervated. Innervation is an important feature of PNECs and may be essential for their function as oxygen sensors (Adriaensen et al., 2003; Brouns et al., 2012). Immunofluorescent staining on serial sections across *Apc*$^{Nkx2.1}$ lungs with antibodies against Uchl1 and neuron-specific beta3-tubulin (Tubb3 or Tuj1) (McKenna and Lwigale, 2011), revealed that the Uchl ^{positive} epithelial layer was enriched in nerve fibers signifying neuronal innervation (Figure 3). Innervation of target tissue is regulated by nerve growth factor, NGF, which mediates axonal growth and branching in peripheral nervous system (Bachar et al., 2004). Expression of an isoform of NGF, NGF-beta, was significantly increased in *Apc*$^{Nkx2.1}$ lungs (2.2+/−0.6 folds).

Apc deficiency is not sufficient for complete neuroendocrine cell differentiation

The above experiments demonstrated that epithelial cells with Apc deficiency expressed a PNEC marker, Uchl1, and were innervated. To what extent *Apc* deficiency was capable of specifying cell fate commitment toward NEC lineage was addressed by determining the developmental expression pattern of Uchl1 and comparing that with the pattern of other NEC markers including Calcitonin Gene-Related Peptide, Cgrp and Synaptophysin, Syp. Syp is a synaptic vesicle glycoprotein that participates in synaptic transmission. In wild type E15 embryos, only Uchl1 was detectable in the PNECs. By E18, all three markers of PNEC differentiation, Uchl1, Cgrp and Syp were detectable in the wild type control lung (Figure 4). These data indicate that PNEC differentiation involves sequential activation of Uchl1 as an early marker, followed by Cgrp and Syp. In the mutant lungs, while Cgrp was readily detectable, Syp expression was never observed in epithelial cells with Ctnnb accumulation that were also Uchl1^{positive} (Figure 2). The increase in Cgrp was further confirmed by western blot analysis (Figure 4).

Ito et al reported that Mash1 is required for PNEC differentiation (Ito et al., 2000). In the present study Realtime PCR analysis indicated Mash1 expression in the mutant lungs was significantly increased in E18 (2.4+/− 0.4 folds), but not in E15 mutant lungs. Collectively, these data indicate that *Apc* deficiency may specify cell fate commitment to PNEC

phenotype (Uchl1, Mash1 and Cgrp), but is not sufficient to drive PNEC differentiation to its completion (expression of Syp).

Canonical Wnt signaling is not required for PNEC differentiation

Activation of canonical Wnt signaling via either Apc inactivation or Ctnnb stabilization by *Nkx2.1-cre* promotes PNEC cell fate commitment and partial differentiation (Li et al., 2009). Whether canonical Wnt signaling is necessary for PNEC cell differentiation was addressed by generating *Nkx2.1-cre; Ctnnb*$^{f/f}$ (*Ctnnb*$^{Nkx2.1}$ for abbreviation) mice in which exon 3 to 6 (Huelsken et al., 2001; Mucenski et al., 2003) of the Ctnnb gene are flanked by loxP sequences (Figure 5). Deletion of exon3 to 6 by recombination results in Ctnnb deficiency and inhibition of canonical Wnt signaling (Huelsken et al., 2001; Mucenski et al., 2003). Immunohistochemistry revealed that Ctnnb was depleted in airway epithelial cells of the mutant lungs. However, both Uchl1 and Cgrp were detectable in Ctnnb-depleted cells (Figure 5). Therefore, after lung primordium formation canonical Wnt signaling is not essential for differentiation of pulmonary neuroendocrine cells.

Nkx2.1 is required for neuroendocrine cell differentiation

As shown in Figure 5 (panels K and S), Ctnnb is depleted in *Ctnnb*$^{Nkx2.1}$ PNECs. Obviously this occurs due to activation of Nkx2.1-cre in these cells or their progenitors. To determine whether Nkx2.1 is required for PNEC differentiation, we characterized the lungs of *Nkx2.1* null (*Nkx2.1*$^{-/-}$) E18 mouse embryos (Kimura et al., 1996; Minoo et al., 1999). Deletion of Nkx2.1 blocked PNEC differentiation as represented by absence of Uchl1 and Cgrp (Figure 5).

Apc deficiency represses Nkx2.1

To further characterize the impact of Apc deficiency on lung epithelial cells, we examined Nkx2.1 expression in *Apc*$^{Nkx2.1}$ lungs. Interestingly, as shown in Figure 6, Nkx2.1 was undetectable in the mutant lung epithelial cells in which Ctnnb accumulated. To determine the timing of Nkx2.1 inhibition and Uchl1 activation in *Apc*$^{Nkx2.1}$ lungs, we compared the expression of Nkx2.1 and Uchl1 in E12 lungs. Nkx2.1 is undetectable in epithelial cells of E12 *Apc*$^{Nkx2.1}$ lungs in which accumulated Ctnnb was found (Figures 6B, 6D, 6F). Likewise, Uchl1 immunoreactivity was absent in E12 mutant lungs. Therefore, activation of Uchl1 occurs subsequent to repression of Nkx2.1. Collectively, the observations from the *Nkx2.1*$^{-/-}$ and *Apc*$^{Nkx2.1}$ lungs indicate that the role of Nkx2.1 in PNEC differentiation may be stage dependent (please see Discussion for details).

Wnt-independent function of APC represses Nkx2.1

Inactivation of Apc via *Nkx2.1-cre* that leads to hyper-accumulation of Ctnnb and consequent activation of canonical Wnt signaling also results in loss of Apc-specific functions that may not be connected to its regulation of Wnt signaling. To distinguish between the two functions in Nkx2.1 repression, we used both in vivo and ex vivo approaches. The in vivo analysis was conducted using the Ctnnb gain-of-function model, *Ctnnb(ex3)*$^{Nkx2.1}$, in which exon 3 of the Ctnnb gene was deleted and canonical Wnt signaling was activated in lung epithelial progenitors(Li et al., 2009). Activation of canonical Wnt signaling represented by accumulation of truncated Ctnnb (Figure 7H) failed to repress Nkx2.1 (Figures 7G, 7H, 7I). In fact, realtime PCR analyses revealed what appeared as increased Nkx2.1 in the mutant lungs (Figure 7N). The relationship between Nkx2.1 and canonical Wnt signaling was further examined ex vivo by mesenchyme-free lung endoderm. Treatment of wild-type endoderm with recombinant Wnt3a activated canonical Wnt signaling, as evidenced by increased Lef1 levels. Nkx2.1 levels however, remained largely unchanged (Figure 7). Therefore, both in vivo and ex vivo analyses

indicate that activation of canonical Wnt signaling per se does not repress Nkx2.1 and suggest a Wnt-independent mechanism for the observed repression of Nkx2.1 in $Apc^{Nkx2.1}$ lungs.

Apc deficiency activates TGF-beta signaling

Previous studies have demonstrated that Nkx2.1 is negatively regulated by TGF-beta1 (Napolitano et al., 2000; Xing et al., 2008). To determine whether TGF-beta signaling is involved in the inhibition of Nkx2.1 by *Apc* deficiency, we examined the $Apc^{Nkx2.1}$ lungs for expression of TGF-beta ligands as well as their functional downstream target, Pai-1. As shown in Figure 8, while TGF-beta3 showed a modest increase, the expression of TGF-beta2 was markedly increased in the mutant lungs (2.3+/−0.5 folds). The overall activity of TGF-beta signaling in the mutant lungs, as represented by Pai-1 expression, was also robustly increased (2.6+/−0.3 folds). To determine whether increased TGF-beta signaling spatially correlated with accumulation of Ctnnb (where Apc deficiency and Nkx2.1 depletion occur), we conducted co-immunostaining with antibodies against phospho-smad2 and Ctnnb. Phospho-smad2 was specifically increased in epithelial cells in which Ctnnb was accumulated (i.e. Apc deficient cells) (Figure 8).

The validity of the above findings was further tested in an ex vivo experimental model, in which we treated mesenchyme-free epithelial explants isolated from E12 wild-type embryonic lungs with recombinant TGF-beta2. The mesenchyme-free epithelial explants cultured in Matrigel, supplemented with FGF10 underwent budding morphogenesis (Figure 8F) and expressed Nkx2.1 (Figure 8G). Treatment of the explants with TGF-beta2 blocked Nkx2.1expression (Figure 8K), thus validating the in vivo findings. Interestingly, in addition to the impact on Nkx2.1, recombinant TGF-beta2 altered the morphology of the epithelial explants. Treatment with FGF10 alone stimulated budding of the epithelial explants, whereas treatment with FGF10 plus TGF-beta2 failed to form new buds. Histological analysis revealed that the epithelial explants cultured in presence of FGF10 and TGF-beta2 formed inwardly folded structures similar to the observed polyps in $Apc^{Nkx2.1}$ lungs (Figure 8L).

Differential impact of Apc deficiency and Ctnnb stabilization on Nkx2.1 expression and TGF-beta signaling activity

As shown by the above-described studies, Nkx2.1 was inhibited in $Apc^{Nkx2.1}$ but not in $Ctnnb(ex3)^{Nkx2.1}$ lungs (Figure 7). To determine whether the differential impacts of the latter two models were associated with or caused by difference in TGF-beta signaling, we examined the expression of TGF-beta ligands in $Ctnnb(ex3)^{Nkx2.1}$ lungs. Unexpectedly, mRNA levels of TGF-beta2 and TGF-beta3 were increased in both $Apc^{Nkx2.1}$ and $Ctnnb(ex3)^{Nkx2.1}$ lungs (Figure 9A). We therefore determined the functional TGF-beta signaling activity in the two models by measuring Pai-1 expression. Paradoxically, in the face of increased TGF-beta ligands, the level of Pai-1 mRNA in fact decreased in $Ctnnb(ex3)^{Nkx2.1}$ lungs, indicating that TGF-beta signaling activity is differentially regulated in $Apc^{Nkx2.1}$ versus the $Ctnnb(ex3)^{Nkx2.1}$ lungs (Figure 9A). In support of this hypothesis, increased phospho-Smad2 (p-Smad2) was only observed in $Apc^{Nkx2.1}$ (Figures 8B-8D) but not in $Ctnnb(ex3)^{Nkx2.1}$ lungs (Figures 9B-9D). Differential Smad2 phosphorylation was further confirmed by western blot analysis (Figures 9E-9G). Interestingly, total Smad2 was also increased in the $Apc^{Nkx2.1}$ lungs, suggesting that Apc deficiency may regulate levels of Smad2. Levels of phospho-JNK (p-JNK) were also increased in $Apc^{Nkx2.1}$, but not in $Ctnnb(ex3)^{Nkx2.1}$ lungs.

To further characterize the differential activation of TGF-beta signaling in the two models, we analyzed the expression of TGF-beta receptors Tgfbr1 (Alk5) and Tgfbr2 (TBRII). Real

time PCR analysis showed that both Tgfbr1 and Tgfbr2 were significantly decreased in *Ctnnb(ex3)*$^{Nkx2.1}$, but remained unchanged in *Apc*$^{Nkx2.1}$ lungs. Therefore, Apc deficiency and Ctnnb stabilization display different impacts on TGF-beta signaling activity accompanied by differential regulation of TGF-beta receptor expression.

Discussion

In this study, we investigated the potential role of Apc in lung epithelial progenitor cell fate determination and differentiation during development. We found that interruption of Apc led to disruption of lung morphogenesis, blocked Clara and ciliated cell differentiation and promoted commitment to PNEC cell differentiation. Further studies showed that while activation of canonical Wnt signaling promoted cell fate commitment to a PNEC phenotype, its inhibition after lung primordium formation was not sufficient to block PNEC differentiation. Importantly, Apc deficiency also caused inhibition of Nkx2.1 through increased TGF-beta signaling. This novel observation revealed a mechanism that connects Apc function with TGF-beta signaling and Nkx2.1, the key transcriptional factor for lung development and homeostasis. By comparing the Apc deficient lungs with that of Ctnnb gain-of-function model, it is clear that the two mutations function differently in regulating TGF-beta signaling activity and Nkx2.1 gene expression.

PNEC differentiation: the role of canonical Wnt signaling and Nkx2.1

The observations made in this study showed that inactivation of Apc and consequent activation of canonical Wnt signaling promotes PNEC fate commitment and differentiation. This is supported by the observation that in *Apc*$^{Nkx2.1}$ lungs, epithelial cells (originating from Nkx2.1^{positive} cells) with Ctnnb accumulation failed to differentiate into Clara or ciliated cells, but expressed the PNEC marker, Uchl1. The observation that PNECs in the wild-type lungs express higher levels of Ctnnb, compared to neighboring epithelial cells is consistent with the latter conclusion (Figure 2H). Importantly, in a previous study we also showed that activation of canonical Wnt signaling by Ctnnb gain-of-function led to a similar impact (Li et al., 2009). However, even though both Apc deficiency and Ctnnb stabilization lead to expansion of Uchl1 positive cells, neither pathway is sufficient to guide the cells to a fully differentiated state as evidenced by lack of Syp expression. This indicates that PNEC differentiation entails a more complex mechanism beyond simple activation of canonical Wnt signaling. In addition, as PNEC cell lineage is absent in *Nkx2.1* null lungs, we propose that Nkx2.1^{positive} cells may serve as progenitors of PNECs. Validation of this possible model requires the application of a cell fate analysis approach using a regulable cre, such as *Nkx2.1-creER*. Nevertheless, the results of the current study establish the critical role of early *Nkx2.1* expression in PNEC differentiation.

An important and entirely novel finding of the present study is the observation that lack of Apc activity represses Nkx2.1 gene expression in lung epithelial progenitors. Interestingly, this occurred before development of neuroendocrine features (expression of Uchl1, Cgrp and innervation). Nkx2.1 defines the origin of the pulmonary endodermal derivatives within the anterior foregut at E9.5, and throughout early lung morphogenesis (Minoo et al., 1999). Thus, Apc deficiency appears to reverse the early commitment of lung endodermal cells by silencing Nkx2.1 in their epithelial descendents. These Nkx2.1^{negative} cells served as progenitors and differentiated into Nkx2.1^{negative}Uchl1^{positive} cells in an environment of Apc deficiency in E15 embryos (Figure 6). By E18, the epithelial cells further differentiated and activated Cgrp, a late marker of PNEC differentiation program. Thus, these observations also indicate that, in presence of over-activated Wnt signaling, Nkx2.1 is not required for progression of PNEC differentiation, and, to some extent differentiation subsequent to specification of the lung primordium. Collectively, the results suggest a stage dependent role of Nkx2.1 in PNEC differentiation; early Nkx2.1 expression during lung primordial

specification is strictly necessary for PNEC cell fate specification and differentiation. Subsequent to this event, emergence of PNECs as observed in the $Apc^{Nkx2.1}$ lungs can occur in the absence of Nkx2.1 activity.

Nkx2.1 regulation by TGF-beta and Wnt signaling

Nkx2.1 is the earliest marker for lung epithelial progenitor identity and is essential for lung morphogenesis and cell differentiation (Minoo et al., 1999). During lung homeostasis, Nkx2.1 controls the expression of surfactant genes in alveolar type II cells, which is essential for reducing alveolar surface tension and host defense mechanisms. In humans, mutations of Nkx2.1 cause serious conditions including benign hereditary chorea, infant respiratory distress syndrome and congenital hypothyroidism (brain-lung-thyroid syndrome) (Carre et al., 2009). Recently, Nkx2.1 was identified as a dual function molecule in lung cancer. Therefore, mechanisms underlying Nkx2.1 gene regulation are critical for many aspects of lung biology.

In this study, we found that Nkx2.1 expression was inhibited in *Apc* deficient lungs, but not in Ctnnb gain-of-function lungs. Detailed analysis revealed that activity of TGF-beta signaling was specifically increased in $Apc^{Nkx2.1}$, but not in $Ctnnb(ex3)^{Nkx2.1}$ lungs. Thus suggesting that TGF-beta signaling, triggered by absence of Apc may be the mechanism by which Nkx2.1 inhibition is brought about. To verify this, we conducted ex vivo experiments using a mesenchyme-free lung endodermal explant model and found that TGF-beta2 indeed inhibited Nkx2.1 expression. This is consistent with cell culture data in which TGF-beta1 was found to inhibit Nkx2.1 at transcriptional level (Napolitano et al., 2000).

Understanding the functional relationship between TGF-beta signaling and Nkx2.1 may help to elucidate the mechanisms underlying a number of key biological processes in lung development, homeostasis and cancer. For example, TGF-beta exhibits dual functions in carcinogenesis (Wakefield and Roberts, 2002). In normal tissue, TGF-beta functions as tumor suppressor; disruption of TGF-beta signaling leads to tumor formation (Wakefield and Roberts, 2002). Conversely, TGF-beta is also known to promote invasion and metastasis of cancer cells, indicating an oncogenic function. Coincidently, Nkx2.1 also displays both tumor suppressive and oncogenic functions in lung cancer. While Nkx2.1 is commonly used as a diagnostic marker for lung adenocarcinomas (Jagirdar, 2008), it also serves as a suppressor of malignant progression in Kras and p53 initiated lung adenocarcinoma development (Winslow et al., 2011). The inhibitory effect of TGF-beta signaling on Nkx2.1 may have functional implications in the dual functions of Nkx2.1 and TGF-beta in lung adenocarcinoma. Nkx2.1 gene regulation is woefully complex and information regarding the identity of potential regulators remains scant. However, stabilization of Ctnnb (Ctnnb exon3 deletion) using *Shh-cre* in endodermal progenitors prior to specification of lung primordium within the anterior foregut was shown to promote differentiation toward lung epithelial progenitors and resulted in expansion of the Nkx2.1 expression domain into the digestive tract (Goss et al., 2009; Harris-Johnson et al., 2009). In support of this finding, Gilbert-Sirieix et al showed that Wnt signaling directly activates Nkx2.1 via TCF/LEF response elements located at −798/−792 within the Nkx2.1 promoter in human papillary thyroid carcinoma cells (Gilbert-Sirieix et al., 2011). Consistent with the latter reports, we found Nkx2.1 levels increased in $Ctnnb(ex3)^{Nkx2.1}$ lungs. Interestingly, in $Apc^{Nkx2.1}$ lungs, even though Apc deficiency caused activation of Wnt signaling, expression of Nkx2.1 was inhibited. Given the observation in the present study that Apc deficiency also activates TGF-beta signaling which inhibits Nkx2.1, the negative impact of TGF-beta signaling appears to be functionally dominant over the positive impact of canonical Wnt signaling on Nkx2.1 regulation. This implies a novel mechanism whereby interactions between TGF-beta and Wnt signaling determine the eventual outcome of Nkx2.1 gene regulation. Finally, the transcription factor Grainyhead-like 2, Grhl2 was reported to activate Nkx2.1 expression in

lung epithelial cells in culture (Varma et al., 2012). In addition, Nkx2.1 was found to positively regulate Grhl2, thereby forming a positive feed-back loop. However, as shown in the supplemental data (Figure S2), we found no significant changes in Grhl2 in $Apc^{Nkx2.1}$ epithelial cells with accumulation of Ctnnb. This indicates that inhibition of Nkx2.1 by Apc deficiency was not mediated by inhibition of Grhl2.

Differential impact of Apc deficiency and Ctnnb stabilization on lung epithelial cell fate

Apc and Ctnnb mutations are frequently observed in colorectal tumors (Morin et al., 1997). Experimentally, Apc deletion and Ctnnb exon3 deletion are the two widely used models for ectopic activation of canonical Wnt signaling. In this study, we show that these two models have distinct impacts on regulating Nkx2.1 expression. This difference is reflected in, and mediated by differential activation of the TGF-beta signaling pathway. Therefore, even though the latter two models share many common features, particularly, activation of canonical Wnt signaling, they are not functionally identical. Detailed mechanisms underlying this difference may involve the Ctnnb- or Wnt-independent functions of Apc, or potential functional differences between wild-type Ctnnb, stabilized in $Apc^{Nkx2.1}$ lungs, and the truncated Ctnnb in $Ctnnb(ex3)^{Nkx2.1}$ lungs. These remain to be determined.

Materials and Methods

Mouse Breeding and Genotyping

All animals were maintained and housed in pathogen-free conditions according to the protocol approved by The University of Southern California Institutional Animal Care and Use Committee (IACUC) (Los Angeles, CA, USA). *Nkx2.1-cre; Apc*$^{f/f}$ (***Apc***$^{\mathbf{Nkx2.1}}$) mice were generated by breeding the *Nkx2.1-cre* (Xu et al., 2008) and $Apc^{f/f}$ (Kuraguchi et al., 2006) mice on C57/BL6 genetic background. *Nkx2.1-cre; Ctnnb*$^{f/f}$ (***Ctnnb***$^{\mathbf{Nkx2.1}}$) mice were generated by breeding the *Nkx2.1-cre* (Xu et al., 2008) and $Ctnnb^{f/f}$ (Huelsken et al., 2001) mice on C57/BL6 genetic background. ***Nkx2.1***$^{-/-}$ mice were described previously (Minoo et al., 1999). *Nkx2.1-cre; Ctnnb[+/lox(ex3)]* [***Ctnnb(ex3)***$^{\mathbf{Nkx2.1}}$] mice were generated as described previously (Li et al., 2009). Genotyping of the transgenic mice were determined by PCR with genomic DNA isolated from mouse tails or embryo tissue as described (Hogan, 1994). The forward (F) and reverse primers (R) for transgenic mouse genotyping are listed below.

Nkx2.1-cre: (creF) 5′-TAA AGA TAT CTC ACG TAC TGA CGG TG-3′ and (creR) 5′-TCT CTG ACC AGA GTC ATC CTT AGC-3′; *Ctnnb (loss-of-function)*: (Forward) 5′-AAGGTAGAGTGATGAAAGTTGTT-3′ and (Reverse) 5′-CACCATGTCCTCTGTCTATTC-3′; *Ctnnb(ex3) (gain-of-function)*: (Forward) 5′-CAT TGC GTG GAC AAT GGC TAC TCA-3′ and (Reverse1) 5′-CTA AGC TTG GCT GGA CGT AAA CTC-3′ for mutant, (Reverse2) 5′-GGC AAG TTC CGC GTC ATC C -3′ for Wild-type; Apc: (Forward) 5′-GAGAAACCCTGTCTCGAAAAAA-3′ and (Reverse) 5′-AGTGCTGTTTCTATGAGTCAAC-3′.

The “Ctrl” lungs used in each experiment were from cre negative littermates of the corresponding mutants.

Realtime Polymerase Chain Reaction (qPCR)

Quantification of selected genes by Realtime PCR was performed using a LightCycler with LightCycler Fast Start DNA Master SYBR Green I Kit (Roche Applied Sciences, IN) as previously described (Li et al., 2005). Relative ratio of a target gene transcript between mutant and control lungs was calculated with the DDCt method (Li et al., 2009). All primers

for qPCR were designed by using the program of Universal ProbeLibrary Assay Design Center from Roche Applied Sciences (IN).

Western Blot

Protein extracts were prepared from embryonic lungs with RIPA buffer (Sigma, MO) and separated on 7% SDS-PAGE gels. Proteins were then blotted to the Immobilon-P transfer membrane (Millipore Corp.). The membranes were probed with specific antibodies and analyzed with the ECL western blot analysis system as described by the manufacturer (Amersham Biosciences, PA). Primary antibodies used are: rabbit anti Apc (Anaspec, CA); rabbit anti p-Smad2; rabbit anti Smad2/3, rabbit anti p-JNK, rabbit anti p-AKT (Cell Signaling); rabbit anti-a-tubulin (Abcam, MA). Antibodies for Ctnnb, Uchl1 and Cgrp are described in Immunofluorescent staining section.

Immunofluorescent staining

Immunofluorescent staining was performed as previously described (Li et al., 2009). Primary antibodies used are: mouse anti b-catenin (Transduction Laboratories, KY); rabbit anti-b-catenin (Anaspec, Inc); mouse anti-Nkx2.1 (Lab Vision, CA); rabbit anti-Uchl1 & rabbit anti-a-tubulin (Abcam, MA); goat anti CC10 (Santa Cruz,CA); mouse anti-Syp (Millipore, MA); rabbit anti-pro SpC (Seven Hills Bioreagents, OH); mouse anti beta3-tubulin, rabbit anti Cgrp and mouse anti beta4-tubulin (Sigma); mouse anti p-H3 (Cell signaling),

Proliferation assay

Proliferating cells in control or mutant lung sections were labeled with p-H3 or Ki67 antibody and the nuclei were counterstained with Dapi. The sections were then photomicrographed. The labeled cells as well as the total number of epithelial cells along the proximal airways, photomicrographed at 40x, were counted. Ratio of proliferating cells in about 300 epithelial cells was calculated. The average ratio was then calculated from at least 6 ratios for each tissue. Significance of the difference in proliferation ratios between control and mutant proximal airway epithelial cells was determined by Student's t test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Apc deficiency in lung epithelial progenitors promotes PNEC differentiation.
- Apc deficiency represses Nkx2.1, earliest marker of lung endodermal cell commitment.
- Apc deficiency activates TGF-beta signaling, which inhibits Nkx2.1 expression.
- Apc deficiency and Ctnnb exon 3 deletion have a differential impact on Nkx2.1.

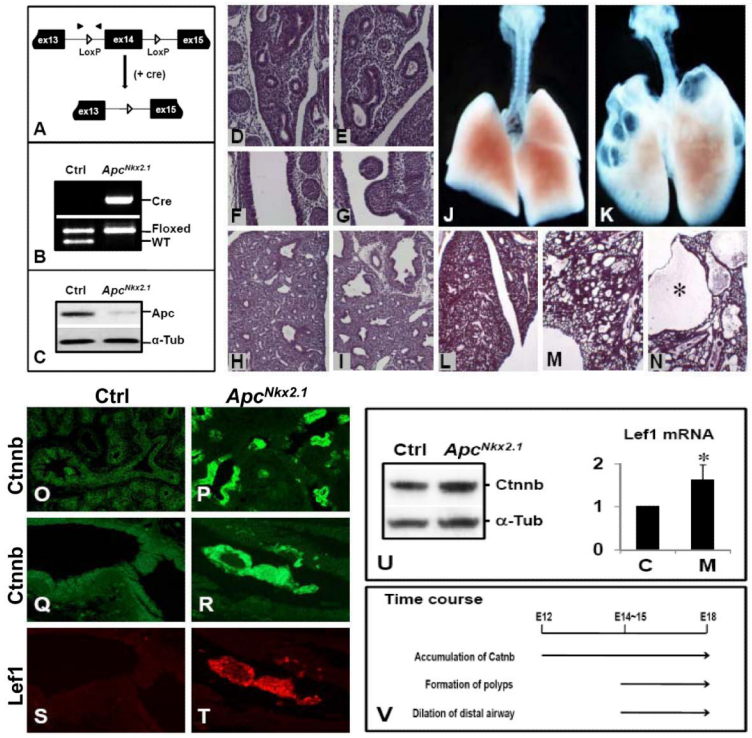

Figure 1.

(A) Illustration of exon 14 of the Apc gene that is flanked by the LoxP sequences. **(B)** Genotyping of $Apc^{Nkx2.1}$ embryos. **(C)** Western blot analysis shows that levels of Apc were decreased in $Apc^{Nkx2.1}$ lungs. **(D&E)** Histology of E12 control & $Apc^{Nkx2.1}$ lungs. **(F-I)** Histology of E15 control & $Apc^{Nkx2.1}$ trachea (F&G) and lungs (H&I). **(J&K)** Gross morphology of E18 control & $Apc^{Nkx2.1}$ lungs. **(L-N)** Histology of E18 control (L) & $Apc^{Nkx2.1}$ lungs (M&N). Panel N shows the severely dilated airspace (*). **(O&P)** Immunofluorescent staining of Ctnnb in control & $Apc^{Nkx2.1}$ lungs. **(Q-T)** Co-immunofluorescent staining of Ctnnb (green) and Lef1 (red) in control (Q&S) & $Apc^{Nkx2.1}$ (R&T) main-stem-bronchi (MSB) indicates increased Lef1 expression in epithelial cells with Ctnnb accumulation. **(U)** Increases of Ctnnb were shown by western blot analysis. Increases of Lef1 mRNA were shown by realtime PCR analysis. Asterisk indicates $p<0.05$. **(V)** Illustration of the time course of Ctnnb accumulation, polyp formation and airspace dilation in the $Apc^{Nkx2.1}$ lungs.

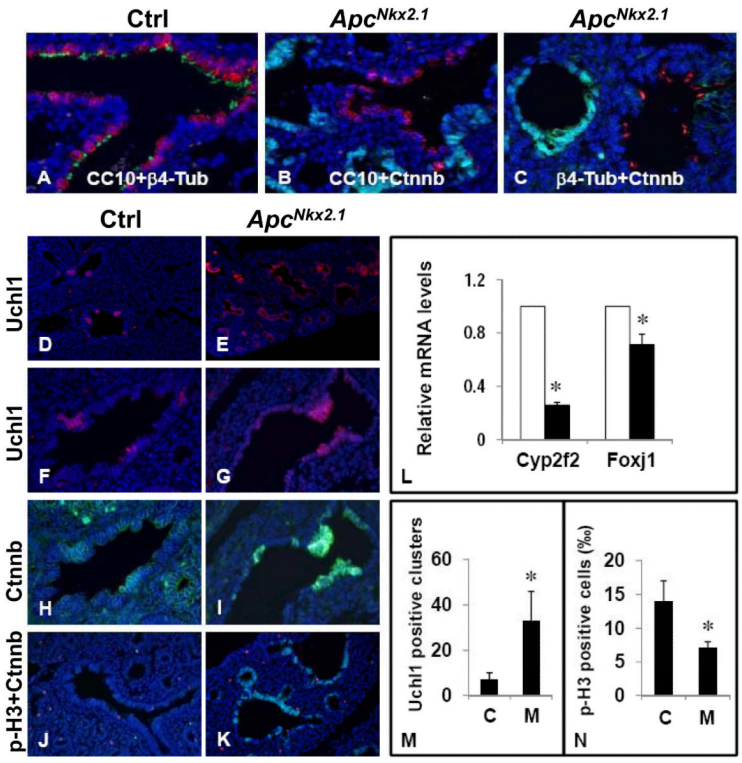

Figure 2.

(A) Co-immunofluorescent staining of CC10 (red, Clara cell marker) and beta4-tubulin (green, ciliated cell marker) in E18 control lungs. **(B&C)** Co-immunofluorescent staining of CC10 (red) & Ctnnb (green), and beta4-tubulin (red) & Ctnnb (green), respectively, in E18 $Apc^{Nkx2.1}$lungs. **(D&E)** Immunofluorescent staining of Uchl1 in E15 control & $Apc^{Nkx2.1}$ lungs, respectively. Please note the expansion of Uchl1 positive cell clusters in the mutant lungs (E). **(F-I)** High magnification of co-immunofluorescent staining of Uchl1 (red) and Ctnnb (green) in control & $Apc^{Nkx2.1}$lungs. **(J-K)** Co-immunofluorescent staining of p-H3 (red) and Ctnnb (green) in E15 control & $Apc^{Nkx2.1}$lungs. Nuclei are shown by Dapi (blue). **(L)** Relative levels of Cyp2f2 (Clara cell marker) and Foxj1 (ciliated cell marker) between $Apc^{Nkx2.1}$ (black bars) and control (white bars) lungs were determined by realtime PCR analysis. **(M)** Numbers of Uchl1 positive epithelial clusters in 0.7 mm^2. Data are averages of at least 10 different areas. **(N)** Ratio of p-H3 positive cells in epithelial cells with Ctnnb accumulation in $Apc^{Nkx2.1}$lungs (M) was reduced as compared to the ratio of p-H3 positive cells in normal lung epithelium (C). Asterisk indicates $p<0.05$.

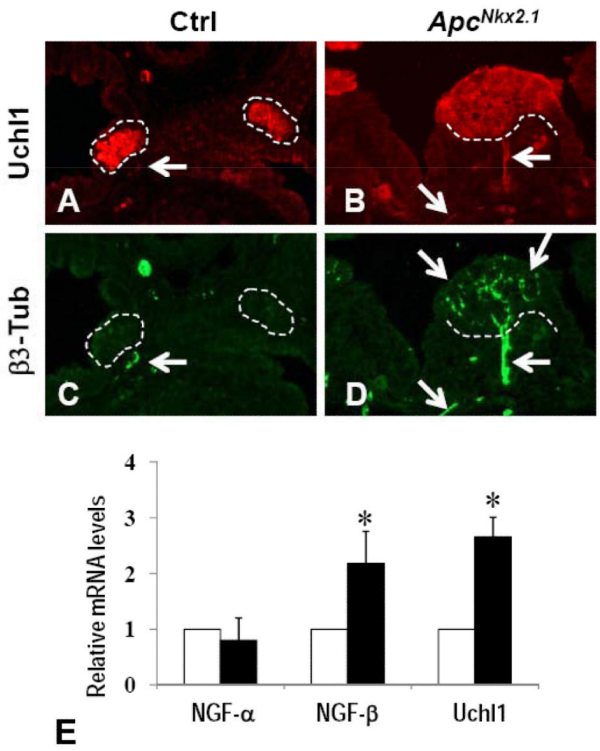

Figure 3.

(A-D) Co-immunofluorescent staining of Uchl1 (red) and beta3-tubulin (green) in E15 control and *Apc*$^{Nkx2.1}$ lungs. Arrowheads indicate nerve fibers. Dotted lines outline the junction between epithelium and mesenchyme. **(E)** Relative levels of NGF-alpha, NGF-beta, and Uchl1 between *Apc*$^{Nkx2.1}$ (close bars) and control lungs (open bars) were determined by realtime PCR analysis. Asterisk indicates $p<0.05$.

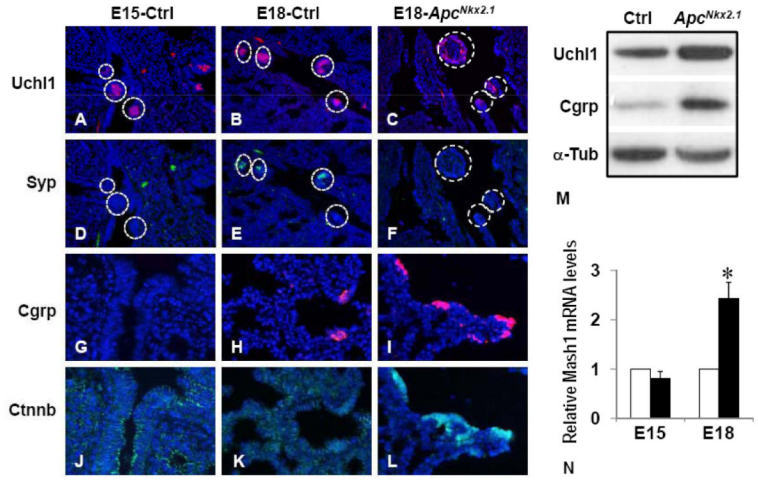

Figure 4.

(A-F) Co-immunofluorescent staining of Syp (green) and Uchl1 (red) in E15 (A&D) and E18 (B&E) control and E18 $Apc^{Nkx2.1}$lungs (C&F). **(G-L)** Co-immunofluorescent staining of Cgrp (red) and Ctnnb (green) in E15 (G&J) and E18 (H&K) control and E18 $Apc^{Nkx2.1}$lungs (I&L). **(M)** Increases of Uchl1 and Cgrp were shown by western blot analysis. **(N)** Increases of Mash1 mRNA were shown by realtime PCR analysis. Asterisk indicates $p<0.05$.

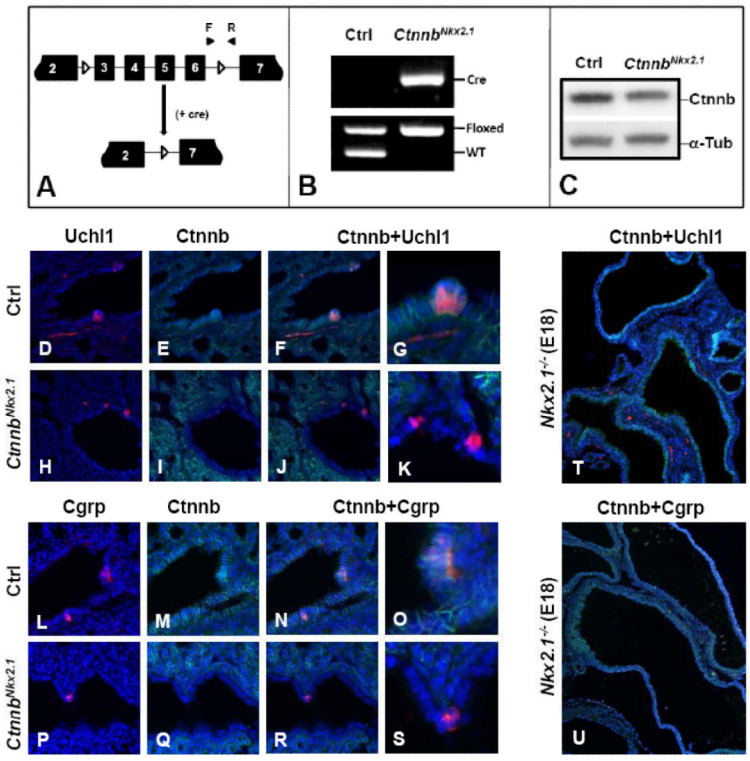

Figure 5.

(A) Illustration of deletion of exons 3-6 of the Ctnnb gene. **(B)** Genotyping of the *Ctnnb*$^{Nkx2.1}$ embryos by PCR. **(C)** Western blot analysis shows that levels of Ctnnb were decreased in E18 *Ctnnb*$^{Nkx2.1}$ lungs. **(D-K)** Co-immunofluorescent staining of Ctnnb (green) and Uchl1 (red) in E18 control and *Ctnnb*$^{Nkx2.1}$lungs. **(L-S)** Co-immunofluorescent staining of Ctnnb (green) and Cgrp (red) in E18 control and *Ctnnb*$^{Nkx2.1}$lungs. **(T)** Co-immunofluorescent staining of Ctnnb (green) and Uchl1 (red) in E18 *Nkx2.1*$^{-/-}$ lung. Results (not shown) from control lungs are similar to panels D-G. **(U)** Co-immunofluorescent staining of Ctnnb (green) and Cgrp (red) in E18 *Nkx2.1*$^{-/-}$ lung. Results (not shown) from control lungs are similar to panels L-O.

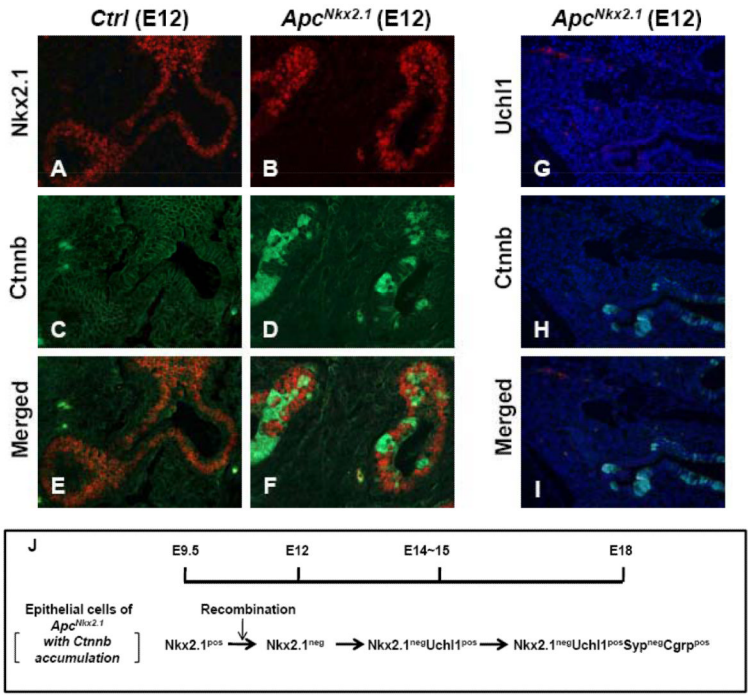

Figure 6.

(A-F) Co-immunofluorescent staining of Nkx2.1 (red) and Ctnnb (green) in E12 control and *Apc*$^{Nkx2.1}$ lungs. Please note that Nkx2.1 and Ctnnb accumulation (bright green) are mutually exclusive in *Apc*$^{Nkx2.1}$ lungs. **(G-I)** Co-immunofluorescent staining of Uchl1 (red) and Ctnnb (green) in E12 *Apc*$^{Nkx2.1}$ lungs. Nuclei are shown by Dapi (Blue). **(J)** Illustration of differentiation process of the *Apc* deficient epithelial cells.

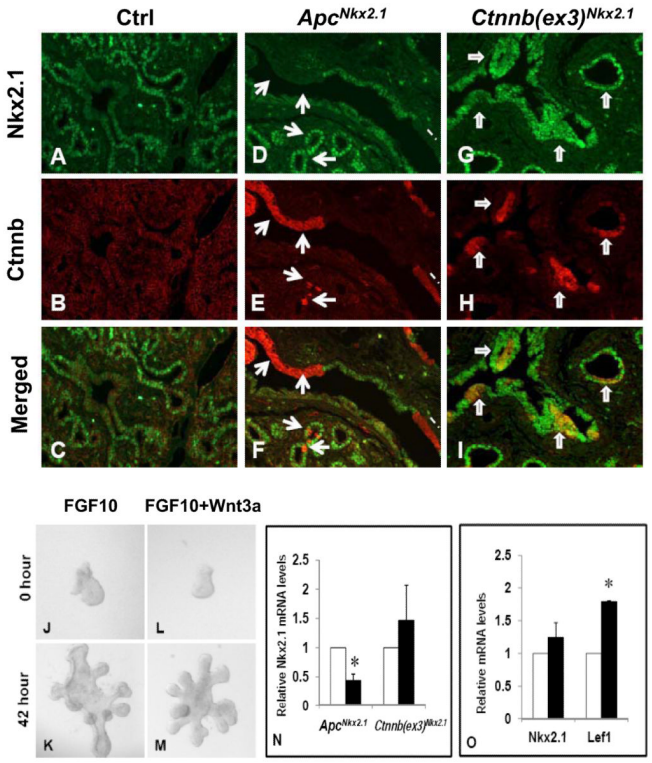

Figure 7.

(**A-I**) Co-immunofluorescent staining of Nkx2.1 (green) and Ctnnb (red) in E15 control (Ctrl), *Apc*$^{Nkx2.1}$ and *Ctnnb(ex3)*$^{Nkx2.1}$ lungs. Please note that Nkx2.1 and Ctnnb accumulation (bright red) are mutually exclusive in *Apc*$^{Nkx2.1}$ lungs (arrows, D-F), whereas they overlap in *Ctnnb(ex3)*$^{Nkx2.1}$ lungs (arrows, G-I). (**J-M**) Mesenchyme-free lung epithelial explants (wild-type) cultured in presence of FGF10 (J & K) or FGF10 plus Wnt3a (L & M). **(N)** Relative levels of Nkx2.1 in *Apc*$^{Nkx2.1}$ and *Ctnnb(ex3)*$^{Nkx2.1}$ lungs were determined by realtime PCR. **(O)** Relative levels of Nkx2.1 and Lef1 were compared by realtime PCR between explants cultured in FGF10 (white bars) and FGF10 plus Wnt3a (black bars). Asterisk indicates $p<0.05$.

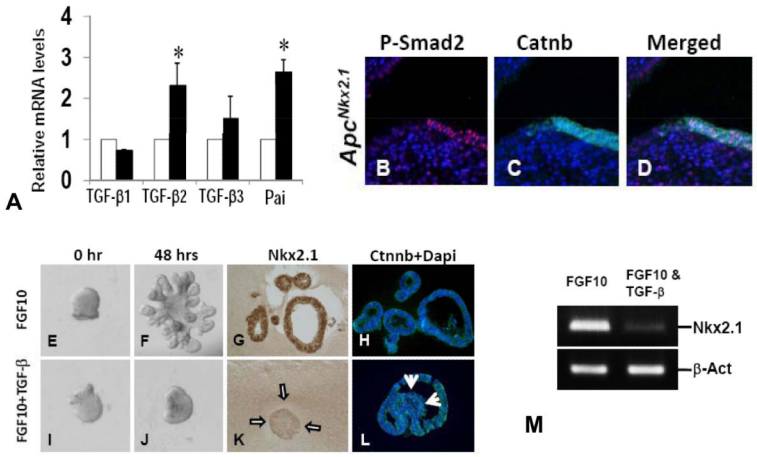

Figure 8.

(**A**) Relative mRNA levels of TGF-beta isoforms and Pai-1 in *Apc*$^{Nkx2.1}$ lungs as compared to the control lungs were determined by realtime PCR. **(B-D)** Co-immunofluorescent staining of Ctnnb (green) and p-Smad2 (red) in *Apc*$^{Nkx2.1}$ lungs. (**E-L**) Mesenchyme-free lung epithelial explants (wild-type) cultured in presence of FGF10 (E-H) or FGF10 plus TGF-beta2 (I-L). (**E, F, I & J**) Gross morphology of explants cultured for 0 or 48 hrs. (**G&K**) Immunostaining of Nkx2.1 in cultured explants. (**H&L**) Comparison of morphology of explants treated with FGF10 alone and FGF10 plus TGF-beta. Sections were stained with Ctnnb and Dapi for visualizing the morphology. (**M**) PCR products of Nkx2.1 and beta-actin (as control) amplified by realtime PCR from embryonic lung endoderm cultured with either FGF10 or FGF10 plus TGF-beta2. Asterisk indicates $p<0.05$.

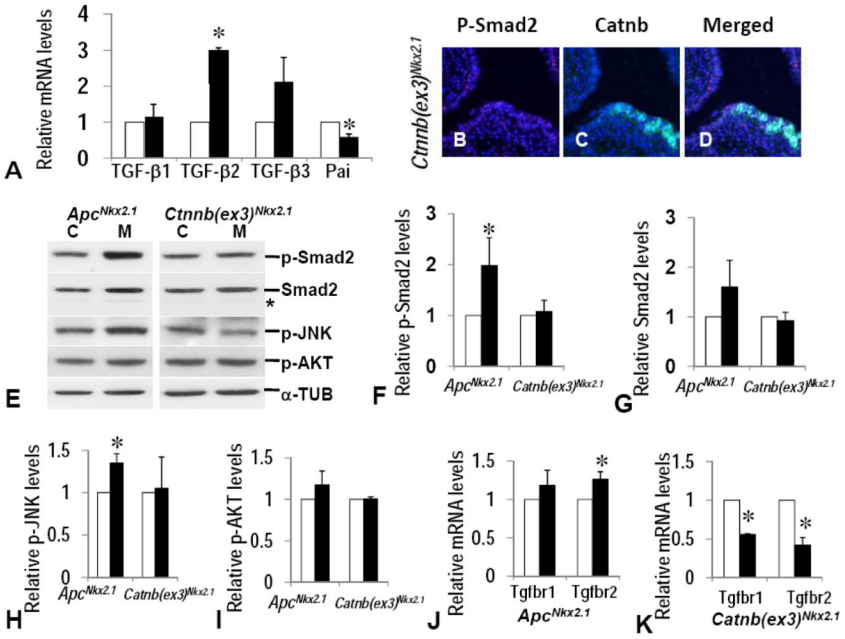

Figure 9.

(**A**) Relative mRNA levels of TGF-beta isoforms and Pai-1 in *Ctnnb(ex3)*$^{Nkx2.1}$ lungs as compared to the control lungs were determined by realtime PCR. **(B-D)** Co-immunofluorescent staining of Ctnnb (green) and p-Smad2 (red) in *Ctnnb(ex3)*$^{Nkx2.1}$ lungs. (**E-I**) Relative levels of p-Smad2, Smad2, p-JNK and p-AKT between mutant (close bars) and control lungs (open bars) were determined by western blot analysis. Asterisk in panel E indicates Smad3. (**J&K**) Realtime PCR analysis shows that levels of TGF-beta receptors were decreased in *Ctnnb(ex3)*$^{Nkx2.1}$, but not in *Apc*$^{Nkx2.1}$ lungs. Data represent means+/−SD of at least 3 independent experiments. Asterisk indicates $p<0.05$.

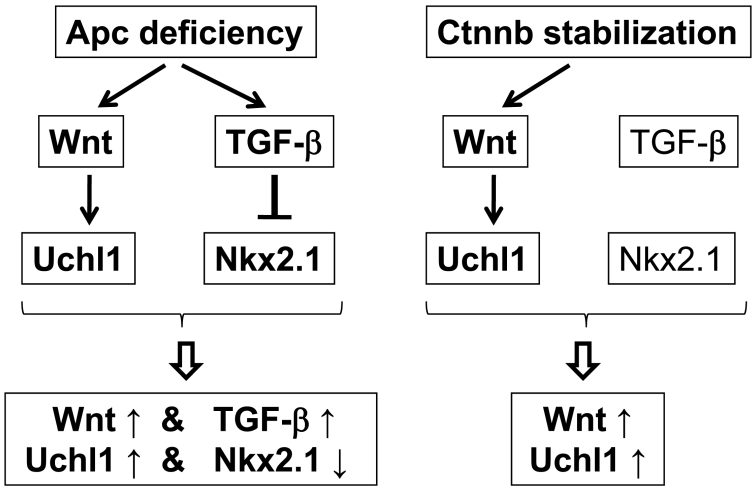

Figure 10.

Comparison and illustration of major impacts of Apc deficiency and Ctnnb gain-of-function in embryonic lungs. “Wnt” and “TGF- ” indicate corresponding signaling activities. “↑” indicates increase. “↓” indicates decrease.