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# Wnt ligand/Frizzled 2 receptor signaling regulates tube shape and branch-point formation in the lung through control of epithelial cell shape

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**Changing the morphology of a simple epithelial tube to form a highly ramified branching network requires changes in cell behavior that lead to tissue-wide changes in organ shape. How epithelial cells in branched organs modulate their shape and behavior to promote bending and sculpting of the epithelial sheet is not well understood, and the mechanisms underlying this process remain obscure. We show that the Wnt receptor Frizzled 2 (Fzd2) is required for domain branch formation during the initial establishment of the respiratory tree. Live imaging and transcriptome analysis of lung-branching morphogenesis demonstrate that Fzd2 promotes changes in epithelial cell length and shape. These changes in cell morphology deform the developing epithelial tube to generate and maintain new domain branches. Fzd2 controls branch formation and the shape of the epithelial tube by regulating Rho signaling and by the localization of phospho-myosin light chain 2, in turn controlling the changes in the shape of epithelial cells during morphogenesis. This study demonstrates the importance of Wnt/Fzd2 signaling in promoting and maintaining changes in epithelial cell shape that affect development of a branching network.**

Development of many epithelial-derived organs requires a process of bending, folding, and reorganization of a primitive epithelial sheet or tube to generate a functional 3D organ. The mammalian lung is derived from a simple endoderm tube through a complex series of morphological changes that generates the highly arborized airways required for postnatal respiration. In humans, the first 16 generations of branching are thought to be genetically hard-wired; this notion is supported by work on mouse lungs showing that the branching pattern across multiple mouse strains is highly reproducible (1, 2). Despite such insight, little is understood about the genetic control of the molecular and cellular mechanisms underlying branching morphogenesis in the lung.

The epithelial cells that line tubular branching networks can be thought of as a large planar epithelial surface that must undergo changes in cell morphology in specific subregions for proper branch formation to occur. Several pathways, including the Wnt signaling pathway, have been implicated in regulating epithelial cell behavior in a plane. Although the canonical Wnt signaling pathway regulates gene expression through nuclear translocation of β-catenin and its subsequent coactivation of LEF/TCF transcription factors, noncanonical Wnt signaling involves a less well-defined signaling network that leads to alterations in epithelial cell shape and cytoskeletal structure. Noncanonical Wnt signaling is known to regulate changes in epithelial cell shape in convergent–extension movements (3, 4) and bending of the neural plate (5), but whether this pathway regulates the development of branched organs is unknown.

In the current study we show that the Wnt receptor Frizzled 2 (Fzd2) plays a key role in regulating the epithelial cell behavior and tube morphology necessary for formation of new branch points during airway morphogenesis. Fzd2 is essential for regulating changes in epithelial cell shape and cell lengthening along the apical–basal axis which we show are critical for formation of new domain branch points and maintaining proper airway tube shape in the developing lung. Loss of Fzd2 leads to decreased apical expression of phospho-myosin light chain 2 (pMLC2) indicative of the decreased Rho signaling that is required for thickening of the lung epithelium before new branch formation. Importantly, activation of Rho signaling can rescue the loss of Fzd2 signaling during lung branching morphogenesis. Together, our data highlight a previously unappreciated mechanism in the formation of branched networks by the control of epithelial cell shape through Wnt signaling.

## Results

**Loss of Fzd2 in the Lung Epithelium Causes the Formation of Distal Cysts in the Lung.** Fzd2 is a Wnt receptor expressed at high levels in the developing lung epithelium and has been implicated in regulating epithelial differentiation downstream of Gata6 (6). To assess further the role of Fzd2 in the developing lung, we generated mice carrying the *Fzd2*$^{flox/flox}$ allele and crossed these mice into the *Shh*$^{cre}$ allele (7) to delete Fzd2 specifically in the lung epithelium (Fig. S1). Fzd2 expression in the *Shh*$^{cre}$:*Fzd2*$^{flox/flox}$ mutants is reduced efficiently in the lung epithelium by embryonic day (E) 12.5, as observed by in situ hybridization and quantitative real-time PCR (qPCR) (Fig. 1 *A* and *B* and Fig. S1*B*). Although initial lung formation occurs in *Shh*$^{cre}$:*Fzd2*$^{flox/flox}$ mutants, by E14.5 mutant lungs contain multiple large cysts in the distal regions of the lung (Fig. 1 *C* and *D*) that persist into adulthood.

In normal lung development, new lateral branches are added along a main founder branch in a stereotyped pattern in

### Significance

We generated a conditional mouse allele for the Wnt receptor Fzd2 and used it to assess the role of Fzd2-mediated Wnt signaling in the lung. Loss of Fzd2 specifically in the developing lung epithelium results in defects in domain branch-point formation which alter the primary branching program of the lung. We show that Fzd2 is required to sculpt the developing epithelium in the lung through activation of the small GTPase RhoA and control of epithelial cell shape. These results reveal the importance of Wnt/RhoA signaling in altering the shape of the developing epithelium of branched organs such as the lung. Such studies highlight the interconnectedness of signaling pathways during the formation of a branched network.

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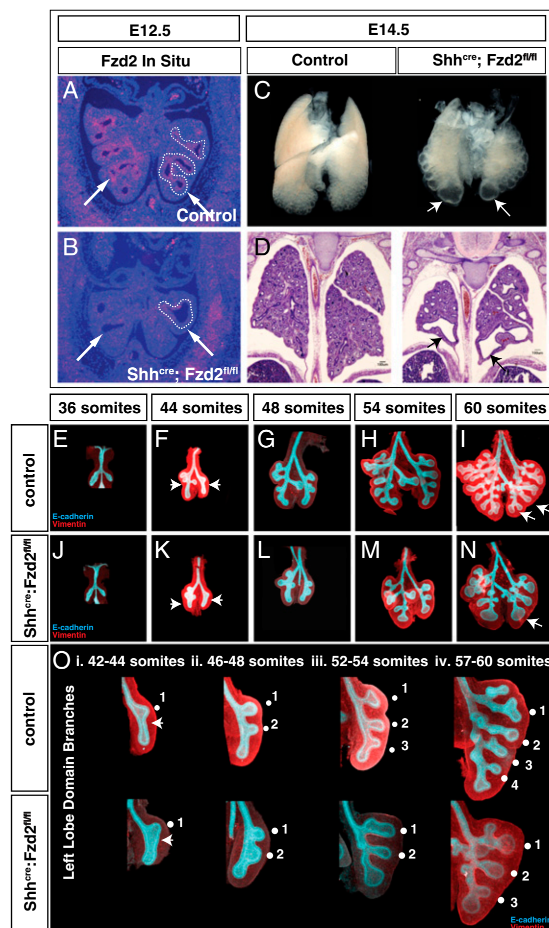


**Fig. 1.** Loss of Fzd2 in developing lung epithelium leads to formation of cysts in distal airways and defective branching morphogenesis. (*A* and *B*) Expression of Fzd2 in *Shh*$^{cre}$*:Fzd2*$^{flox/flox}$ mutants is decreased specifically in the developing lung epithelium at E12.5 (arrows and dotted outlines). (*C* and *D*) At E14.5, *Shh*$^{cre}$*:Fzd2*$^{flox/flox}$ mutants exhibit cysts in the distal airway region of the whole-mount lung (*C*, arrows) and in H+E-stained histological tissue sections (*D*, arrows). Controls in all experiments are *Shh*$^{cre}$ mice. (*E*–*N*) Whole-mount immunohistochemistry using vimentin to mark mesenchyme (red) and E-cadherin to mark epithelium (cyan) was used to characterize branching morphogenesis in control (*E*–*I*) and *Shh*$^{cre}$*:Fzd2*$^{flox/flox}$ mutants (*J*–*N*) from approximately E10.5 (36 somites) through E13.5 (60 somites). The development of cysts in the distal regions of *Shh*$^{cre}$*:Fzd2*$^{flox/flox}$ mutants correlates with a decrease in formation of domain branches (*I* and *N*, arrows). (*O*) The deficiency in domain branch formation is apparent by the 57–60 somite stage, as enumerated in *O*. (Scale bars: 500 μm.)

a process described as "domain branching" (2). To determine if there were defects in branching morphogenesis in *Shh*$^{cre}$*:Fzd2*$^{flox/flox}$ mutant lungs, we examined embryonic lungs from E10.5 to E12.5 using whole-mount immunostaining. At E11.5 (48 somites) defects in epithelial tube morphology are notable: The *Shh*$^{cre}$*:Fzd2*$^{flox/flox}$ mutant airways are shorter and wider than those in control lungs (Fig. 1 *F* and *G* and *J*–*L*). At E12.5 (54–60 somites), *Shh*$^{cre}$*:Fzd2*$^{flox/flox}$ mutant lungs have failed to undergo proper lateral domain branch formation, and large cysts have formed instead of new distal branches (Fig. 1 *H*, *I*, *M*, and *N*). The defects in tube morphology can be observed in the left lobe (arrowheads in Fig. 1*O*), and defects in new domain branch formation can be seen and quantified. Quantification of the branching defect reveals an approximate 50% decrease in new domain branch formation in *Shh*$^{cre}$*:Fzd2*$^{flox/flox}$ mutants (Fig. S2). These results suggest that distal cysts in the *Shh*$^{cre}$*:Fzd2*$^{flox/flox}$ mutants reflect a morphological defect in the initial branching program. In addition to defects in domain branch formation during the establishment of the respiratory tree, we also observe defects in later branching modalities, including planar bifurcation, which likely contribute to cyst formation.

**Loss of Fzd2 Does Not Change Wnt/β-Catenin Signaling Significantly in Lung Epithelium.** Fzd2 activates both β-catenin–dependent and –independent Wnt signaling, depending on cellular context (8–11). Therefore we evaluated the effect of loss of Fzd2 expression on canonical Wnt signaling in vivo using two Wnt reporter mouse lines and qPCR for endogenous Wnt target genes (12, 13). *Shh*$^{cre}$*:Fzd2*$^{flox/flox}$*:TOPGAL* mutant lungs did not exhibit an appreciable change in LacZ histochemical staining compared with control lungs (Fig. S3 *A*–*F*). qPCR for known Wnt targets in the developing lung did not reveal a significant change in canonical Wnt signaling activity (Fig. S3*G*). These findings are consistent with a previously reported global Fzd2-null allele that demonstrated that the loss of Fzd2 does not significantly affect canonical Wnt/β-catenin signaling (8). Previous work established a role for Wnt/β-catenin signaling in regulating cell-fate decisions in the lung during development (14–16). However, we do not observe any change in proximal-to-distal patterning or cell differentiation with the loss of Fzd2 (Fig. S4). These data suggest that Wnt/Fzd2 signaling has a previously unappreciated role in regulating morphological changes independent of Wnt/β-catenin signaling or cell specification.

**Loss of Fzd2 Disrupts the Molecular Branching Program of the Lung.** To assess further the molecular changes that underlie the branching defects described above, we determined the changes in the transcriptome of *Shh*$^{cre}$*:Fzd2*$^{flox/flox}$ mutant lungs at E12.5. These data revealed changes in signaling factors known to regulate lung branching morphogenesis, including Fgf10, Bmp4, Fgfr2, and Shh (Fig. 2 *A* and *B* and Dataset S1). Previous work has proposed a model for lung branch formation that includes roles for Fgf10 signaling as well as Bmp4 and Shh (Fig. 2*C*) (17, 18). This model describes a role for Fgf10 in establishing the site of new bud formation, although recent work has questioned whether focal Fgf10 expression is required for proper branching morphogenesis to occur (19). Furthermore, it remains unclear how Fgf10 mediates changes in cell behavior to give rise to new buds. In situ hybridization analysis shows that Fgf10 expression is elevated and its domain is expanded in *Shh*$^{cre}$*:Fzd2*$^{flox/flox}$ mutant lungs at E12.5 (Fig. 2 *D* and *H*). In contrast, the pattern of Fgfr2b, Bmp4, and Shh expression is not altered in *Shh*$^{cre}$*:Fzd2*$^{flox/flox}$ mutants (Fig. 2 *E*–*G* and *I*–*K*). We assessed a number of described downstream effects of Fgf10 signaling (20–22) in *Shh*$^{cre}$*:Fzd2*$^{flox/flox}$ mutant lungs and found no significant change in proliferation, orientation of cell division (Fig. S5), or Sprouty2 expression (Fig. 2*B*). To determine whether the decrease in Fgfr2 expression in *Shh*$^{cre}$*:Fzd2*$^{flox/flox}$ mutants could reduce sensitivity to Fgf ligands, we exposed control and *Shh*$^{cre}$*:Fzd2*$^{flox/flox}$ mutant lung buds to Fgf10-soaked beads and measured epithelial growth toward these beads. The epithelium from lung buds in *Shh*$^{cre}$*:Fzd2*$^{flox/flox}$ mutants extends toward the Fgf10 beads, but the mutant airways had an increased diameter and a less distinct tubular morphology than the control lung buds (Fig. S6). These results demonstrate that Fzd2-deficient lung epithelium is able to respond to the chemoattractant effects of Fgf10 but exhibits a defect in the ability of the epithelium to organize and maintain a distinct tubular shape while extending toward a focal region of Fgf10 expression; these findings are consistent with the defects observed in Fzd2-deficient lung epithelium during development (Fig. 1 *M* and *N*).

**Fzd2 Is Required for Maintaining Tube Dimension and New Branch Formation.** Using *Shh*$^{cre}$*:R26R*$^{mTmG}$ control and *Shh*$^{cre}$*:Fzd2*$^{flox/flox}$*:R26R*$^{mTmG}$ mutants to label the lung epithelium with GFP upon recombination driven from the *Shh*$^{cre}$ allele, we imaged newly forming domain branches using a lung explant model system (23). At the time of explantation on E11, the two major airways, or bronchi, of the lung have formed initial buds corresponding to the five lobes of the lung (Fig. 3*A*). Over the course of the next 18 h

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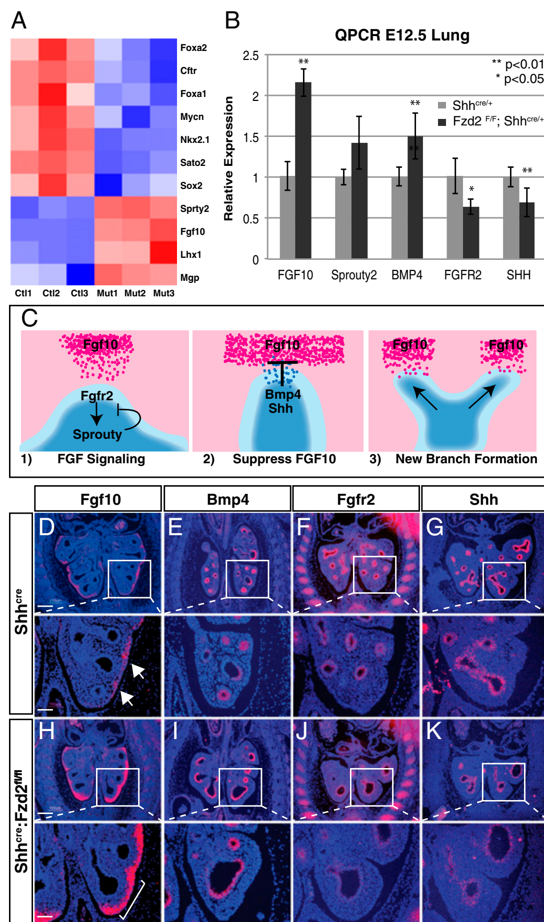


Fig. 2. Fzd2 regulates the components of the signaling niche that controls branching morphogenesis. (*A*) Microarray analysis reveals disruption in the expression of multiple signaling factors, including Fgf10, that are important for branching morphogenesis. (*B*) qPCR analysis of multiple components of the branching signaling niche reveals increased expression in Fgf10 and Bmp4 and decreased expression of Fgfr2 and Shh. (*C*) Diagram showing how the Fgf10, Bmp4, and Shh pathways are thought to interact to control branching morphogenesis; Fgf10 induces the outgrowth of a new bud, and Bmp4 and Shh inhibit Fgf10 activity to form a cleft, leading to bifurcation and two new bud points. (*D–K*) In situ hybridization showing expanded and increased Fgf10 expression, denoted by the bracket in *H* as compared to the arrows in *D*, increased Bmp4 expression, and decreased Fgfr2 and Shh expression in control and $Shh^{cre}$:$Fzd2^{flox/flox}$ mutants). (Scale bars: 100 μm.)

these buds grow and extend from the main bronchi as the more proximal region of the bud narrows and constricts (Fig. 3 *A* and *B*). During this time period new domain branches form in the right caudal and left lobes (Fig. 3*A*, arrowheads). A kymograph of the control left lobe over the 18-h period shows distinct outgrowth at the bud tips and at the first and second domain branches (Fig. 3*A*). In contrast to the control lung, $Shh^{cre}$:$Fzd2^{flox/flox}$:$R26R^{mTmG}$ mutants exhibit reduced bud outgrowth and decreased constriction along the epithelial tube, and the main lobes of the lung increase in diameter but not in length (Fig. 3 *A* and *B*). This growth pattern leads to a failure to form new domain branches in $Shh^{cre}$:$Fzd2^{flox/flox}$:$R26R^{mTmG}$ mutant lungs (Fig. 3*A*, brackets).

We measured branch growth in the explant cultures and found that the extension of the airway epithelial tube and newly formed branches is decreased significantly in the $Shh^{cre}$:$Fzd2^{flox/flox}$:$R26R^{mTmG}$ mutants as compared with control (Fig. S7). These live-imaging experiments demonstrate that loss of Fzd2 affects the maintenance of epithelial tube morphology, new bud formation, and branch extension.

**Formation of New Branch Points Requires Epithelial Thickening and Changes in Epithelial Cell Shape.** During our analysis of early branching, we observed that the epithelium undergoes a number of stereotyped changes before the formation of new branches. At the site of new bud formation, the lung epithelium thickens, and the epithelial tube begins to alter its shape (Fig. 3*C*, *i*). Then a distinct bend in the epithelium forms (Fig. 3*C*, *ii*), and the newly formed bud grows away from the main epithelial tube (Fig. 3*C*, *iii* and *iv*). To visualize the changes in shape of individual cells that occur during domain branching, we generated $Shh^{creERT2}$:$R26R^{mTmG}$ mice to label individual cells in the developing epithelium of the lung (Fig. 3*D*). Using this system, we

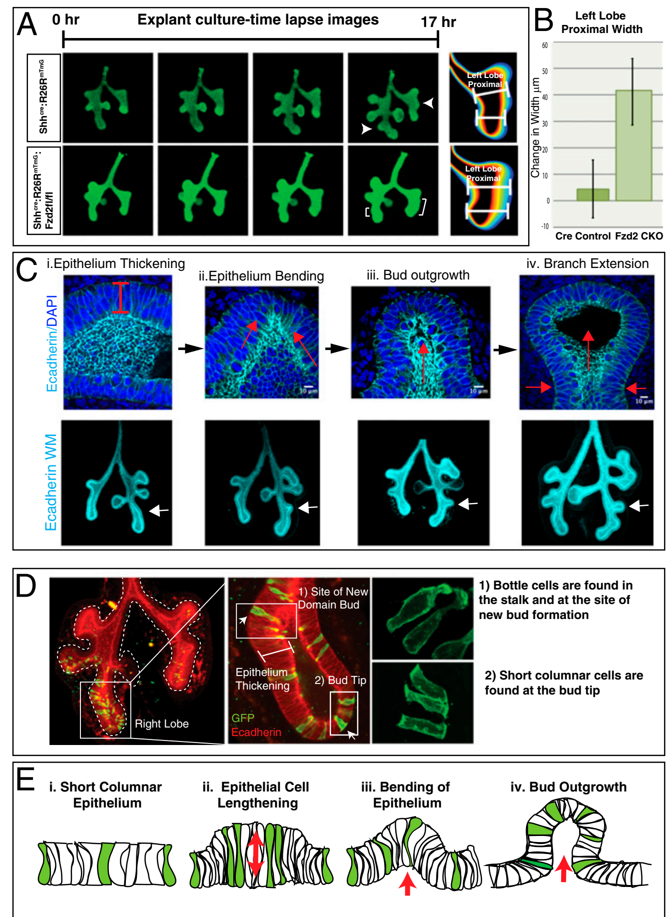


Fig. 3. Real-time imaging and single-cell analysis show the cellular and morphological changes required for new branch formation. (*A* and *B*) Real-time imaging of control and $Shh^{cre}$:$Fzd2^{flox/flox}$ mutants ex vivo shows that although control lungs form new domain branch points through extension at predictable sites of bud formation, $Shh^{cre}$:$Fzd2^{flox/flox}$ mutant lung epithelium expands without bending of the epithelium, leading to a wider airway tube. (*C*) At sites of new domain branch-point formation, the epithelium first thickens (*C*, *i*), then buckles at a distinct point (*C*, *ii*), and then buds and extends away from the founder epithelial tube (*C*, *iii*); the epithelial tube constricts at the base of this new branch as extension proceeds (*C*, *iv*). (*D*) Using the $Shh^{creERT2}$:$R26R^{mTmG}$ reporter to mark individual epithelial cells, we found that epithelial cells at sites of new branch points have a broad basal surface and an elongated and narrow apical region. Conversely, epithelial cells at the growing tip are more columnar and shorter with fairly equivalent apical and basal surface areas. (*E*) Diagram showing how the epithelial sheet that composes the developing lung airways deforms at sites of new branch-point formation: Epithelial cells lengthen along the apical–basal axis, creating a bend in the epithelial sheet. This process results in the formation of new bud or branch point in the epithelial tube. As the new bud extends, the cells in the bud tip once again adopt a short columnar morphology.

observed two spatially distinct cell morphologies in the developing epithelium at E11.5: bottle-shaped cells located in both the stalk region of the tube and at sites where new domain branch points form and short columnar cells located at the bud tip (Fig. 3*D*). Although these are the two most distinct cell shapes observed in the epithelium, we also observed mitotic cells, elongated columnar cells, and epithelial cells adjacent to the mitotic cells (Fig. S8) throughout the epithelium. Quantification of the appearance of labeled cells in distinct regions of the epithelium demonstrates an increased frequency of bottle cells with basally localized nuclei at sites of new bud formation (Fig. S8).

The presence of bottle cells and apical–basal cell lengthening are common features of folding epithelial sheets as seen in gastrulation in *Xenopus* (24, 25), neural tube bending (26, 27), optic vesicle formation (28), and *Drosophila* salivary gland development (29). Consistent with these models, at the site of new bud formation the lung epithelium thickens and adopts a pseudostratified morphology, suggesting that the epithelial thickening is the result of epithelial cell lengthening (Fig. 3*C*, *i* and Fig. S8). Based on these observations, we propose a model in which the lung epithelium bends to produce new domain branches from the epithelial tube. At the region where a new bud will form, lung epithelial cells lengthen along their apical–basal axes, causing a thickening of the epithelium, and this process, with the resulting change in cell morphology, is required for the bending of the epithelial tube (Fig. 3*E*).

**Wnt/Fzd2 Is Required for Sculpting New Domain Branch Points by Promoting Apical–Basal Lengthening Through the RhoA Pathway.** Using the model described above, we evaluated lung epithelial cell behavior and shape in $Shh^{cre}:Fzd2^{flox/flox}$ mutants. Using ImageJ, we determined the apical surface area by outlining and quantifying the apical-most surface of E-cadherin–stained epithelial cells. $Shh^{cre}:Fzd2^{flox/flox}$ mutant cells had an increased apical surface area, suggesting a failure to undergo cell shape remodeling at the apical surface (Fig. 4 *A*–*C*). Moreover, in contrast to what is observed in the control lungs, $Shh^{cre}:Fzd2^{flox/flox}$ epithelium does not thicken at predicted sites of new branch formation (Fig. 4 *D*–*F*). E-cadherin immunostaining was used to outline individual lung epithelial cells in both control and $Shh^{cre}:Fzd2^{flox/flox}$ mutants, and cell shape, length, and volume were assessed (Fig. 4*G*). As expected, control lung epithelial cells exhibit bottle-cell morphology at sites of new branch-point formation (Fig. 4*H*), elongated columnar morphology in the stalk region, and short columnar morphology in the bud tip (Fig. S9). In contrast, epithelial cells in $Shh^{cre}:Fzd2^{flox/flox}$ mutants displayed a squat columnar shape at the bud tip and at sites of new bud formation (Fig. 4*I* and Fig. S9). These changes did not affect the overall cell volume of $Shh^{cre}:Fzd2^{flox/flox}$ mutant cells significantly but did result in a 30% decrease in apical–basal cell length (Fig. 4 *J* and *K*). These results demonstrate that, upon loss of Fzd2, epithelial cells fail to lengthen along the apical–basal axis and adopt a bottle-cell morphology; as a result, the epithelial sheet fails to thicken and bend at the region of new branch formation.

To define further the underlying mechanism by which Fzd2 regulates the formation of new branch points, we examined our transcriptome analysis for pathways and factors that regulate cell shape and cytoskeletal organization. Notably, we found that expression of Celsr1 and Arhgef19, two factors implicated in affecting the noncanonical Wnt signaling via the Rho pathway (5, 30), was decreased in $Shh^{cre}:Fzd2^{flox/flox}$ mutant lungs (Fig. 5 *A* and *B*). To evaluate further whether changes in the Rho pathway could be changed with loss of Fzd2, we evaluated the expression of pMLC, a downstream target of Rho signaling (31). In control lung epithelium we found that apical pMLC2 expression was increased in regions undergoing bending as compared with the bud tip (Fig. S10), indicating increased apical RhoA activity at sites of new bud formation. In contrast, we found that pMLC2 levels were decreased at the apical surface of Fzd2-deficient lung epithelium (Fig. 5 *C*–*E*); this finding is consistent with the decreased epithelial RhoA signaling activity in $Shh^{cre}:Fzd2^{flox/flox}$ mutant lungs.

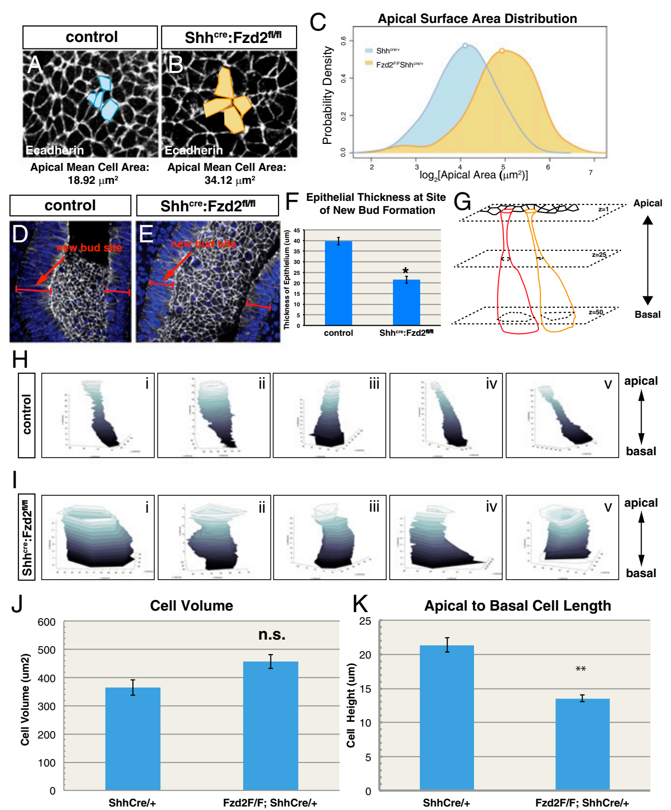


**Fig. 4.** Loss of Fzd2 causes defects in epithelial cell shape. (*A*–*C*) The average apical surface area was increased in $Shh^{cre}:Fzd2^{flox/flox}$ mutant lung epithelial cells as measured by outlining the apical surface delineated by E-cadherin immunostaining. (The apical region is diagrammed in *G*). (*D*–*F*) The epithelial thickening observed in control lungs at sites of new branch-point formation was absent in $Shh^{cre}:Fzd2^{flox/flox}$ mutant lungs. (*G*) To visualize control and $Shh^{cre}:Fzd2^{flox/flox}$ mutant lung epithelial cells individually, confocal images of E-cadherin–stained epithelium were analyzed using EDGE (44) as diagrammed. (*H* and *I*) Representative images of individual tracings of five control (*H*) and $Shh^{cre}:Fzd2^{flox/flox}$ mutant (*I*) lung epithelial cells are shown. (*J* and *K*) Although overall cell volume was not changed (*J*), apical–basal cell length (*K*) was reduced in $Shh^{cre}:Fzd2^{flox/flox}$ mutants. n.s., not significant. $**P < 0.001$.

Previous reports have suggested that Rho kinase (ROCK) and pMLC inhibitors can affect branching morphogenesis in lung explants (23, 32, 33). Therefore we evaluated whether inhibiting Rho effectors caused changes in lung epithelium at the cellular and tissue level similar to those we observed with loss of Fzd2. We found that inhibition of ROCK by fasudil (34) and of pMLC2 by ML7 (23) caused defects in branching morphogenesis and an increase in cyst formation similar to those seen in $Shh^{cre}:Fzd2^{flox/flox}$ mutants (Fig. 5*F*). On the cellular level, lung explants treated with fasudil and ML7 had lung epithelium with greatly expanded regions of short columnar epithelial cells (Fig. S11), with cell morphology similar to that observed with loss of Fzd2 (Fig. 5 *G*–*J*). To determine whether activation of RhoA signaling could rescue some or part of the $Shh^{cre}:Fzd2^{flox/flox}$ mutant phenotype, we treated $Shh^{cre}:Fzd2^{flox/flox}$ mutant lung explants with the RhoA activator, calpeptin (35). Treatment of $Shh^{cre}:Fzd2^{flox/flox}$ mutant explants with calpeptin rescued significant aspects of the phenotype in 67% of the explants (Fig. 5 *J* and *K*). In the calpeptin-treated explants, the number of branch tips exhibiting a cystic phenotype was reduced, and we observed an increase in the number of new domain branches and new bud tips added over a 24-h period (Fig. S12). Epithelial cells in the calpeptin-treated $Shh^{cre}:Fzd2^{flox/flox}$ lung epithelium regained their bottle-cell morphology as observed in control DMSO-treated explants (Fig. 5 *H*, *i* and *K*, *i*). Taken together, these data reveal a molecular pathway by

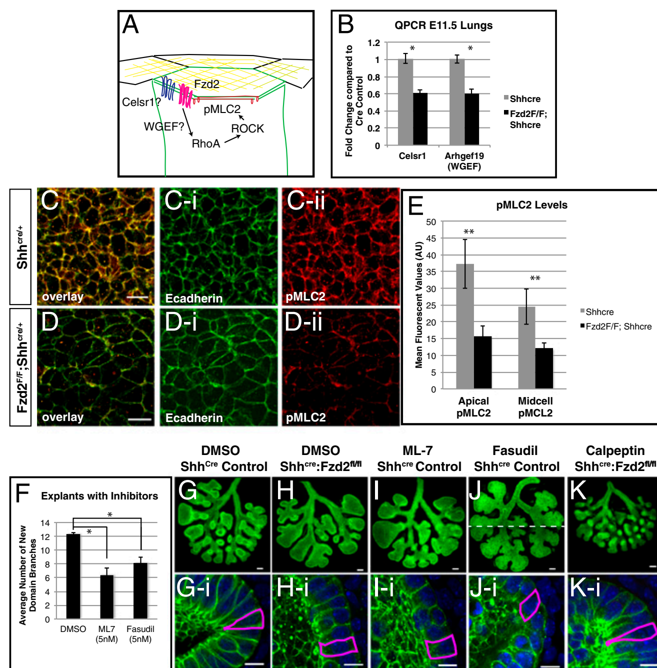


**Fig. 5.** Changes in the RhoA/pMLC2 pathway cause branching defects and cystic lungs. (*A*) Components of the noncanonical Wnt signaling pathway have been shown to affect changes in cytoskeletal behavior via the Rho pathway. (*B*) qPCR data showing decreased levels of Celsr1 and Arhgef19 expression in $Shh^{cre}$:$Fzd2^{flox/flox}$ mutant lungs at E11.5. (*C* and *D*) Expression of pMLC2 in $Shh^{cre}$ (*C*, *ii*) and $Shh^{cre}$:$Fzd2^{flox/flox}$ (*D*, *ii*) mutant lungs. (*E*) Expression of pMLC2 is quantitatively decreased in $Shh^{cre}$:$Fzd2^{flox/flox}$ mutant lungs. (*F*) Quantification of domain branch numbers in lung explants treated with ML7 or fasudil. (*G*–*J*) E-cadherin whole-mount immunostaining of $Shh^{cre}$ or $Shh^{cre}$:$Fzd2^{flox/flox}$ mutant lung explants treated with DMSO (*G* and *H*), ML7 (*I*), or fasudil (*J*). Lower panels (*G*, *i*–*J*, *i*) show cell outlines after each treatment regime. (*K* and *K*, *i*). Automated tile-scanning was required to capture the entire lung in the fasudil treated $Shh^{cre}$ sample (*J*), and the dashed line indicates the region stitched by the Zeiss software due to the size of these control lung explants. $n$ = 9–12 lungs treated per condition. (Scale bars: 10 μm.) *$P < 0.05$, **$P < 0.01$.

which Fzd2 regulates RhoA signaling, which, along with proper expression of Fgf10, is required for changes in epithelial cell morphology to coordinate formation of new branch points (Fig. S13).

## Discussion

Changing the morphology of the primitive endoderm tube to form the highly ramified respiratory tree requires alterations in individual cells that lead to dramatic changes in organ shape. We show that Wnt/Fzd2 signaling is necessary for controlling changes in cell shape through regulating RhoA signaling. The loss of apical–basal lengthening in Fzd2 mutants results in a more uniform lung epithelial cell shape that leads to a failure in the epithelium to thicken. Failure to form a thickened, pseudostratified epithelium in the Fzd2 mutant lungs results in both a widened epithelial tube and a defect in formation of new branches. Our studies indicate that Wnt/Fzd2 signaling plays a critical role in altering epithelial sheet morphology by promoting the changes in cell shape that are required for generating complex tissue structures such as the branched network of the lung.

In this study, we have focused on the changes in the epithelium during new branch formation when we specifically deleted Fzd2 in the lung epithelium. New branch formation and maintenance of epithelial tube morphology likely require coordinated changes between the epithelium and the surrounding mesenchyme. Although we observed specific changes in epithelial cell biology with loss of Fzd2, we did not see any changes in mesenchymal cell biology as assessed by SM22α staining or actin localization, suggesting that the changes in cell biology are restricted to the epithelium. However, we did observe changes in mesenchymal Fgf10 expression that likely contribute to the overall defects observed on the loss of Fzd2 in the developing lung. Previous work has suggested that Fgf10 signaling from the mesenchyme acts to promote specific outgrowth where a new branch forms (17, 36, 37), although new research has called into question whether focal localization of Fgf10 is required for proper branching morphogenesis to occur (19). Interestingly, the previously reported effects of Fgf10 signaling, including changes in cell proliferation or orientation of cell division, were not changed in Fzd2-deficient lung epithelium, suggesting either that this ligand has a different role in regulating branch-point formation or that the Fzd2-deficient epithelium lacks responsiveness to Fgf signaling (20–22). The increase in levels and expansion in the domain of Fgf10 expression could result from the decreased expression of Shh or Fgfr2, which provide important feedback signals that control the proper expression of Fgf10 (38, 39). However, even in the context of a precise focal Fgf10 signal, the Fzd2-deficient epithelium cannot maintain proper branch morphology. Together, these results suggest that the Fzd2 mutant lung epithelium is intrinsically defective in the epithelial morphology required for new branch formation, but alterations in Fgf10 signaling could contribute to the overall phenotype in a non–cell-autonomous manner.

The formation of new branch points in the developing respiratory tree requires changes in cell behavior in a specific region of the lung epithelium that lead to the bending of the epithelial tube. Thickening of the epithelium in a specific region precedes bending of the epithelium, a change in morphology that is observed in other systems where a sheet of cells invaginates to promote complex tissue reorganization required for development (24, 25, 40). Importantly, Wnt/Fzd signaling has been implicated in promoting these processes during gastrulation (27, 41–43). Although we observe a defect in the thickening of the lung epithelium with loss of Fzd2, it is unclear at this point if the thickening of the epithelium is required for the bending process to occur or instead is a secondary effect of volume conservation in a cell with a decreased apical surface area (44). Because both these processes are defective with loss of Fzd2, we cannot determine whether the failure to form new domain branches is caused by a failure to change cell shape that results from remodeling at the apical surface or by a failure of the lung epithelial cells to elongate along the apicobasal axis.

Noncanonical Wnt signaling can modify epithelial cell shape and behavior in epithelial sheets through multiple downstream pathways (5, 43). In the Fzd2-deficient lung epithelium, the epithelial cells failed to adopt a bottle shape, and there was a decrease in the components of the actomyosin contractile network at the apical surface as evidenced by a decrease in pMLC2 apical localization. pMLC2 is a target of the RhoA pathway (45), which has been identified as a component of noncanonical Wnt signaling that can regulate changes in cell morphology by modifying cytoskeletal dynamics (46–50). In $Shh^{cre}$:$Fzd2^{flox/flox}$ mutants we found decreased expression of two factors, Celsr1 and Arhgef19, which have been implicated in both noncanonical Wnt and Rho signaling (5, 30). Along with decreased apical expression of pMLC2, these data indicate decreased RhoA signaling upon loss of Fzd2 expression. Inhibition of either ROCK or pMLC2 causes defects in cellular organization and branching morphogenesis, and a Rho activator could partially rescue the loss of Fzd2, supporting a role for RhoA signaling in orchestrating the cell changes needed for new branch formation.

Although the importance of branching morphogenesis has been known for some time, little has been revealed about how changes in cell shape can alter and sculpt the shape of the growing lung epithelial sheet that composes the branching airways. Our data show that Wnt/Fzd2 signaling is required for regulating lung epithelial cell shape and can contribute to bending of the epithelial tube to generate and maintain new branch points. Such alterations have an important impact on postnatal lung structure that may impede respiration. These data provide both a new model for how changes in cell shape

promote branching morphogenesis and show that Wnt/Fzd2 is essential for these changes.

## Materials and Methods

See *SI Materials and Methods* for a full description of methods.

**Mouse Strains and Breeding.** Animals were housed under US Department of Agriculture- and Association for Assessment and Accreditation of Laboratory Animal Care International-approved conditions, with free access to food and water, and maintained in accordance with the Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania. All experiments were approved and performed in accordance with IACUC guidelines and regulations. The mouse $Fzd2^{flox/+}$ allele was generated using homologous recombination in R1 ES cells as described (51, 52). Schematic of targeting construct, Southern blot probes, and position of PCR genotyping primers are shown in Fig. S1.

**Histological Preparation.** Embryos were dissected, fixed in 4% paraformaldehyde in PBS at 4 °C, dehydrated through a series of descending ethanol washes, embedded in paraffin wax, and sectioned at 6 μm. Histological procedures were performed as described (51, 53).

**Immunohistochemistry and in Situ Hybridization.** Immunofluorescent staining of paraffin sections, vibratome sections, and whole-mount lungs was performed as previously described (2).

**Quantitative Real-Time PCR.** Total RNA was isolated from E12.5 lungs and prepared as previously described (51). qPCR was performed using the SYBR Green system with primers listed in Dataset S2.

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