SUPPLEMENTAL INFORMATION INVENTORY

The provided Supplemental Information includes 1. Supplemental Data, 2. Supplemental Experimental Procedures with Supplemental References.

1. The supplemental data includes additional controls and primary data that support the main conclusions of the paper.
    a. Figure 1 goes with Supplemental Figure 1.
    b. Figure 3A,B goes with Supplemental Figure 2.
    c. Figure 3C,D goes with Supplemental Figure 3.
    d. Figure 3E,F goes with Supplemental Figure 4.
2. The supplemental experimental procedures include a complete list of the detailed methods and reagents required to reproduce the experiments in the paper. The supplemental references provide additional references that are unique to the supplemental experimental procedures.

**SUPPLEMENTAL METHODS AND DATA**

# Primary Cilia Regulate Branching Morphogenesis During Mammary Gland Development.

Kimberly M. McDermott, Bob Y. Liu, Thea D. Tlsty and Gregory J. Pazour

## SUPPLEMENTAL EXPERIMENTAL PROCEDURES

**Mice.** Mice used in this study were F1 hybrids generated by crossing $Tg737^{orpk}$ heterozygotes on two different inbred backgrounds as well as *Kif3a* and *Ift20 mutant mice*. One inbred line of $Tg737^{orpk}$ mice was on the C57B6J background (greater than 10 backcrosses) and the other was originally derived from a C3H by FVB cross but maintained by brother-sister matings for more than 10 generations. Unlike other murine models of ciliary dysfunction (e.g. complete knockout of Ift88/Tg737, Pkd2 and Inversin), which have early embryonic or postnatal lethality [1-3], these $Tg737^{orpk}$ -/- (mutant) F1 mice survive to adulthood. Thus, mammary gland development could be analyzed in the $Tg737^{orpk}$ mutant mice. Because there is no detectable ciliary dysfunction in the heterozygotes, we used both $Tg737^{orpk}$ +/+ and +/- mice as littermate wild type controls. Estradiol levels were assayed in serum by the University of Virginia Center for Research Reproduction (NICHD Grant U54-HD28934).

**Immunocytochemical Analysis.** Mammary tissue sections were double stained with the antibodies that recognize acetylated α-tubulin (clone 611-ß-1, Sigma, 1:500) and γ-tubulin (clone GTU88, Sigma, 1:1000), double stained with antibodies recognizing acetylated α-tubulin and

IFT88 [4] (1:100) or triple stained with the antibodies that recognize acetylated α-tubulin, γ-tubulin and with an antibody specific for α-smooth muscle actin (clone 1A4, Sigma, 1:1000). Additional antibodies used to stain mammary tissue sections include detyrosinated tubulin (catalog number ab48389, Abcam, 1:500), unphosphorylated β-Catenin (clone 8E7, Upstate Biotechnologies, 1:100,), Ki67 (clone TEC-3, Dako, 1:100) and cleaved Caspase-3, Asp 175 (catalog number 9661, Cell Signaling Technologies, 1:200). Species and isotype specific secondary antibodies fluorescently labeled with fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC) (1:1000, Southern Biotechnologies) and Alexa 647 (1:1000, Molecular Probes) were used for detection of primary antibodies.

Immunocytochemical staining was performed using a standard staining protocol. In brief, paraffin tissue sections were deparaffinized in xylene and rehydrated in isopropanol and water. Antigens were retrieved by heating in 10 mM EDTA. Tissues were blocked with Antibody Blocking Buffer (ChemMate) for one hour followed by incubation with primary and secondary antibodies diluted in the same buffer. DNA was counter stained using Hoechst dye (Molecular Probes). Samples were mounted with Prolong antifade reagent (Molecular Probes). Fluorescence was analyzed on a Zeiss 510 LSM Confocal Microscope using an Argon12 laser (excitation 488 nm), a HeNe laser (excitation 543 nm) a HeNe laser (excitation 633) and a Titanium:Sapphire dual photon laser (tuned to 790 nm for excitation of Hoechst). Images are maximum projections of equal confocal z images taken 0.38 μm apart. Images were taken under identical conditions and post-acquisition manipulations were also identical. The total thickness of the z stacks was kept constant throughout the experiments at 11 μm each to assure that accurate comparisons were being made.

The frequency of cilia in the mammary gland was determined by counting the number cilia in a field of view (containing ductal structures) and dividing that by the total number of nuclei. Luminal epithelial cells were identified by localization adjacent to lumens, basal epithelial cells were identified by basal position as well as staining with smooth muscle actin antibody and stromal cells were identified as all cells outside of ductal epithelium. Only cilium >0.5 μm were included in the analysis. Statistical significance was determined using the two-sided Student's t-Test.

**Mammary Gland Whole Mount Preparation and Analysis.** The #4 inguinal mammary glands were fixed in Carnoy's Fixative (3:1 ethanol to glacial acetic acid) and whole mounted onto glass slides, stained with alum carmine, and cleared of fat with Histo-Clear (National Diagnostics). Whole mounts were photographed on a bright field microscope (model DMLB; Leica) and images transferred to Adobe Photoshop or Velocity (Improvision) for analysis. Velocity software was used for morphometric analysis. Extent of ductal extension was determined based on the average of the length of three straight lines measured from the nipple to the ends of the three longest ducts of each mammary gland. Quantitation of branch points was determined based on the average number of branches originating from three ducts (3.5 mm long each).

**Transplantation of cleared mammary fat pads.** To isolate mammary epithelial cells, mammary glands were dissected, chopped, and incubated in digestion media (RPMI+ 0.8 mg/mL of collagenase I (Sigma), 0.35mg/mL of hyaluronidase (Sigma), and 5% FBS with rocking for 2 hours at 37$^{o}$C). After digestion, mammary cells remained in clusters known as organoids. For transplantation of *Tg737*$^{orpk}$ mutant and control tissue, the organoids were washed twice with

MEGM and stained with Hoechst dye for counting of cell nuclei. For transplantation of from *Ift20* $^{flox/flox}$ and *Kif3a* $^{flox/flox}$ cells, the organoids were plated on 6-well plates in DMEM/F12 media containing 10% FBS. Epithelial cells were allowed to grow out of the organoids for 48 hours prior to infection with 10 MOI of adenovirus (GFP alone or GFP-Cre). 48 hours after infection the cells were harvested and the GFP positive population was sorted on a FACS Aria. Organoids and GFP positive cells used for transplantation were resuspended in MEGM + Matrigel (5 µg/mL, Becton Dickinson) and loading dye (final concentration: 5% glycerol, 0.5% trypan blue, and 25 mM Hepes) at 4$^{o}$C. The mammary fat pads of immune compromised SCID/Beige recipient females that weighed between 10-13 grams (3-4-week-old) were cleared according the procedures described [5]. 50,000 or 100,000 cells from mutant mice were injected into the empty fat pad, and an equal number of cells from control mice were injected into the contralateral fat pad. Eight to twelve weeks after transplantation, fat pads were prepared for whole mounts. All transplantation surgeries were performed at UCSF in accordance with protocols approved by the UCSF Committees on Animal Research.

***In vitro* organotypic branching assay.** Organotypic branching assays were performed as described [6, 7]. Briefly, organoids were isolated as described above and were resuspended (approximately 2 organoids per µl) in 50 µl growth factor reduced Matrigel (BD Biosciences). The organoid/Matrigel suspension was dropped onto a 4-well coverslip bottom chamber (Nunc) and allowed to setup for 30 minutes at 37°C. Branching media was then added to each well (DMEM/F12, insulin (10 mg/mL), transferrin (5.5 mg/mL), and selenium (5 ng/mL) (ITS medium supplement; Sigma) and 2.5 nM FGF2 (Sigma) and 100 U/mL penicillin/streptomycin (Sigma)). Immunocytochemical staining of ductal structures was performed as described [8].

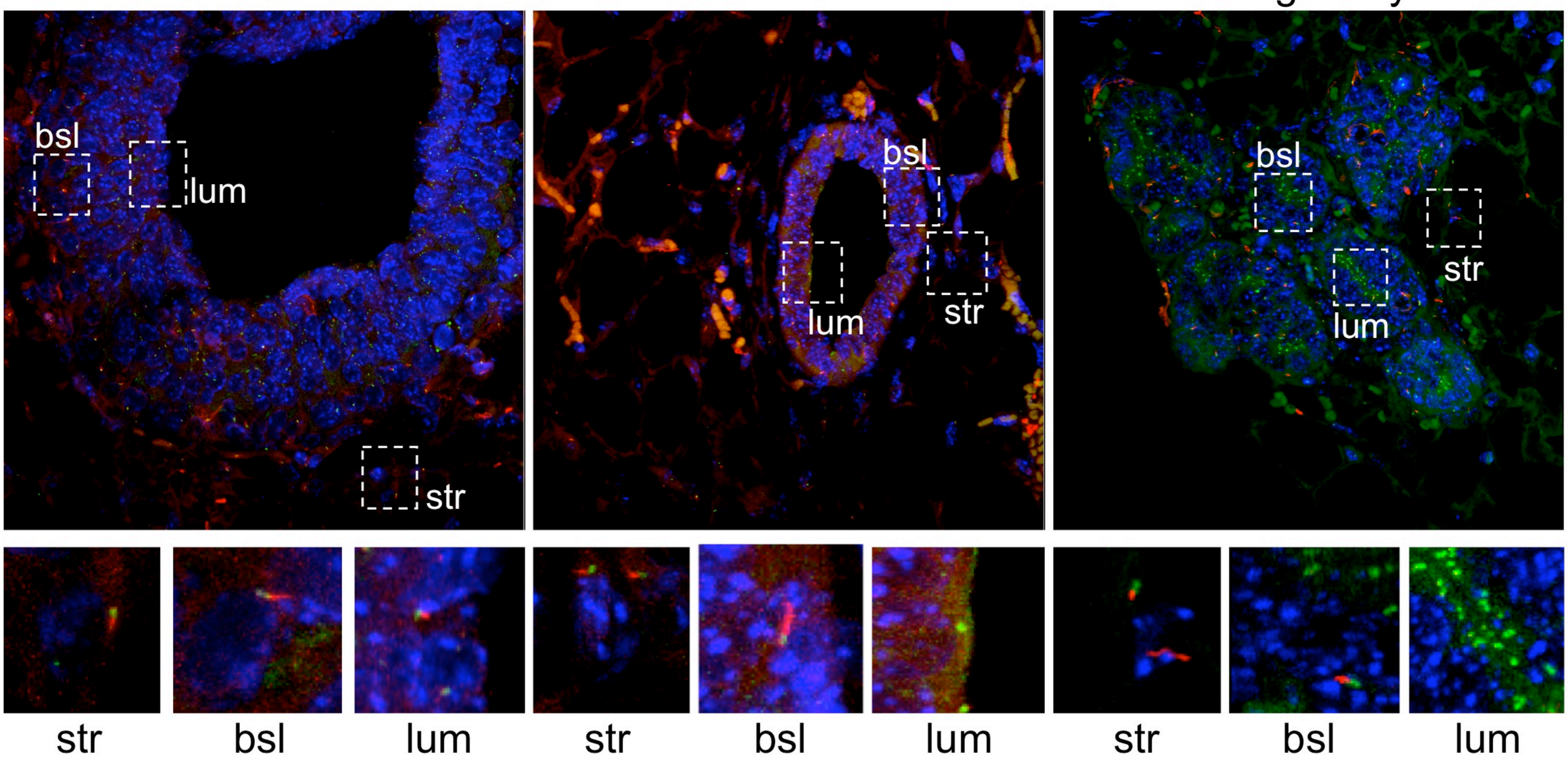
**RNA isolation and quantitative real-time RT-PCR.** RNA was isolated from whole mammary glands using the RNeasy lipid tissue kit (Qiagen) and from 3D cultures using the RNeasy kit (Qiagen). First strand cDNA synthesis was performed on each sample using 2 µg RNA with the $RT^2$ First Strand Kit (SA Biosciences). Quantitative real-time PCR was performed using 5 ng of cDNA in 25 µl reactions with SYBR Green master mix (SA Biosciences). Reactions were performed in the Opticon Real-time PCR system (MJ Research). Mouse-specific primers (supplied by SA Biosciences) used were *Gusb* primers (PPM05490B, NM 010368), *Tcf1* primers (PPM05452B, NM_009331), *Tcf3* primers (PPM05450B, NM_009332), *Axin2* primers (PPM05474B, NM_015732), *Nkd1* primers (PPM05451C, NM_027280) and *Gli1* primers (PPM41530E, NM_010296). Relative level of mRNA was determined by calculating the ΔΔCt value normalized to the mRNA level of the house keeping gene *Gusb* and then further normalized to average Ct of wild type mammary glands. All experiments were performed in triplicate in at least three separate experiments.

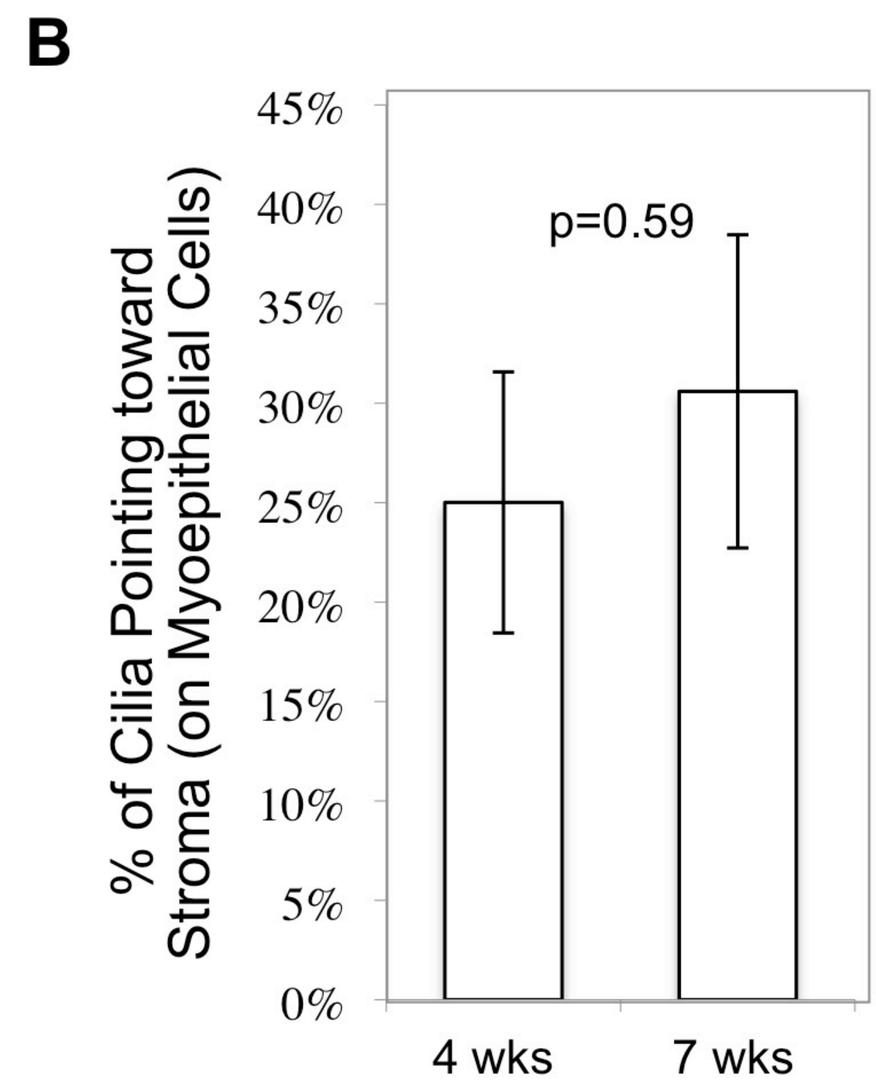
**TCF/LEF Luciferase Reporter Assay.** Organoids from $Tg737^{orpk}$ control and mutant mice were isolated as described above. Cells were allowed to grow out of the organoids and were harvested by trypsinization. The cells were then stained with an FITC-conjugated antibody to CD49f (a marker of basal epithelial cells) and sorted using a FACS Aria (Becton Dickenson). These cells were then propagated in DMEM/F12 media supplemented with 10% FBS, 1% v/v Nutridoma-SP (100X stock; Roche), insulin (10 mg/mL), transferrin (5.5 mg/mL), and selenium (5 ng/mL) (ITS medium supplement; Sigma) , hydrocortisone (0.5 µg/mL; Cascade Biologics), Glutamax (Invitrogen), non-essential amino acids (0.1 mM; Invitrogen), sodium pyruvate (110 µg/mL; Invitrogen) and penicillin/streptomycin (100 U/mL; Sigma). Early passage (passage 3-4) cells

were plated at a density of 1 X $10^4$ cells per well in a 96 well plate. Cells were co-infected with 10 MOI of lentivirus containing a TCF/LEF luciferase reporter construct and lentivirus containing renilla luciferase (SABiosciences). 72 hours after infection, the cells were treated with 100 ng/mL Wnt3a or Wnt5a (R&D Systems) for 18 hours. Luciferase units were measured using a dual luciferase assay (Promega) and read on a GloMax Luminometer (Promega) with two injectors. Cells were harvested and assayed from two wild type and two mutant mice, experiments were run in triplicate and two independent experiments were performed for each.

## SUPPLEMENTAL REFERENCES


1. Morgan, D., Turnpenny, L., Goodship, J., Dai, W., Majumder, K., Matthews, L., Gardner, A., Schuster, G., Vien, L., Harrison, W., et al. (1998). Inversin, a novel gene in the vertebrate left-right axis pathway, is partially deleted in the inv mouse. Nat Genet *20*, 149-156.
2. Wu, G., Markowitz, G.S., Li, L., D'Agati, V.D., Factor, S.M., Geng, L., Tibara, S., Tuchman, J., Cai, Y., Park, J.H., et al. (2000). Cardiac defects and renal failure in mice with targeted mutations in Pkd2. Nat Genet *24*, 75-78.
3. Zhang, Q., Murcia, N.S., Chittenden, L.R., Richards, W.G., Michaud, E.J., Woychik, R.P., and Yoder, B.K. (2003). Loss of the Tg737 protein results in skeletal patterning defects. Dev Dyn *227*, 78-90.
4. Pazour, G.J., Baker, S.A., Deane, J.A., Cole, D.G., Dickert, B.L., Rosenbaum, J.L., Witman, G.B., and Besharse, J.C. (2002). The intraflagellar transport protein, IFT88, is essential for vertebrate photoreceptor assembly and maintenance. J Cell Biol *157*, 103-113.
5. Ip, M.M., and Asch, B.B. (2000). Methods in Mammary Gland Biology and Breast Cancer Research, 1st Edition, (Springer).
6. Fata, J.E., Mori, H., Ewald, A.J., Zhang, H., Yao, E., Werb, Z., and Bissell, M.J. (2007). The MAPK(ERK-1,2) pathway integrates distinct and antagonistic signals from TGFalpha and FGF7 in morphogenesis of mouse mammary epithelium. Dev Biol *306*, 193-207.
7. Ewald, A.J., Brenot, A., Duong, M., Chan, B.S., and Werb, Z. (2008). Collective epithelial migration and cell rearrangements drive mammary branching morphogenesis. Dev Cell *14*, 570-581.
8. Debnath, J., Muthuswamy, S.K., and Brugge, J.S. (2003). Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. Methods *30*, 256-268.

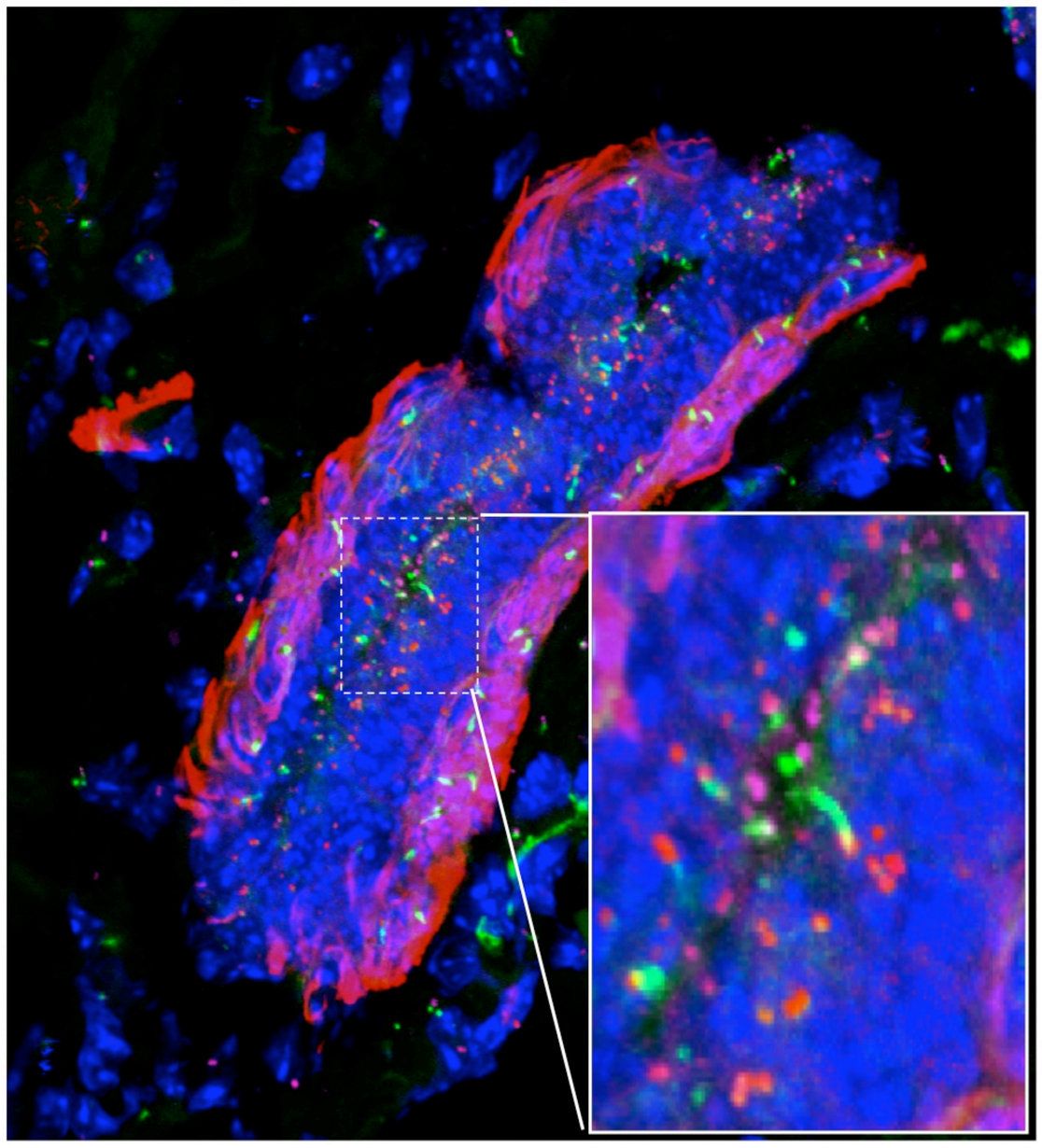


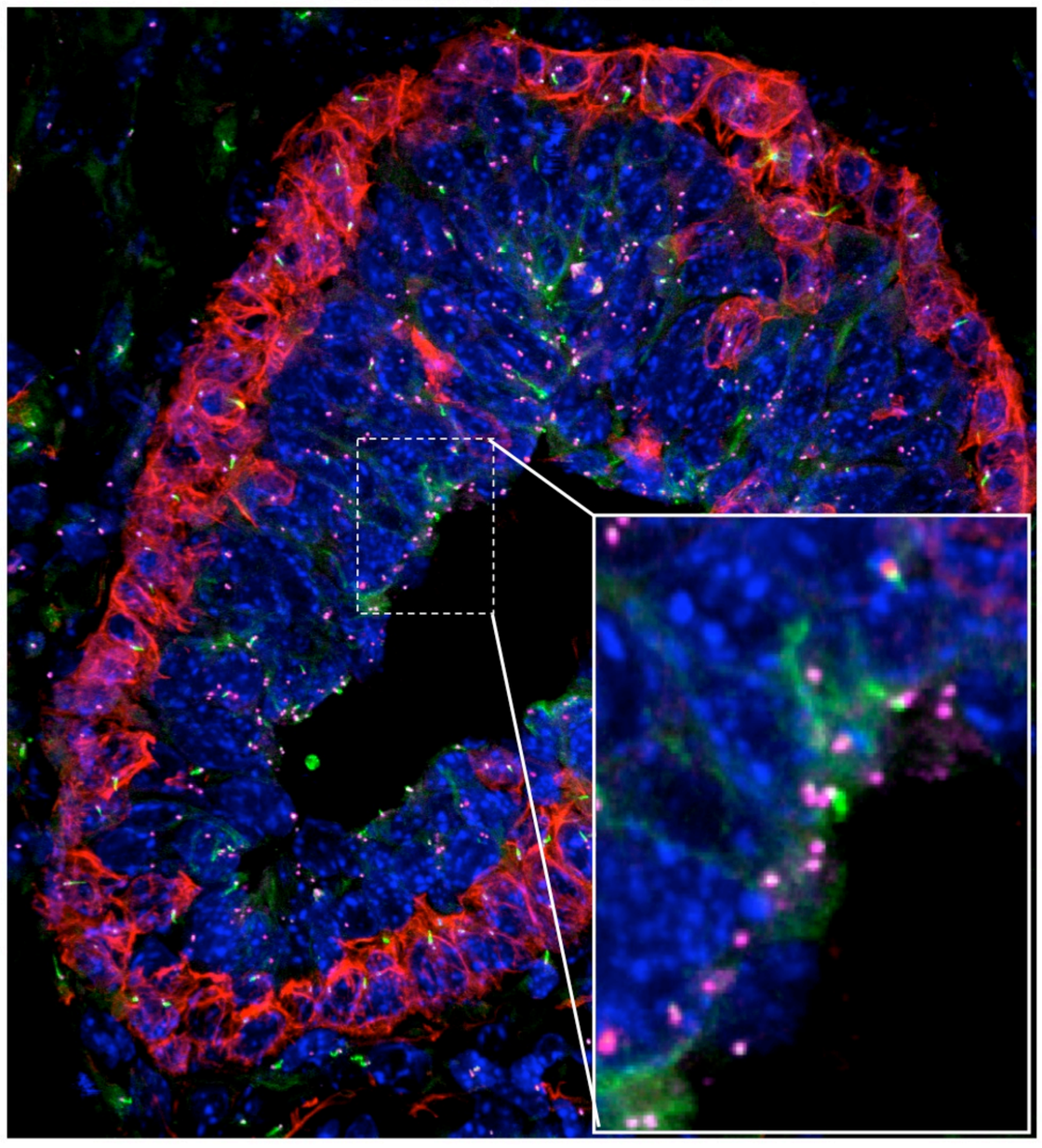

**Supplementary Figure 1. Location of primary cilia during mammary gland development.** **(A)** Mammary glands were harvested at early development (4 weeks), late development (7 weeks) and mid-pregnancy (12 days) from wild type mammary glands. Immunofluorescent confocal projections were acquired for the localization of primary cilia. Cilia were detected by an antibody to detyrosinated α-tubulin (red) and centrosomes were detected by an antibody to γ-tubulin (green). Insets highlight the localization of cilia on stromal (str), basally-located epithelial (bsl) and luminal epithelial (lum) cells. **(B)** Cilia on myoepithelial cells of wild type mammary glands analyzed at 4 and 7 weeks of age were analyzed to determine the percentage of ciliary axonemes extending towards the stroma.

**C**

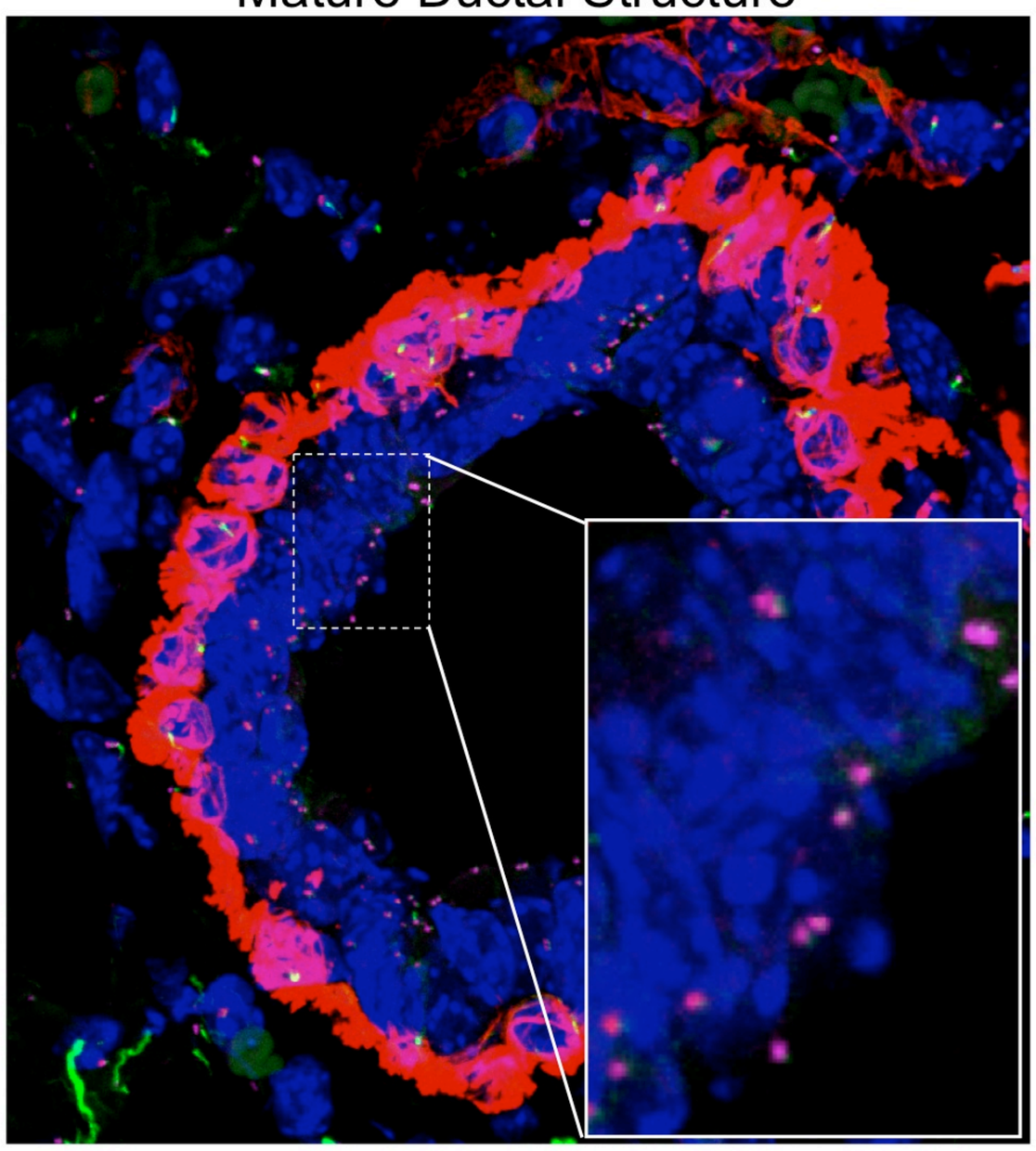
Wt, 4 Weeks
Mature Ductal Structure



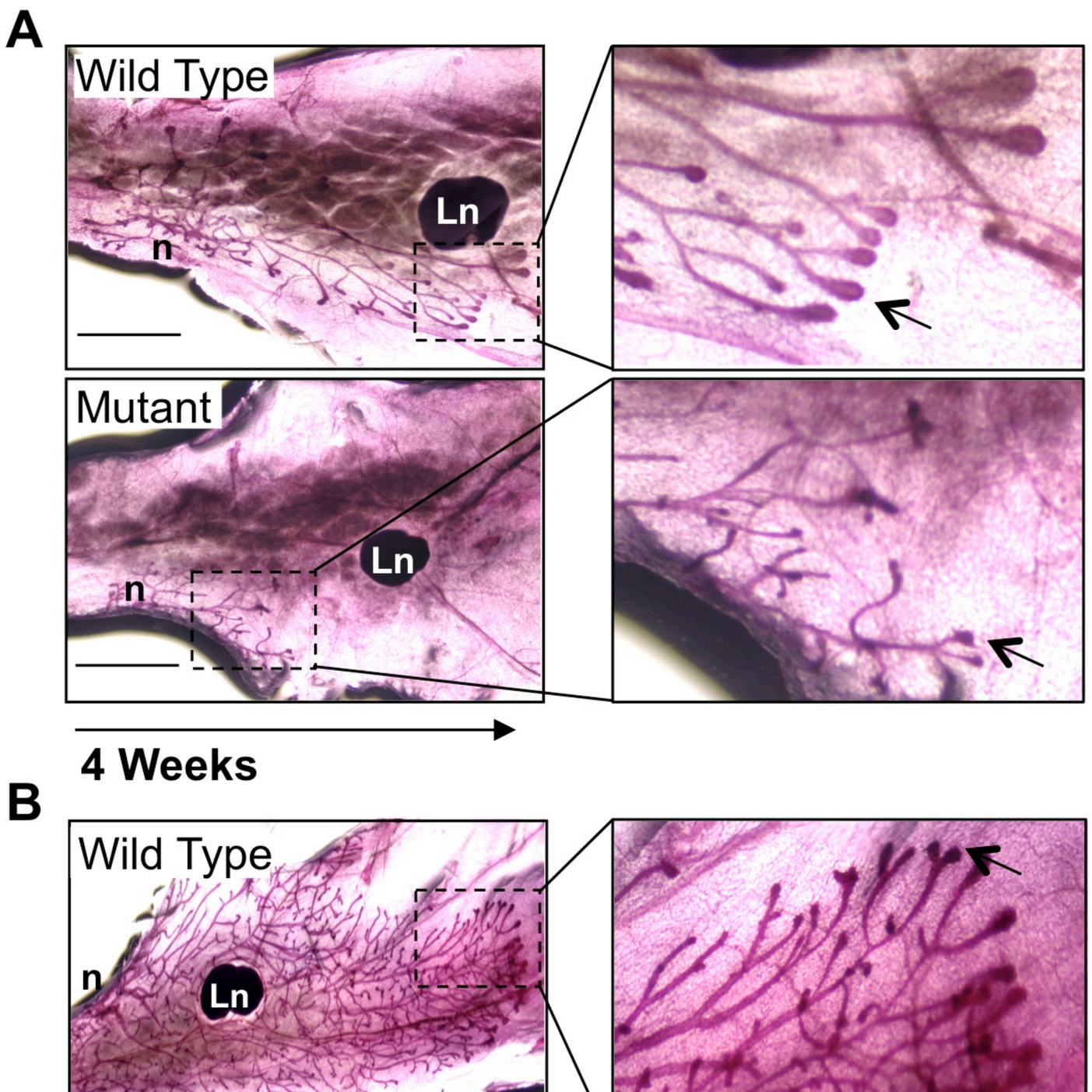
Wt, 4 Weeks
Terminal End Bud

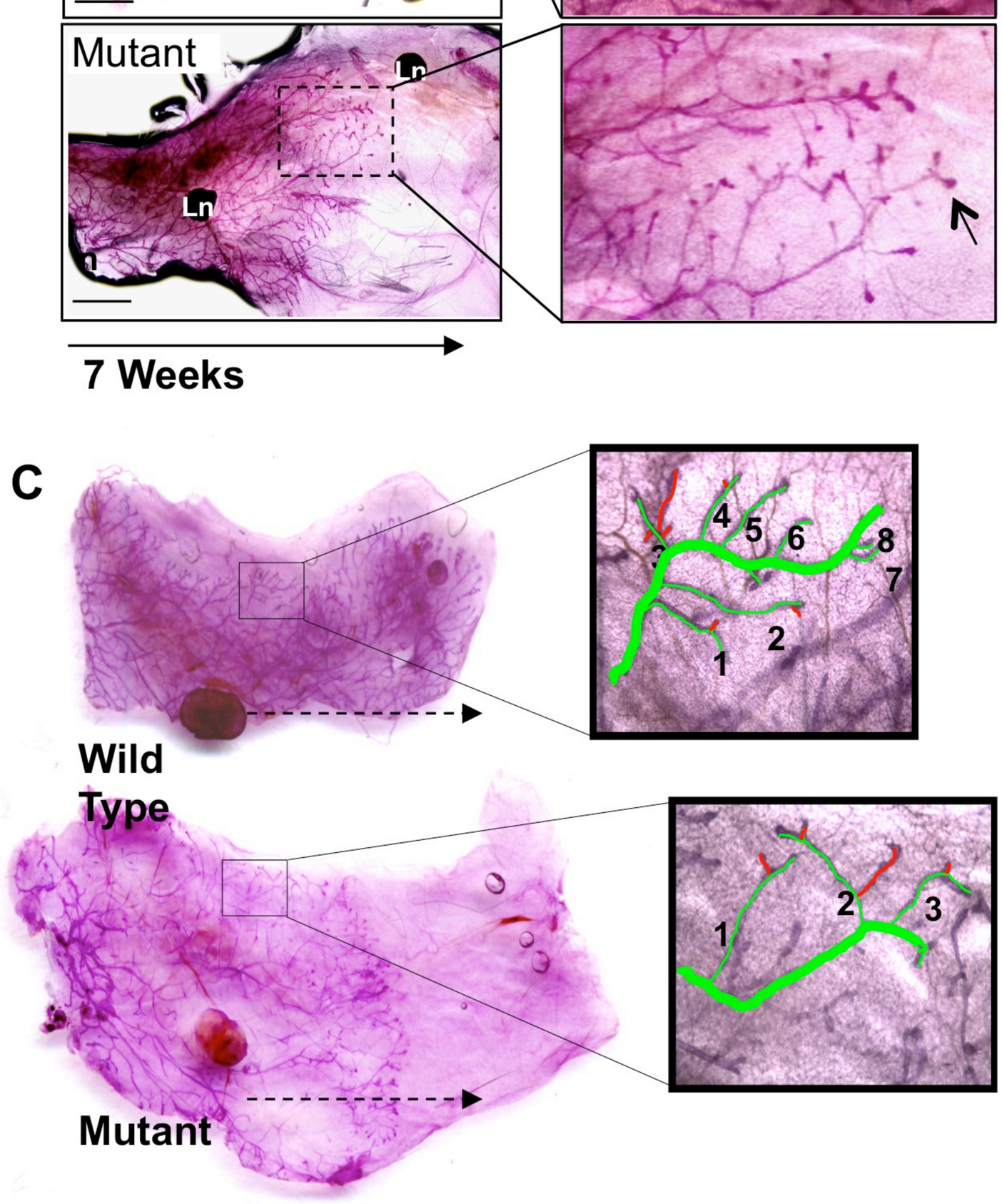


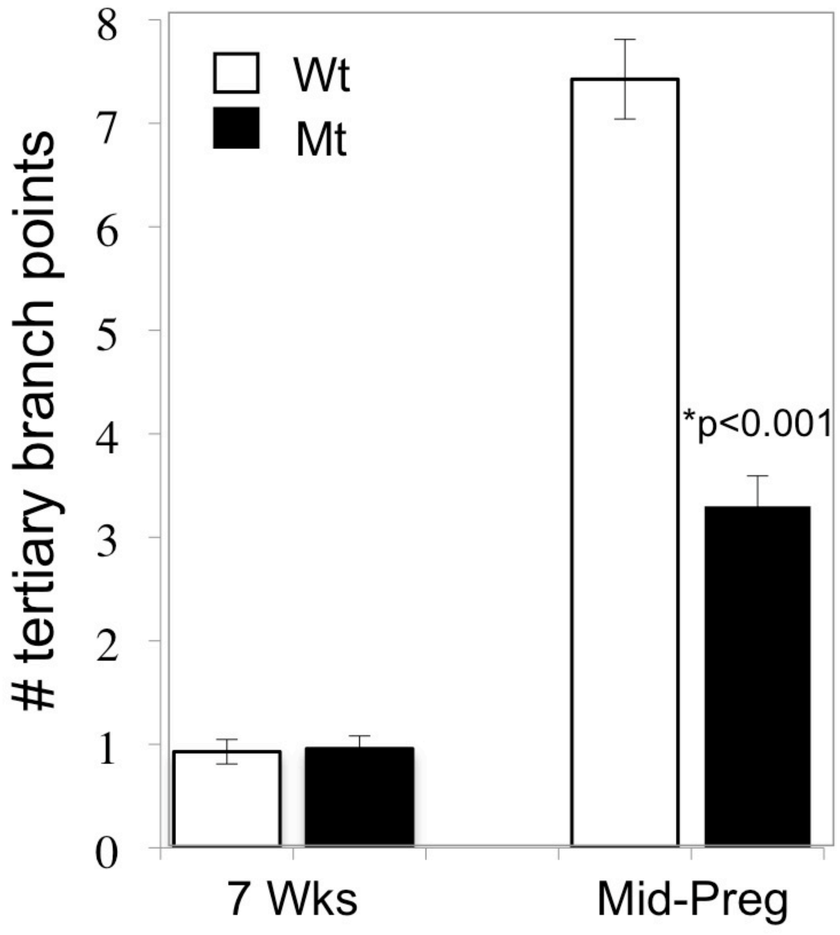
Wt, 7 Weeks
Mature Ductal Structure



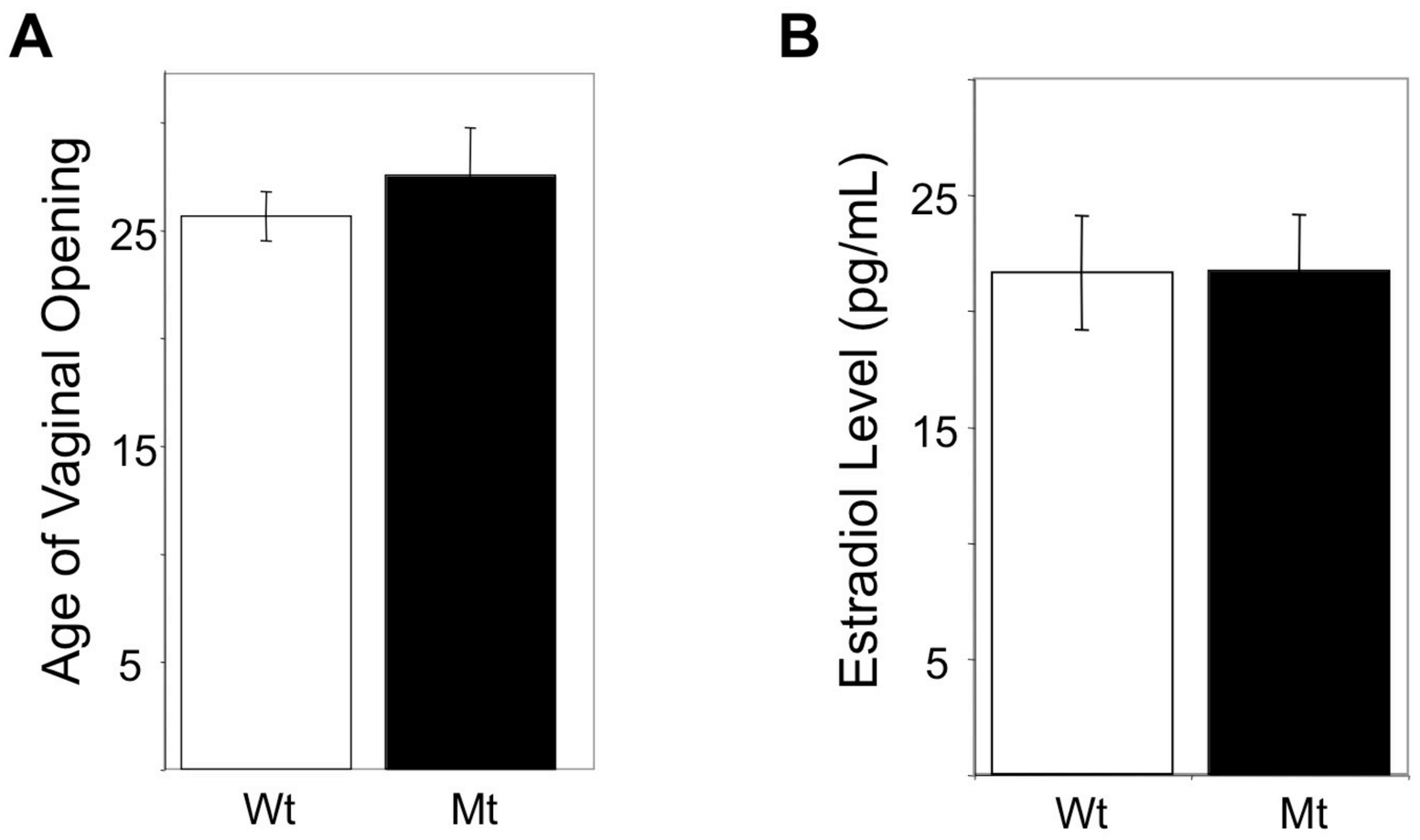
**Supplementary Figure 1 (cont.). Location of primary cilia during mammary gland development.** (C) Immunofluorescent confocal projections were acquired for the localization of primary cilia in the murine mammary glands to compare mature ductal structures and terminal end buds at 4 weeks and to mature ductal structures at 7 weeks of age. Cilia were detected by an antibody to acetylated α-tubulin (green), centrosomes were detected by an antibody to γ-tubulin (purple) and basally-located epithelial cells were identified with an antibody recognizing smooth muscle actin (red). Insets highlight the localization of cilia on luminal epithelial cells.

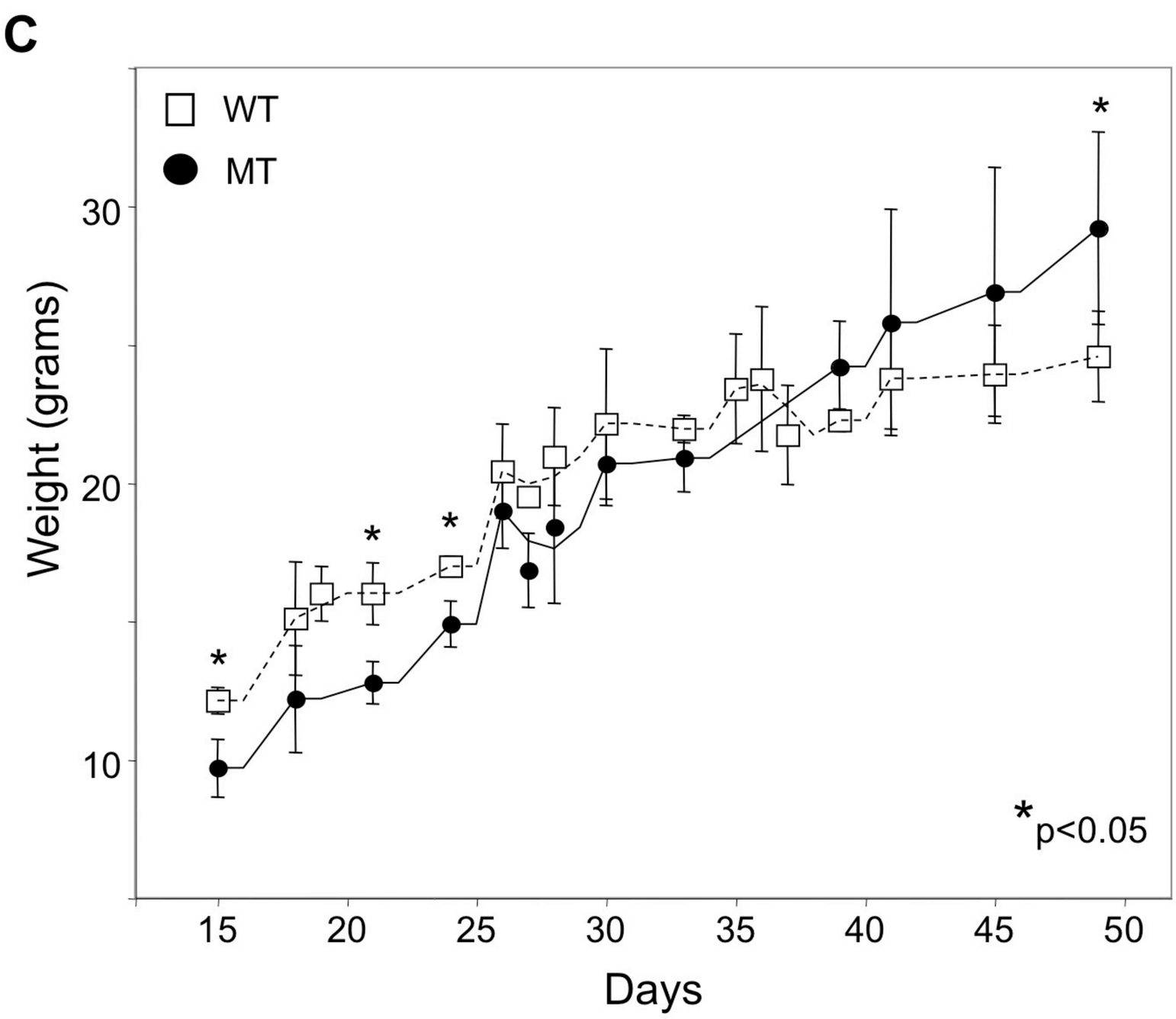


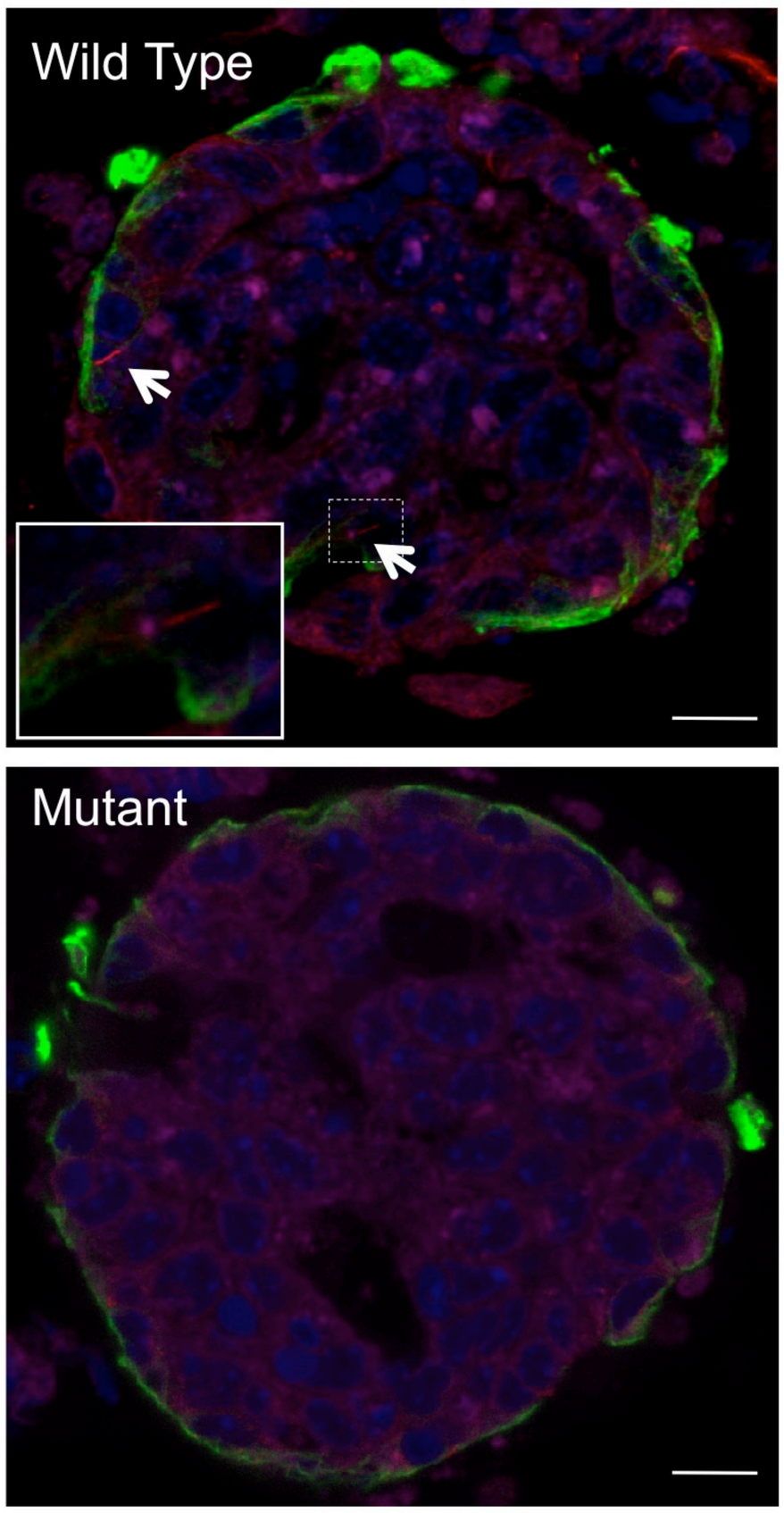

**Supplementary Figure 2. Primary cilia regulate branching morphogenesis.** Whole mount analysis was performed on wild type and mutant mammary glands harvested at **(A)** early development (4 weeks) and **(B & C)** late development (7 weeks). **(A & B)** The boxed insets are zoomed in areas of terminal end buds found at both 4 and 7 weeks in wild type and mutant mammary glands. **(C)** The boxed inset represents quantitation of branching in similar regions of the #4 inguinal fat pad to show a direct comparison between wild type and mutant branching. The green line and numbers represent number of branch points from a single duct and the red lines represent tertiary branch points. The bar graph represents quantitation of tertiary branch points at 7 weeks and mid-pregnancy.



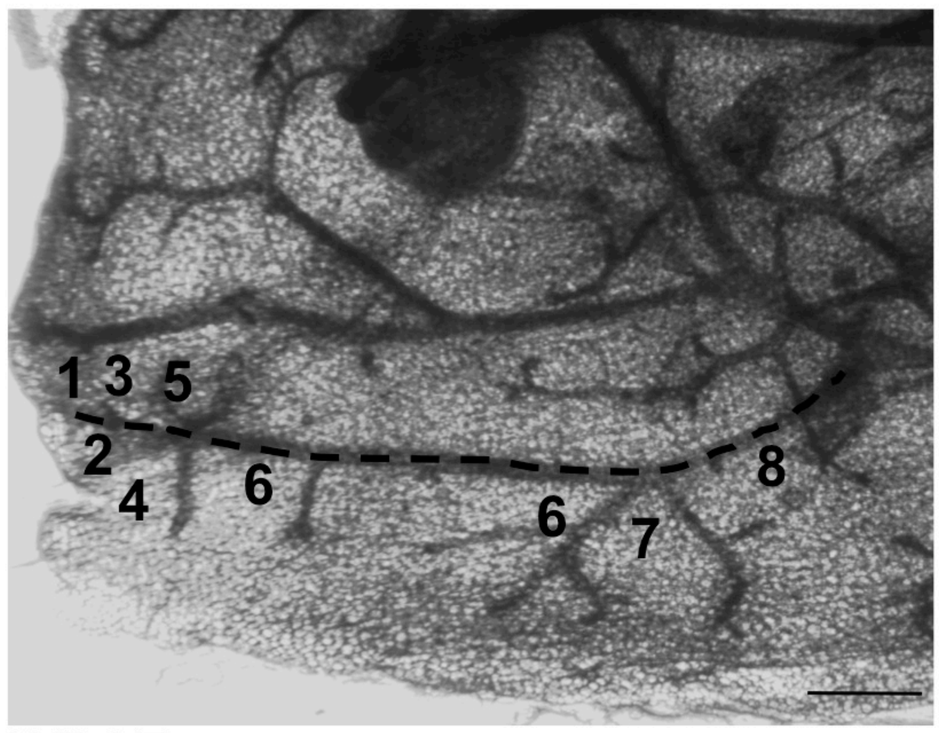




**Supplementary Figure 3. Loss of primary cilia does not affect onset of puberty.** (**A**) Date of vaginal opening, (**B**) estradiol level and (**C**) animal weight was determined for wild type and mutant Tg737$^{orpk}$ mice. Bar graphs represent the quantification of the age (day) of vaginal opening or the level of estradiol (pg/mL) found in the blood stream. Line graph plotted to visualize the wild type (white squares) and mutant (black circles) animal weights (grams) over time (days). Student's t-Test (2-sided) was performed to determine statistical significance (*). (n values for age of vaginal opening of Wt = 8 mice and Mt = 10 mice. n values for weights of Wt = 9 mice and Mt = 8 mice.)

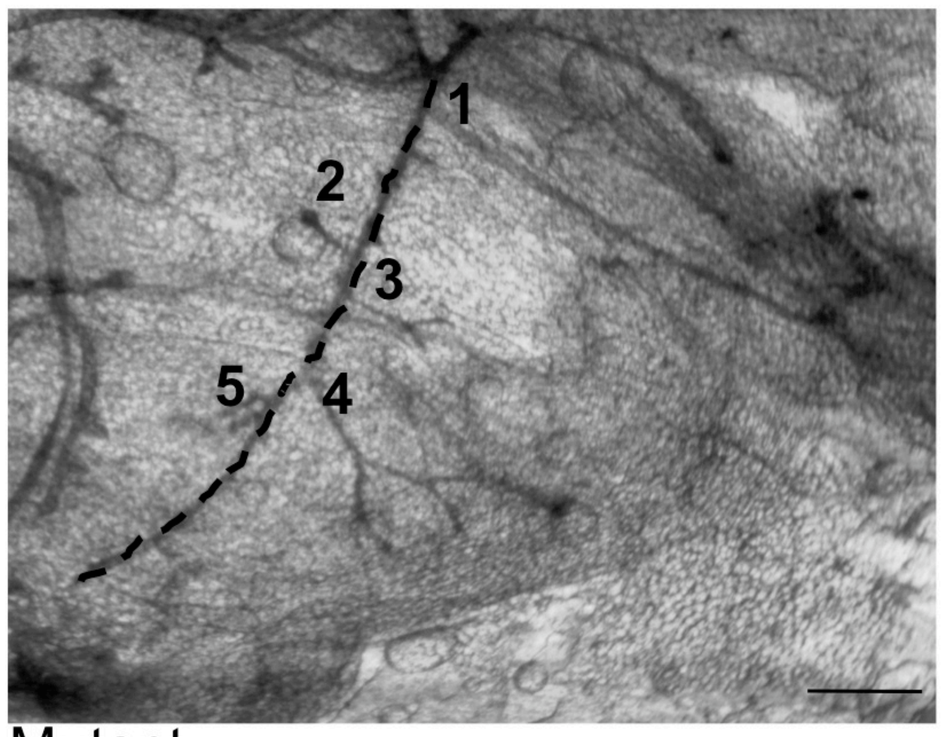
**A**




**B**



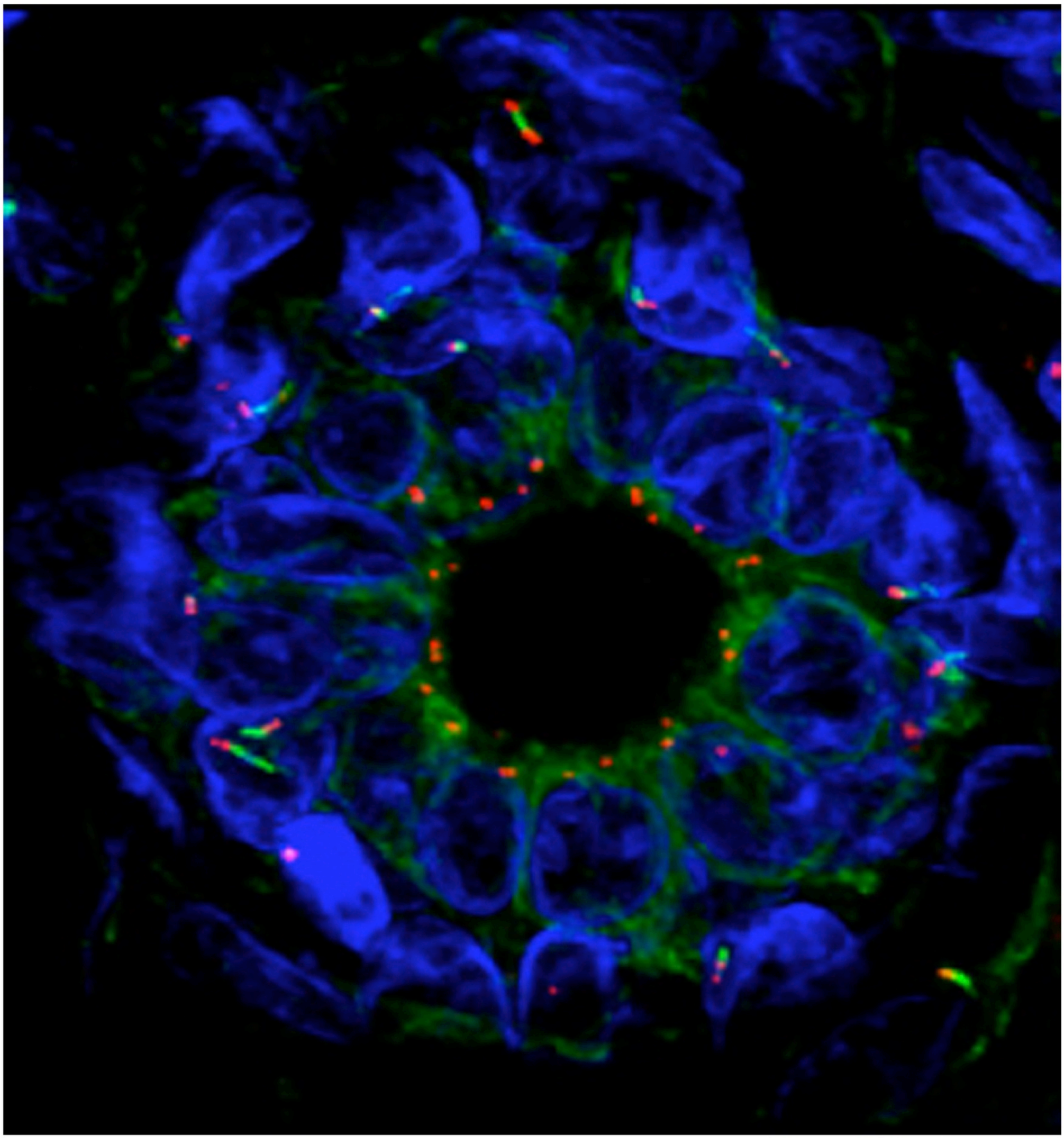

Wild Type




Mutant

**IFT20 Transplants**

**Supplementary Figure 4. Location of primary cilia *in vitro* and branching morphogenesis *in vitro*.** **(A)** Immunofluorescent confocal projections were acquired for the localization of primary cilia in the mammary tissue grown in *in vitro* 3D cultures. Cilia were detected by an antibody to acetylated α-tubulin (red), centrosomes were detected by an antibody to γ-tubulin (purple) and basally-located epithelial cells were identified with an antibody recognizing smooth muscle actin (green). Inset highlight the localization of cilia on basally located epithelial cells.
**(B)** Whole mount analysis was performed on wild type and mutant mammary glands harvested from mammary glands transplanted with wild type or Ift20 mutant mammary tissue. The dashed line and numbers represent number of branch points from a single duct.



**McDermott et. al., Cover Submission.**

**Primary Cilia in the Human Mammary Gland.** Immunofluorescent confocal projection of adult human mammary tissue for localization of primary cilia. Primary cilia were detected by an antibody to acetylated $\alpha$-tubulin (green), centrosomes were detected by an antibody to $\gamma$-tubulin (red) and the nuclei were identified with Hoechst dye (blue).