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Report

# Primary Cilia Regulate Branching Morphogenesis during Mammary Gland Development

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## Summary

**During mammary gland development, an epithelial bud undergoes branching morphogenesis to expand into a continuous tree-like network of branched ducts [1]. The process involves multiple cell types that are coordinated by hormones and growth factors coupled with signaling events including Wnt and Hedgehog [2–5]. Primary cilia play key roles in the development of many organs by coordinating extracellular signaling (of Wnt and Hedgehog) with cellular physiology [6–8]. During mammary development, we find cilia on luminal epithelial, myoepithelial, and stromal cells during early branching morphogenesis when epithelial ducts extend into the fat pad and undergo branching morphogenesis. When branching is complete, cilia disappear from luminal epithelial cells but remain on myoepithelial and stromal cells. Ciliary dysfunction caused by intraflagellar transport defects results in branching defects. These include decreased ductal extension and decreased secondary and tertiary branching, along with reduced lobular-alveolar development during pregnancy and lactation. We find increased canonical Wnt and decreased Hedgehog signaling in the mutant glands, which is consistent with the role of cilia in regulating these pathways [6–11]. In mammary gland and other organs, increased canonical Wnt [12–14] and decreased Hedgehog [15, 16] signaling decrease branching morphogenesis, suggesting that Wnt and Hedgehog signaling connect ciliary dysfunction to branching defects.**

## Results and Discussion

### Primary Cilia Are Abundant on Multiple Cell Types in the Mammary Gland

In the mammary gland, the ductal epithelium consists of a luminal layer of secretory cells and an outer layer of myoepithelial cells embedded in an extracellular matrix (ECM) and surrounded by a stroma of adipocytes, fibroblasts, immune cells, and vasculature. Prior electron microscopy studies documented primary cilia on basally located myoepithelial cells [17–20], but cilia were rarely observed on the luminal epithelial cells. It is unusual for luminal epithelial cells in vertebrates to not be ciliated, and so we reexamined the distribution of cilia. Cilia were present on myoepithelial and stromal cells at similar percentages throughout development (Figures 1A and 1B; see also Figure S1A available online). Cilia on myoepithelial cells extended from the centrosome toward the stroma and ECM or toward the interstitial space between the myoepithelial cells and luminal epithelial cells (Figure S1B). In contrast to previous reports, we also found cilia on the luminal epithelial cells in terminal end buds and mature ductal structures. Luminal epithelial cilia were more abundant early in development (4 wks: 17% ± 1.9%) and decreased significantly in developed tissues (7 wks: 2% ± 1.1%, $p = 6 \times 10^{-4}$; adult: 4% ± 1.9%, $p = 7 \times 10^{-4}$; pregnancy: 4% ± 1.5%, $p = 1 \times 10^{-4}$) (Figure 1B; Figure S1C). The loss of cilia with maturity is reminiscent of the cilium on hair cells in the rodent ear. In these cells, a cilium forms initially and appears to be required for proper development of the microvilli bundles but regresses once the microvilli have formed [21]. Thus, in these epithelial cell types, it appears that cilia play developmental roles but are not critical once the organ has developed.

### Primary Cilia Regulate Branching Morphogenesis

The conservation of cilia in mammary glands across diverse species suggests that cilia have biological significance in this organ, but this has not been studied. To address this question, we analyzed branching morphogenesis in the mammary glands of the $Tg737^{orpk}$ mouse, which has a ciliary assembly defect because of a mutation in the IFT88 subunit of the intraflagellar transport (IFT) particle [22–26]. IFT88 is found in control mammary cilia but is not detectable in mutant (MT) cilia (Figure 2A). Mutant animals have significantly reduced (4 wks: $p = 0.006$; 7 wks: $p = 7 \times 10^{-5}$; pregnant: $p = 2 \times 10^{-5}$) numbers of cilia as compared to controls (Figures 2B and 2C). The cilia that remained in the mutants were shorter than those in controls, consistent with ciliary dysfunction (Figure 2A).

Branching morphogenesis begins in the mammary gland with the onset of puberty at 3–4 wks and continues until the growth of primary ducts accompanied by secondary and tertiary branching fills the fat pad at 8–10 weeks of age [1]. To test whether cilia play a role in ductal extension and branching morphogenesis, we examined mammary glands from mutant and control animals by whole mounts at early (4 wks) and late (7 wks) development. At 4 wks, the glands of the mutant mice had significantly less ($p = 2 \times 10^{-5}$) ductal extension as compared to controls (Figure 3A; Figure S2A). By 7 wks, ductal extension and branching morphogenesis in the control animals was nearly complete, and the fat pad was almost completely filled (Figure 3B; Figure S2B). In contrast, ductal extension in the mutant animals was significantly reduced ($p = 3 \times 10^{-8}$), and a large portion of the fat pad was unfilled (Figure 3B; Figure S2B). At 7 wks, although the number of tertiary branched points was the same, the number of secondary branch points was significantly less ($p = 5 \times 10^{-5}$), and there was less organization and directionality of the branches in the mutant glands as compared to controls (Figure 3C; Figure S2C). These results demonstrate

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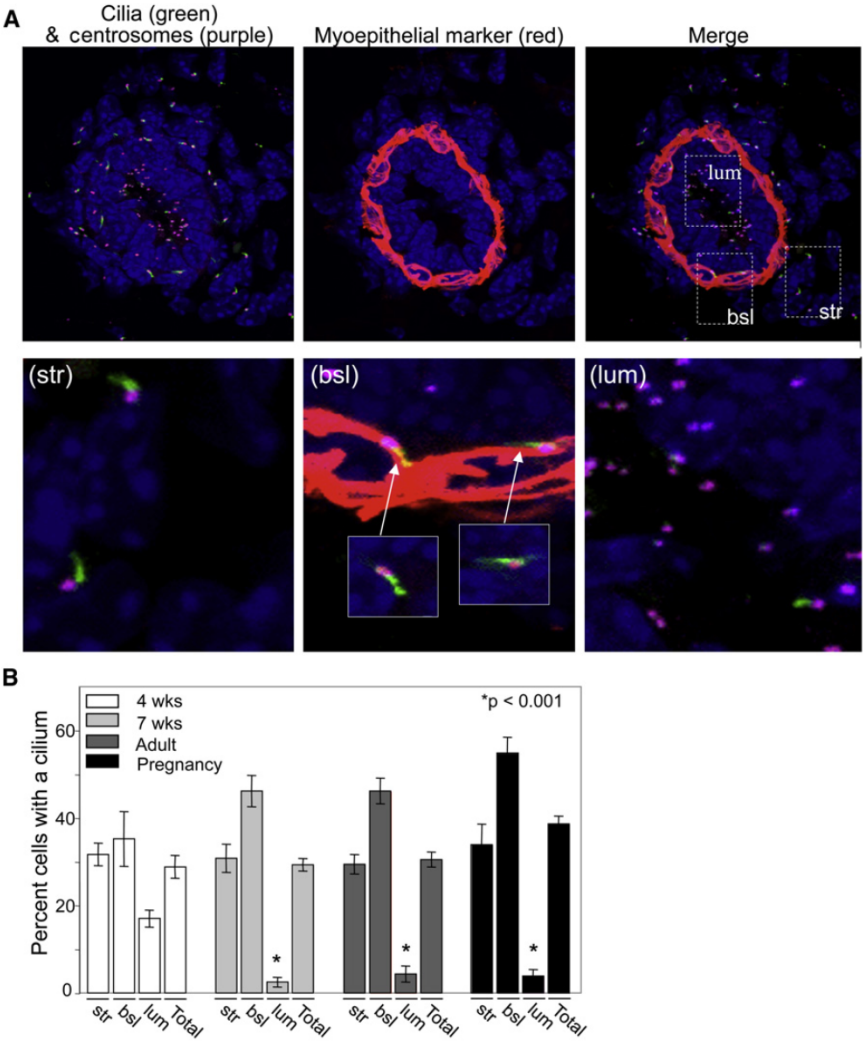


**Figure 1. Localization of Primary Cilia within the Mammary Gland**

(A) Immunofluorescent confocal projections were acquired for the localization of primary cilia in the murine mammary glands (7 wks). Cilia were detected by an antibody to acetylated α-tubulin (green), centrosomes were detected by an antibody to γ-tubulin (purple), and myoepithelial cells (a basally located epithelial cell type) were identified with an antibody recognizing smooth muscle actin (red). Insets highlight the localization of cilia on stromal (str), basal epithelial (bsl), and luminal epithelial (lum) cells.

(B) Bar graph represents quantitation of the number of cells with a primary cilium in stromal (str), basal epithelial (bsl), and luminal epithelial (lum) cells in murine mammary glands of early development (4 wks: n = 4 mice, >500 cells), mature (7 wks: n = 5 mice, >500 cells; adult: n = 4 mice, >500 cells), and midpregnant (12 days: n = 3 mice, >500 cells) mammary glands. Student's t test (two-sided) was performed to determine statistical significance (*). Error bars represent standard error. Statistical significance noted is compared to 4 wk luminal epithelial values. See also Figure S1.

that ductal extension and branching morphogenesis of the mammary ductal tree are decreased by ciliary dysfunction because of reduced IFT88.

Decreased ductal extension and branching morphogenesis in the mammary gland of the Tg737$^{orpk}$ mouse could be due to loss of localized ciliary function in the mammary gland or could be a result of secondary effects of ciliary dysfunction at distant organ sites. For example, the loss of cilia could delay the age of puberty, which in turn would affect mammary development. The age of vaginal opening, which correlates with the onset of puberty [27], was similar in control and mutant animals (Figure S2D), suggesting that onset of puberty is not a factor in the phenotype. In addition, we found no significant difference in the estradiol levels (Figure S2E), indicating that the branching morphogenesis defect is not an indirect result of altered estrogen signaling. Weight is also a factor in the onset of puberty [27], but we found no statistically significant difference between the weights of the Tg737$^{orpk}$ control and mutant mice (Figure S2F) at puberty.

Although the onset of puberty and estrogen signaling did not appear to be contributing factors to the phenotype, the pleiotropic nature of the Tg737$^{orpk}$ mutation raises concerns about other unanticipated secondary effects. To overcome these concerns and more directly assess how the loss of primary cilia affects branching morphogenesis, we transplanted Tg737$^{orpk}$ control and mutant mammary epithelial tissue into fat pads of wild-type (WT) recipient mice that had their mammary epithelial buds removed prior to puberty. By transplanting mutant cells on one side of the animal and control cells on the other, we were able to directly compare the ability of control and mutant cells to populate the fat pad and branch without any concerns about secondary effects of the Tg737$^{orpk}$ mutation. Transplanted control cells grow into the fat pad and branch, similar to what is observed in normal development, but the transplanted mutant cells showed a significant decrease in branching morphogenesis ($p = 1 \times 10^{-9}$) (Figure 3D). This indicates that the defect in branching morphogenesis is caused by the lack of IFT88 in the mammary epithelial cells and not a secondary defect caused by pathology elsewhere in the mutant animals.

As a further test of the cell-intrinsic nature of the defect, we examined branching morphogenesis in an in vitro organotypic culture model. To do this, we cultured fragments of mammary tissue (organoids) from Tg737$^{orpk}$ control and mutant mice in a 3D matrix. Under these conditions, the epithelial cells grow and undergo branching morphogenesis in a process that resembles in vivo development, including the formation of a bilayered epithelium with ciliated myoepithelial cells (Figure S2G). After 7 days of culture, the number of branch points per organoid was significantly ($p = 2 \times 10^{-4}$) decreased in mutant organoids (MT = 2.8 ± 0.5) as compared to controls (WT = 8.5 ± 1.1) (Figure 3E). These results further demonstrate that Tg737$^{orpk}$ mutant mammary epithelial tissue has decreased branching morphogenesis under identical conditions to that of control tissue.

To test whether the decreased branching morphogenesis is due to loss of cilia or is due to a nonciliary function of IFT88, we assessed branching morphogenesis in the *Kif3a* and *Ift20*

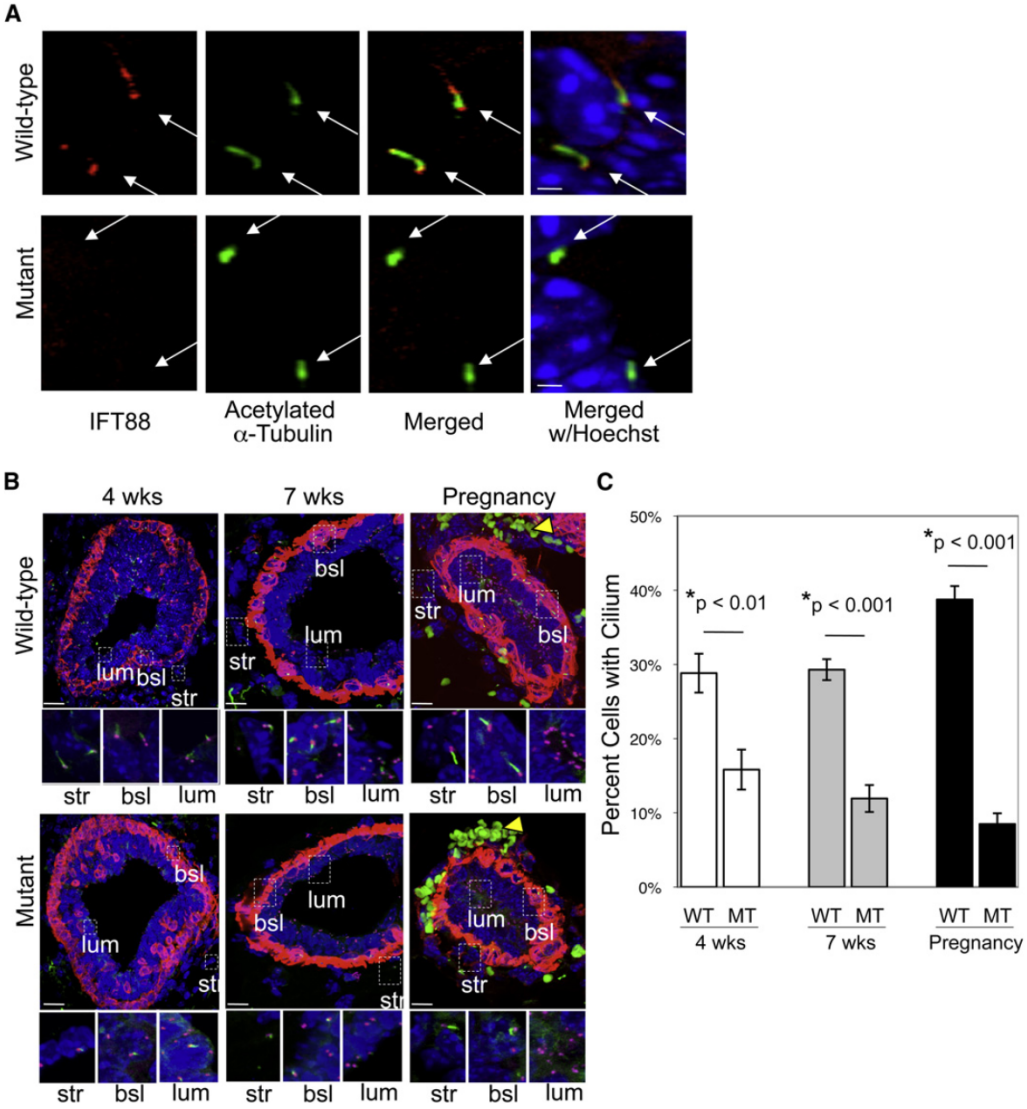


Figure 2. Ciliary Dysfunction in Mammary Glands of the Tg737$^{orpk}$ Mutant Mouse

(A) Immunocytochemistry with antibodies that recognize IFT88 (red) and acetylated α-tubulin (green) was performed to determine whether IFT88 is expressed in the cilium (white arrows) in mammary glands of wild-type and mutant mice.

(B) Mammary glands were harvested at early development (4 wks), late development (7 wks), and midpregnancy (12 days) from wild-type and mutant mammary glands. Immunofluorescent confocal projections were acquired for the localization of primary cilia. Cilia were detected by an antibody to acetylated α-tubulin (green), centrosomes were detected by an antibody to γ-tubulin (purple), and myoepithelial cells (a basally located epithelial cell type) were identified with an antibody recognizing smooth muscle actin (red). Insets highlight the localization of cilia on str, bsl, and lum cells. Note that the green cells (yellow arrowhead) visible during pregnancy are autofluorescent red blood cells.

(C) Immunocytochemistry with antibodies to acetylated α-tubulin and γ-tubulin was performed to determine the number of cilia in mammary glands of the wild-type (WT) and mutant (MT) mice at early development (4 wks), late development (7 wks), and midpregnancy (12 days). Bar graphs represent quantification of the number of cells containing a primary cilium. These results validate the usefulness of the Tg737$^{orpk}$ model for studying ciliary function in mammary gland biology. Student's t test (two-sided) was performed to determine statistical significance (*). Error bars represent standard error. n values: for WT, 4 wks = 5 mice, >500 cells; for MT, 4 wks = 5 mice, >500 cells; for WT, 7 wks = 5 mice, >500 cells; for MT, 7 wks = 5 mice, >500 cells; for WT, pregnant = 4 mice, >500 cells; for MT, pregnant = 2 mice, >500 cells.

genetic models of ciliary dysfunction [28, 29]. Mammary ductal tissue harvested from *Kif3a*$^{flox/flox}$ or *Ift20*$^{flox/flox}$ mice was infected with adenovirus-carrying GFP-Cre to delete *Kif3a* or *Ift20*. Control cells were generated by infecting cells with adenovirus-carrying GFP without Cre. Flow-sorted GFP-positive cells were then transplanted into cleared fat pads of recipient mice. The results were similar to what was observed in the Tg737$^{orpk}$ transplants in that the deletion of *Kif3a* or *Ift20* significantly reduced the number of branch points in the transplanted glands (MT Kif3a: $p = 1 \times 10^{-17}$; MT IFT20: $p = 1 \times 10^{-8}$) (Figure 3F; Figure S2H). Together, this work provides the first genetic evidence that primary cilia play a role in the normal expansion of the mammary ductal tree during development.

During pregnancy, additional secondary and tertiary branches are formed on existing branches. This is followed by differentiation of the branch ends into lobular-alveolar structures that produce milk [1]. At midpregnancy, the number

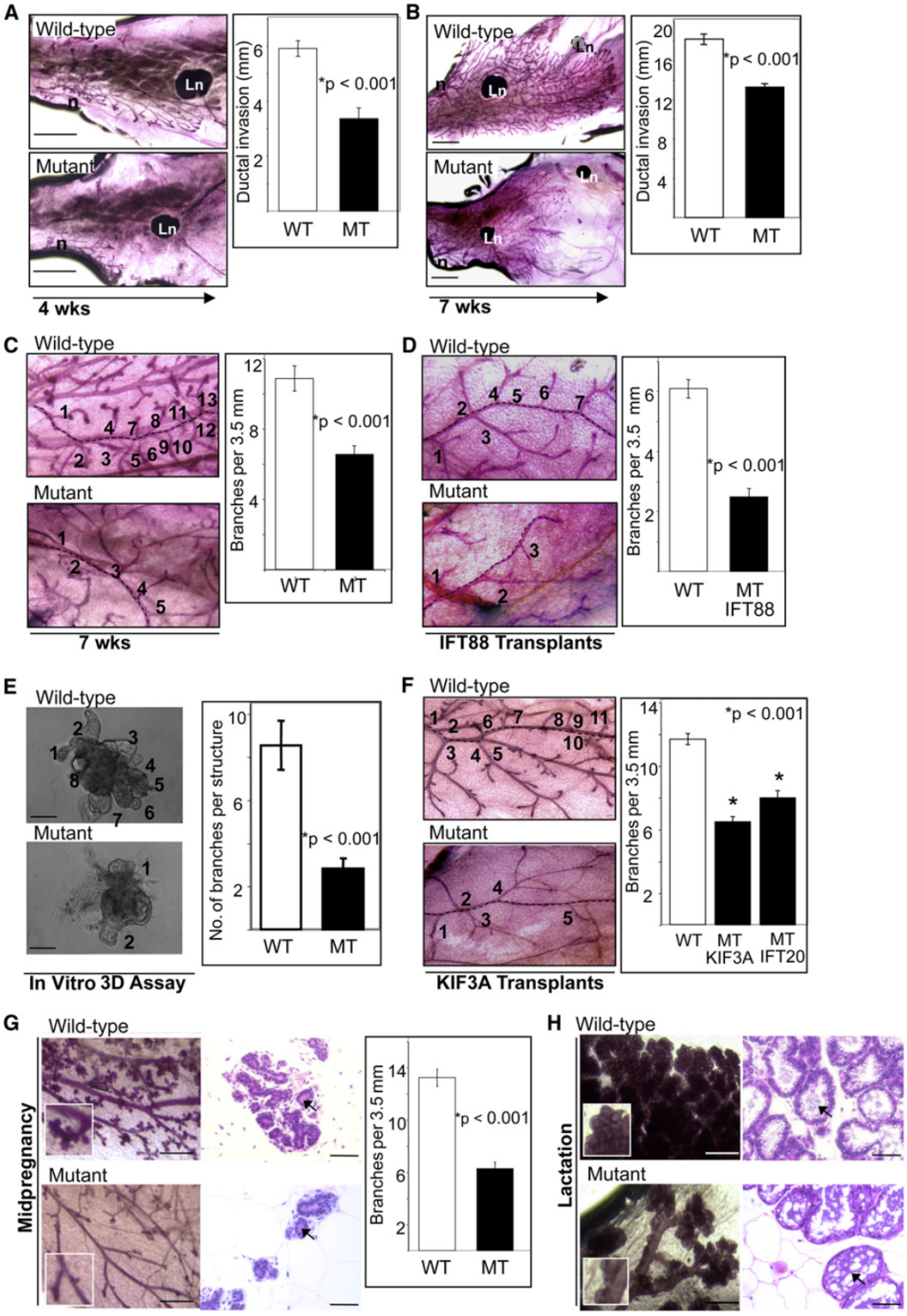


Figure 3. Primary Cilia Regulate Branching Morphogenesis

(A–E) Whole-mount analysis was performed on WT and MT mammary glands harvested at early development (4 wks) (A) and late development (7 wks) (B and C) to determine the extent of ductal elongation and the number of branch points. Whole mounts were also prepared from mammary glands of mice that had their fat pads cleared of endogenous ductal tissue and transplanted with mammary tissue from wild-type or mutant Tg737$^{orpk}$ mice (harvested 8–12 wks post transplantation) (D). Branching morphogenesis was also assayed in a three-dimensional (3D) in vitro basement membrane culture with wild-type or mutant mammary tissue isolated from Tg737$^{orpk}$ mice (E).

(F) Whole mounts of mammary gland transplants from wild-type and Kif3A mutant mice. Quantification of branching morphogenesis in transplants from wild-type, Kif3A, and Ift20 mice is shown in the bar graph.

(G and H) Mammary glands were harvested at midpregnancy (12 days) (G) and lactation (5 days of nursing) (H) from wild-type and mutant mammary glands. Glands were analyzed by whole mount (left panels) and hematoxylin and eosin staining (right panels) at each time point. The following abbreviations are used: Ln, lymph node; n, nipple.

of secondary and tertiary branch points was significantly less (secondary: $p = 6 \times 10^{-10}$; tertiary: $p = 3 \times 10^{-12}$) in the mutant glands compared to controls (Figure 3G; Figure S2C). We were unable to accurately quantitate branching at lactation because of dense ductal tissue in controls. However, the extent of secondary and tertiary branching in the mutant mammary glands during lactation was visibly reduced as compared to controls (Figure 3H). The pregnant and lactating mutant mammary glands also appear to have delayed or altered alveolar development as compared to the littermate controls (Figures 3G and 3H). However, histological analysis suggests that both wild-type and mutant mammary glands produce milk (Figures 3G and 3H), indicating that the cells are able to at least partially differentiate.

### Loss of Primary Cilia Increases Canonical Wnt Signaling and Decreases Hedgehog Signaling

Wnt signaling is crucial for the development of most mammalian organs. This signaling works through canonical and noncanonical pathways [30], and recent studies have implicated cilia in regulating both branches [8, 9, 31–33]. The role of cilia in regulating Wnt in early mouse development is controversial [9, 34] but is more established in organ development [29, 35]. Given the connections between Wnt signaling and cilia, and because Wnt signaling is linked to branching morphogenesis during mammary gland development [3], we investigated whether cilia regulate Wnt signaling during mammary gland development. β-catenin is a key component of canonical Wnt signaling, and increased cytoplasmic levels of the unphosphorylated form correlate with increased signaling. We found increased cytoplasmic levels of unphosphorylated β-catenin (and reduced membrane localization) in Tg737$^{orpk}$ mutant terminal end buds as compared to controls (Figure 4A). To understand how this affects Wnt signaling, we quantitated the downstream transcriptional targets of canonical Wnt signaling, *Axin2*, *Tcf1*, *Tcf3*, and *Nkd1*, by real-time quantitative polymerase chain reaction of RNA isolated from Tg737$^{orpk}$ control and mutant mammary tissue and from organoids grown in 3D culture (Figure 4B). In mammary tissue, *Tcf1* and *Nkd1* were significantly increased (2.5-fold, $p = 0.002$ and 2.0-fold, $p = 0.0002$, respectively) in the mutants (Figure 4B). In 3D organ culture, *Axin2*, *Tcf1*, *Tcf3*, and *Nkd1* were all significantly increased (1.9-fold, $p = 0.01$; 2.4-fold, $p = 1 \times 10^{-7}$; 2.2-fold, $p = 0.003$; 1.4-fold, $p = 0.03$, respectively) in the mutant organoids (Figure 4B). The difference between the two systems is likely due to the large amount of nonepithelial tissue in mammary glands, which may have obscured the signal from the epithelial cells.

We also examined the ability of Tg737$^{orpk}$ mutant mammary ductal tissue to respond to Wnt ligands. Mammary cells isolated from ductal tissue of Tg737$^{orpk}$ wild-type and mutant animals were infected with a lentivirus containing a TCF/LEF luciferase reporter for canonical Wnt signaling. After infection with the virus, the cells were treated with Wnt3a, a ligand expected to activate canonical Wnt signaling, and Wnt5a, a closely related molecule that activates noncanonical Wnt signaling but is not expected to activate the canonical pathway interrogated by our TCF\LEF reporter construct [36]. As expected, untreated cells from wild-type and mutant mammary glands had low luciferase activity, and treatment with the control ligand Wnt5a did not significantly increase activity (Figure 4C). Treatment with Wnt3a increased luciferase activity 5.5-fold in control cells and 18.4-fold in mutant cells (Figure 4C). This indicates that the Tg737$^{orpk}$ mutant cells were significantly ($p = 2 \times 10^{-10}$) more responsive to Wnt3a stimulation than are wild-type cells.

The role of Wnt signaling in mammary gland branching morphogenesis is controversial, with some studies finding that increased canonical Wnt signaling leads to decreased branching morphogenesis, whereas other studies have come to the opposite conclusion. For example, deletion of APC, which leads to increased levels of β-catenin and increased canonical Wnt signaling, results in reduced ductal extension during mammary gland development [12]. Similarly, during early lung development, expression of stabilized β-catenin in the presumptive epithelium partially inhibited branching morphogenesis [13, 14]. In contrast, overexpression of Wnt ligands in mammary tissue or expression of stabilized *Xenopus* β-catenin in luminal epithelial cells caused increased branching [37–40]. The exact reasons for the discrepancies are not known but may reflect differences in how the noncanonical pathway is affected. For example, overexpression of Wnt ligands may also affect the noncanonical Wnt pathway in ways that deletion of APC does not [3]. In addition, the spatial and temporal control of Wnt signaling may be important during branching morphogenesis. Our in vitro expression studies (Figure 4C) suggest that basal canonical signaling is not altered in the cilia-defective cells, but these cells are hyper responsive to activating ligand. It may be expected that hyper responsiveness will give a different phenotype than overexpression of stabilized β-catenin throughout development. Thus, cilia may function to provide spatial and temporal regulation to Wnt signaling to allow for proper branching morphogenesis.

Defects in Hedgehog signaling lead to decreased branching morphogenesis in the lung and salivary gland [15, 16]. Given that ciliary defects decrease signaling through the Hedgehog pathway [41], we sought to determine whether Hedgehog signaling was altered in mammary glands of mice with ciliary dysfunction. Consistent with published studies [6, 7, 10, 11], we found that expression of the Hedgehog target gene *Gli1* was significantly decreased (1.8-fold, $p = 0.01$) in mutant mammary tissue as compared to controls (Figure 4D). Canonical Wnt and Hedgehog signaling often play a role in proliferation [30], but we did not see alterations in proliferation or apoptosis in the mutant cells (Figure 4E). It is possible that only small changes are needed in these parameters to cause the observed phenotypes, but our assays were not sensitive enough to detect these changes.

Our results demonstrate alterations in Wnt and Hedgehog signaling in the mutant mammary gland, but it is likely that primary cilia also regulate additional signaling events. One possibility is communication with the extracellular matrix

Bar graphs represent the quantification of the extent of ductal elongation (arrow designates direction of growth) into the fat pad (A and B), the number of branch points per 3.5 mm ductal length (numbers and dashed line represent number of branch points from a duct) (C, D, F, and G), and the number of branch points per tissue piece (E). Student's t test (two-sided) was performed to determine statistical significance (*). Error bars represent standard error. n values: for ductal extension at 4 wks (A) and 7 wks (B) for WT and MT = 5 mice, 15 ducts each; for branch points at 7 wks (C) for WT and MT = 5 mice, 14 and 13 ducts; for transplanted IFT88 tissue (D) for WT = 4 mice, 12 ducts and for MT = 7 mice, 27 ducts; for 3D assay (E) performed in triplicate, counting WT = 109 tissue pieces and MT = 92 tissue pieces (E); for transplanted Kif3A and IFT20 tissue (F) for WT = 10 mice, 69 ducts, MT Kif3A = 7 mice, 43 ducts, MT IFT20 = 4 mice, 34 ducts (F); for midpregnancy (G) for WT = 4 mice, 19 ducts, and MT = 2 mice, 18 ducts. See also Figure S3.

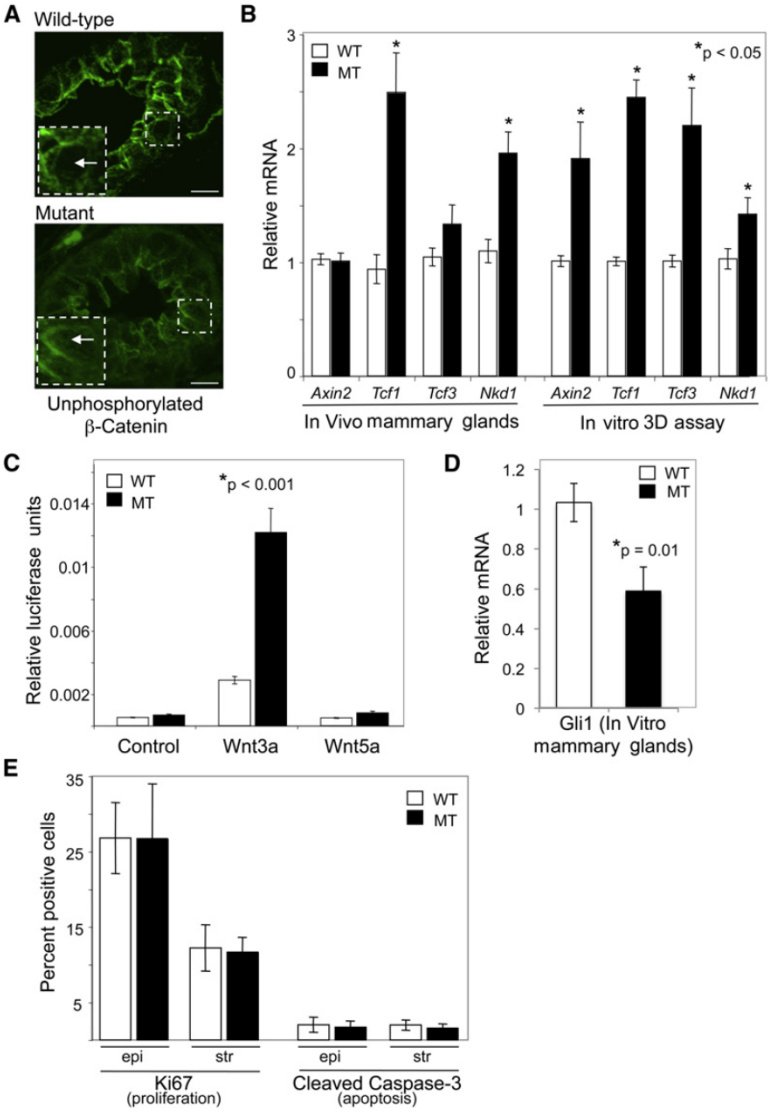


Figure 4. Loss of Primary Cilia Increases Canonical Wnt Signaling and Decreases Hedgehog Signaling during Mammary Gland Development

(A) Mammary tissue from WT and MT Tg737$^{orpk}$ mammary glands were stained with an antibody that recognizes the unphosphorylated form of β-catenin to determine localization of active β-catenin. Unphosphorylated β-catenin cytoplasmic localization (white box inset with arrow) is increased in the epithelium of mutant mammary epithelium as compared to wild-type. Images are maximum projections of equal confocal Z images taken 0.38 μm apart. Images were taken under identical conditions, and postacquisition manipulations were also identical.

(B and D) Real-time quantitative polymerase chain reaction (qPCR) was performed on RNA isolated from whole mammary glands or from organoids cultured in a 3D branching assay of WT and MT Tg737$^{orpk}$ mice. Bar graph represents WT and MT mRNA levels of the *Axin2*, *Tcf1*, *Tcf3*, *Nkd1*, and *Gli1* genes (normalized to the housekeeping gene, *Gusb*).

(C) Wnt signaling measured in cells isolated from mammary glands of WT and MT Tg737$^{orpk}$ mice. Cells were infected with a TCF/LEF luciferase reporter and were untreated (control) or treated with the Wnt ligands (Wnt3a or Wnt5a). Bar graph represents relative luciferase activity (Wnt signaling).

(E) Immunocytochemistry with antibodies that recognize Ki67 (marker of proliferation) and cleaved caspase-3 (marker of apoptosis) was performed on tissue sections from mammary glands harvested at early development (terminal end buds analyzed at 4 wks) from wild-type and mutant Tg737$^{orpk}$ mammary glands. Bar graphs represent quantitation of percent Ki67- and cleaved caspase-3-positive cells. Student's t test (two-sided) was performed to determine statistical significance (*). Error bars represent standard error. (n values: for qPCR from RNA isolated from tissue, WT = 5 mice, MT = 5 mice; for qPCR from RNA isolated from cultured organoids, WT = 3 mice, MT = 3 mice; for luciferase assay, WT and MT = cells isolated from 2 mice each; for Ki67 and caspase-3 analysis, WT = 4 mice, >1000 cells, MT = 3 mice, >1000 cells).

via integrins. Integrin signaling plays an important role in mammary gland development [5], and recently it was demonstrated that integrins localize to primary cilia [42, 43]. Our working model is that mammary epithelial cells utilize primary cilia to receive spatial and temporal signals (Wnt, Hedgehog, etc.) to communicate with other cells of the branching ductal epithelium to control orientation of cell division and cell migration in order to develop into the complex three-dimensional structure of the mature organ.

### Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures and two figures and can be found with this article online at doi:10.1016/j.cub.2010.02.048.

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