Supplementary Notes

Wnt9b^{neo} represents a partial loss of function allele.

The Wnt9b^{neo} allele has a PGK-Neo cassette inserted in the 2nd intron and loxp sites flanking the 2nd exon. *Wnt9b$^{neo/+}$* mice appeared normal at birth, however a small percentage (less than 10%) developed cystic kidneys later in life (data not shown). To test whether the *Wnt9b^{neo}* allele functioned as a partial loss of function (hypomorphic) allele or whether it affected the expression of another gene (neomorph), we examined the kidneys of *Wnt9b$^{neo/-}$* mice. *Wnt9b$^{neo/-}$* mice perished within 24 hours of birth. Histological analysis revealed that the mutant kidneys were severely hypoplastic with few obvious glomeruli but did possess a few mesenchyme-derived tubules (Data not shown). The *Wnt9b$^{neo/-}$* phenotype was more severe than the *Wnt9b$^{neo/neo}$* phenotype but less severe than the *Wnt9b$^{-/-}$* phenotype suggesting that the Wnt9b^{neo} allele is a hypomorph and that, in addition to its early inductive role, Wnt9b plays a second role during kidney tubule morphogenesis.

Wnt9b is expressed in the distal collecting ducts in postnatal and adult kidneys.

While we have previously shown that ureteric bud produced Wnt9b is involved in induction of the mesenchymally derived renal vesicles [1], expression data suggests that it may be involved in additional developmental processes. Section in situ hybridization shows that Wnt9b is expressed throughout the collecting duct epithelium of postnatal day 1(P1) and adult (7 months) kidneys (Supplementary figure 1a and c). Normally, renal vesicle induction is confined to the outer most (cortical) region of the kidney, as indicated by Pax8 positive cells (Supplementary figure 1b) and induction is reported to cease by P5 in mice [2]. However, Wnt9b is expressed in the distal (medullary) P1 collecting ducts (arrowhead in Supplementary figure 1a)

that are remote from areas of active tubule induction, and in adult kidneys after induction has ceased. Based on its expression in the medullary regions of the kidney, it seemed feasible that Wnt9b was also playing a role in tubule morphogenesis, differentiation or maintenance.

Characterization of KspCre;Wnt9b$^{flox/-}$ kidneys

KspCre is active in the distal collecting ducts through E15.5 and throughout the collecting ducts and ureteric bud tips at E17.5 (Supplementary figure 2 f and h). Comparison of KspCre activity and Wnt9b expression indicates that excision of Wnt9b by *KspCre* will ablate this gene in collecting duct stalks but leave its activity intact in the ureteric bud tips prior to E17.5 (Supplementary figure 2). Consistent with this hypothesis, E15.5 *KspCre;Wnt9b$^{-/flox}$* kidneys expressed several Wnt9b target genes including Wnt4, Wnt11, Pax8 and Lhx1 in a manner that was indistinguishable from wildtype littermates (Supplementary figure 3a, b, e and f and not shown). At E17.5, the number of Wnt4, Pax8 and Lhx1 positive renal aggregates/vesicles found in mutants was reduced compared to littermate controls but the ureteric bud tips continued to express Wnt11 (Supplementary figure 3 c, d, g and h and not shown). At P1, a timepoint where KspCre is active throughout the ureteric bud tips, all Wnt9b target genes examined, including Wnt11, were completely absent (data not shown). Although most P1 mutant kidneys were smaller than those of their wild-type littermates, they still contained numerous normally patterned, mature nephrons and the mutant kidneys produced urine (data not shown). At 10 days of age, *KspCre;Wnt9b$^{-/flox}$* mice were present at expected Mendelian ratios and did not display any overt external differences compared to wild-type littermates. However, by P20, mutants were identifiable based on significantly reduced body mass. Physiological analysis of P30 mice suggested that mutants had severe renal dysfunction based on measurements of urinary albumin (data not shown). The majority of *KspCre;Wnt9b$^{-/flox}$*

mice died between P30 and P45 and none survived past P60. Control littermates (*KspCre;Wnt9b*$^{+/flox}$) showed no defects in kidney histology or function (not shown).

Wnt9b mutants do not show defects in cell numbers

In most mouse models of cystic kidney disease, cystogenesis is associated with increased rates of cell proliferation and/or apoptosis[3]. To determine whether these processes contributed to cystogenesis in Wnt9b mutants, we examined rates of cell proliferation and apoptosis in P1 and P15 *Wnt9b* $^{neo/neo}$ kidneys. Surprisingly, although cysts were apparent in mutants at both stages, we were unable to detect significant differences in cell proliferation or cell death between wild type and *Wnt9b* mutants (Supplementary figure 5). This data suggests that the mechanism for cyst formation in *Wnt9b* mutants is independent of changes in cell number.

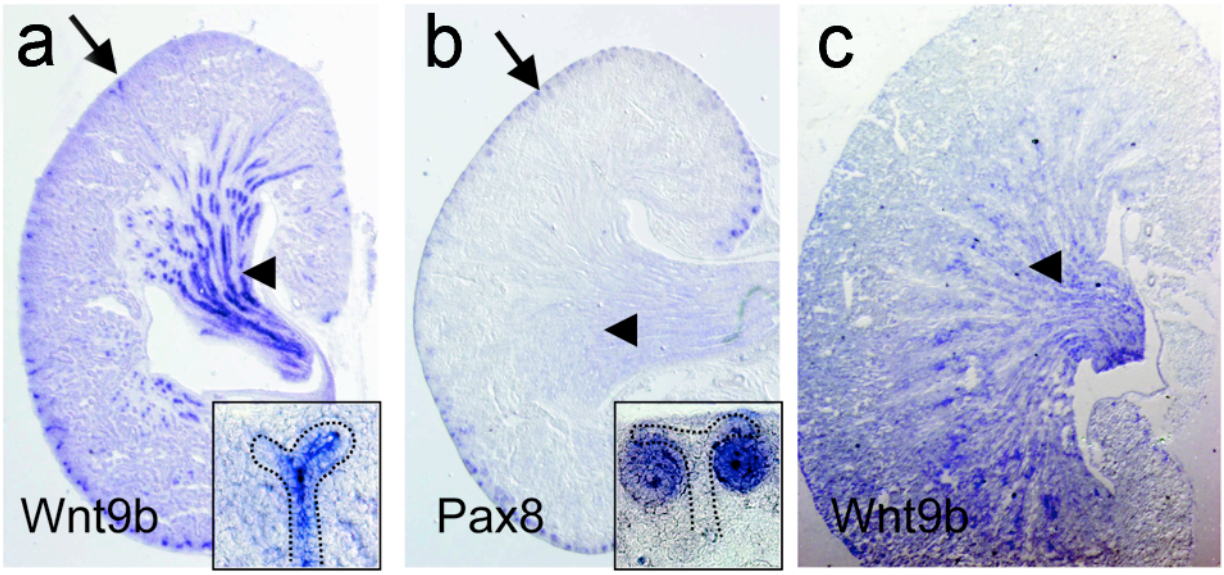
Wnt9b cystic defects are independent of β-catenin signaling.

To determine if the role for Wnt9b in establishing and maintaining tubule diameter was mediated by β−catenin, we first assayed the protein and mRNA levels of downstream components of the canonical pathway. We detected no difference in the level of dephosphorylated β−catenin (an indicator of canonical Wnt signaling) between wild-type and *Wnt9b*$^{neo/neo}$ kidneys at P1 (Figure 6a). To address the possibility that there were regionalized differences in β−catenin stabilization, we also examined the mRNA expression of a β−catenin reporter gene, axin-2, using section in situ hybridization [4-7]. As expected, based on the normal levels of de-phosphorylated β−catenin, axin-2 mRNA was still expressed at or above normal levels in *Wnt9b*$^{neo/neo}$ mutant collecting ducts indicating that canonical signaling was still intact in this tissue at a timepoint where there are clear cellular defects (Figure 6b and c). Of note, we did

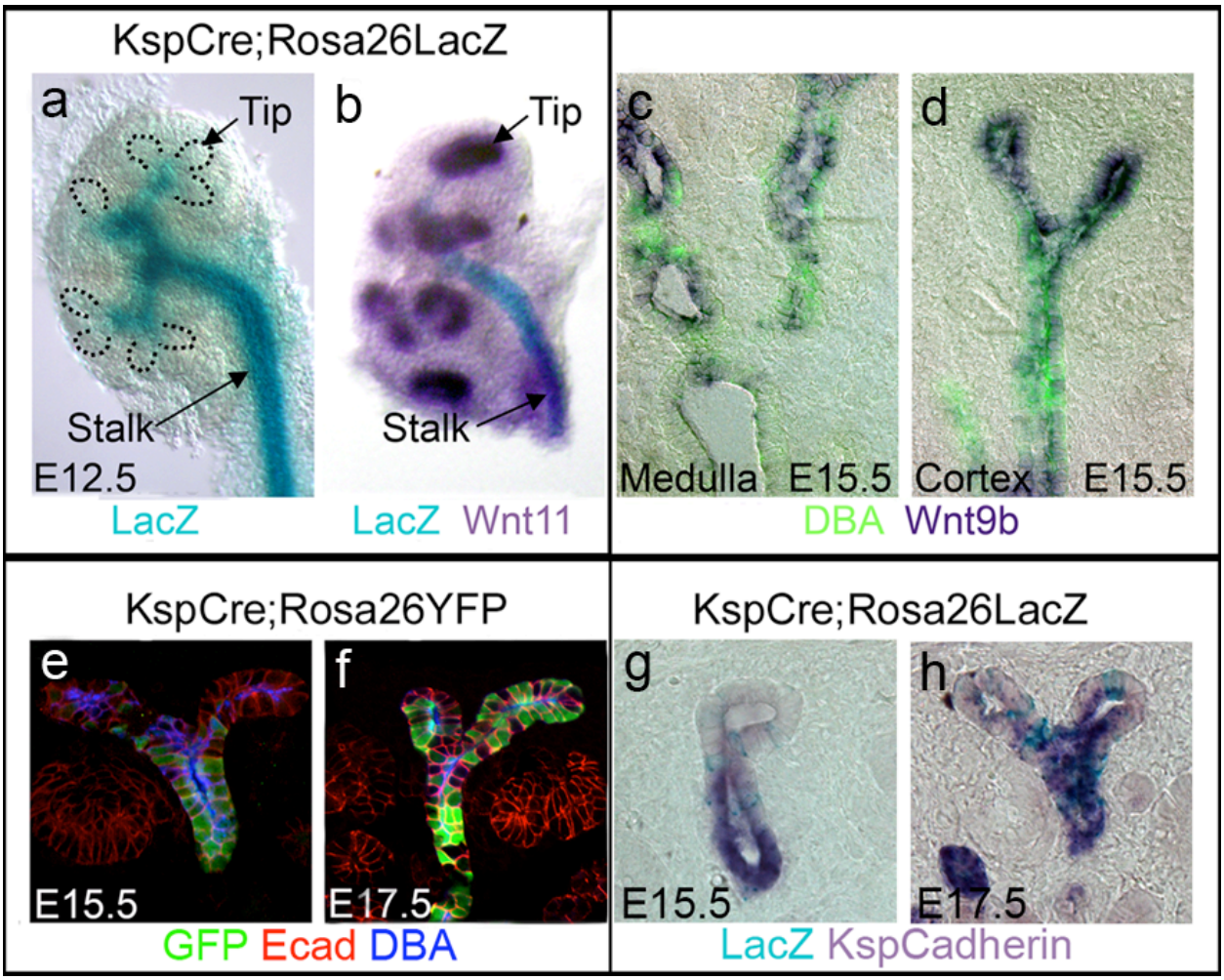
not detect ectopic expression of axin-2 mRNA in cystic proximal tubules suggesting that increased levels of β-catenin were not driving cyst formation (Figure 6c and data not shown).

In addition to the lack of significant change in the expression of β−catenin or β−catenin target genes, we also failed to observe genetic interaction between Wnt9b and β−catenin. Wnt9b/β−catenin compound heterozygotes had no discernible phenotype and removal of a single allele of β−catenin from *Wnt9b*$^{neo/neo}$ mutants had no measurable impact on cyst formation (data not shown).

Finally, we used the *KspCre* transgene to simultaneously ablate Wnt9b and to drive expression of an activated form of β−catenin (*catnb1*^{exon3flox} [8]) in the distal nephron epithelium. If Wnt9b signals through β−catenin, expression of activated β−catenin in the Wnt9b mutant background might rescue the Wnt9b mutant phenotype. Reciprocally, if Wnt9b signals though a non-canonical pathway, expression of activated β−catenin would not rescue the Wnt9b mutant phenotype. In fact, if Wnt9b signals independent of β-catenin, then activating β-catenin while inactivating Wnt9b might result in more severe cysts. Kidney tubules with activated β-catenin alone (*KspCre*;*catnb*^{exon3flox}) are mildly dilated at birth and die by P2 (Supplementary figure 7c and g and data not shown). However, the cystic index for P1 *KspCre*;*Wnt9b*$^{flox/-}$;*catnb*^{exon3flox} kidneys was significantly greater than that of *KspCre*;*Wnt9b*$^{-/flox}$ or *KspCre*;*catnb*^{exon3flox} kidneys further supporting the hypothesis that, in this cellular context, Wnt9b does not signal through β-catenin (Supplemental Figure 7i).



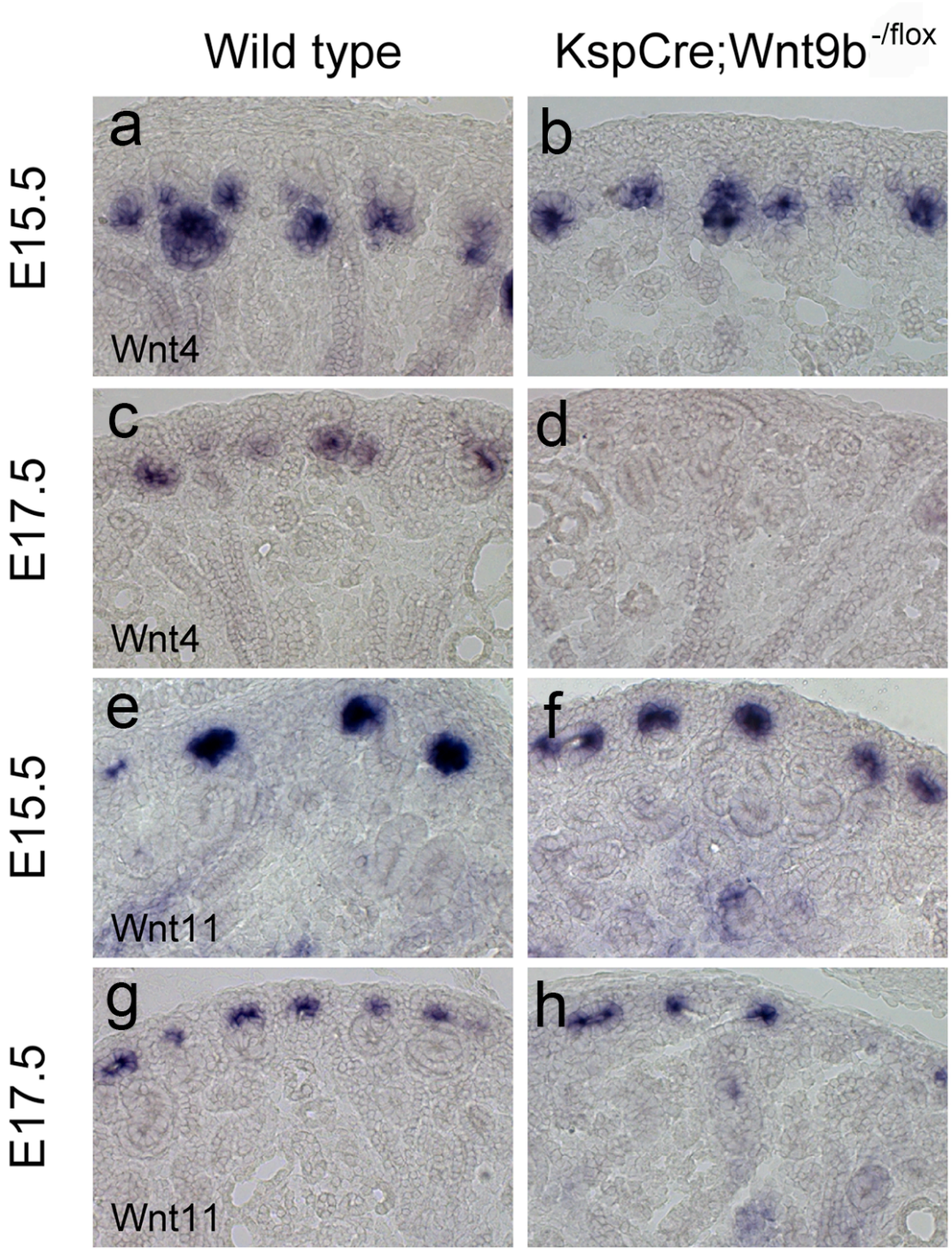
Supplementary figure 1: Expression of Wnt9b in the kidney.
Sections of P1 (a and b) and adult (7 month) kidneys hybridized with riboprobes to Wnt9b (a and c) and Pax8 (b). (a) Wnt9b is expressed in both the tips of the ureteric buds (arrows and inset) and in the distal collecting ducts in the kidney medulla (arrowhead) at P1. (b) Pax8 is expressed in renal vesicles adjacent to the ureteric buds (arrow and inset) but is not expressed in the medullary regions of the kidney (arrowhead) at P1. (c) Wnt9b continues to be expressed in the distal collecting ducts of adult kidneys (arrowhead) after nephrogenesis has ceased.



Supplementary figure 2: *KspCre* is expressed only in the ureteric stalk during early stages of kidney development.

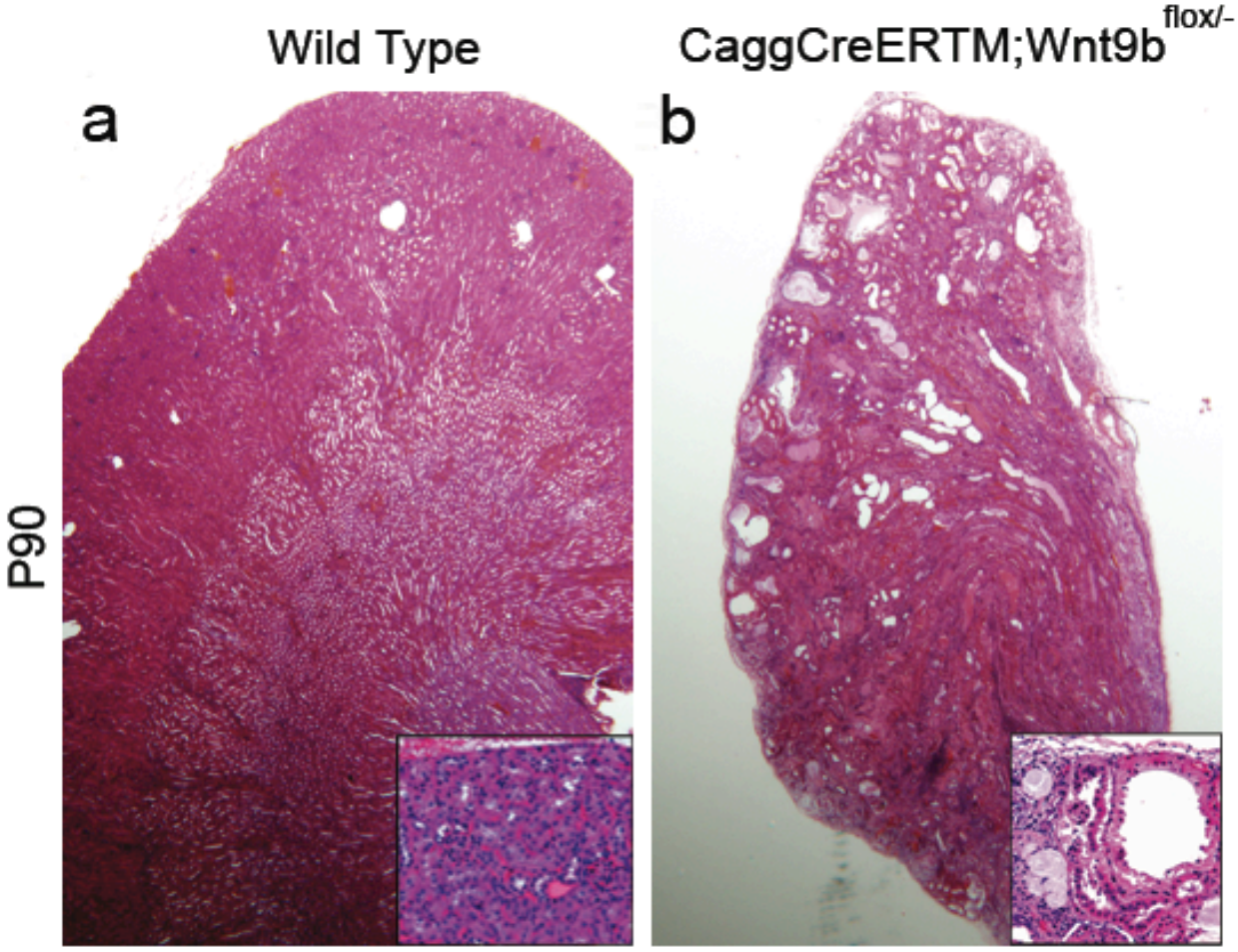
E12.5 *KspCre*;*R26R* kidneys stained with X-gal (light blue in a) or X-gal and an antisense Wnt11 probe (light blue and purple, respectively in b). LacZ staining is apparent in the ureteric stalk (arrows in a and b) but is not observed in the outlined tips of the ureteric bud of (a) or the Wnt11 positive bud tips (b). The images in a and b are from the same kidney before (a) and after hybridization with an antisense Wnt11 probe. Wnt9b expression (dark blue in c and d) co-localizes with DBA lectin (green in c and d) in the medulla (c) and the ureteric bud tips (d) at E15.5. *KspCre;R26YFP* kidneys stained with anti-GFP (green in e and f), anti-E-cadherin (red in e and f), and DBA lectin (blue in e and f). At E15.5, KspCre activity (as shown by GFP expression) co-localizes with DBA lectin in the stalk but not in the tips of the ureteric bud (e). By E17.5, KspCre activity colocalizes with DBA throughout the collecting ducts and ureteric bud (f). *KspCre;R26LacZ* kidneys stained with X-gal and a KspCadherin antisense probe (g and h). At E15.5 (g), KspCadherin expression completely overlaps with LacZ staining in the stalk of the ureteric bud while both are absent from the branching tips. By E17.5 (h), KspCadherin expression and LacZ staining co-localize throughout the ureteric bud suggesting that KspCre

activity in the tips is the result of expanded Ksp expression and not the result of cell mixing or migration.



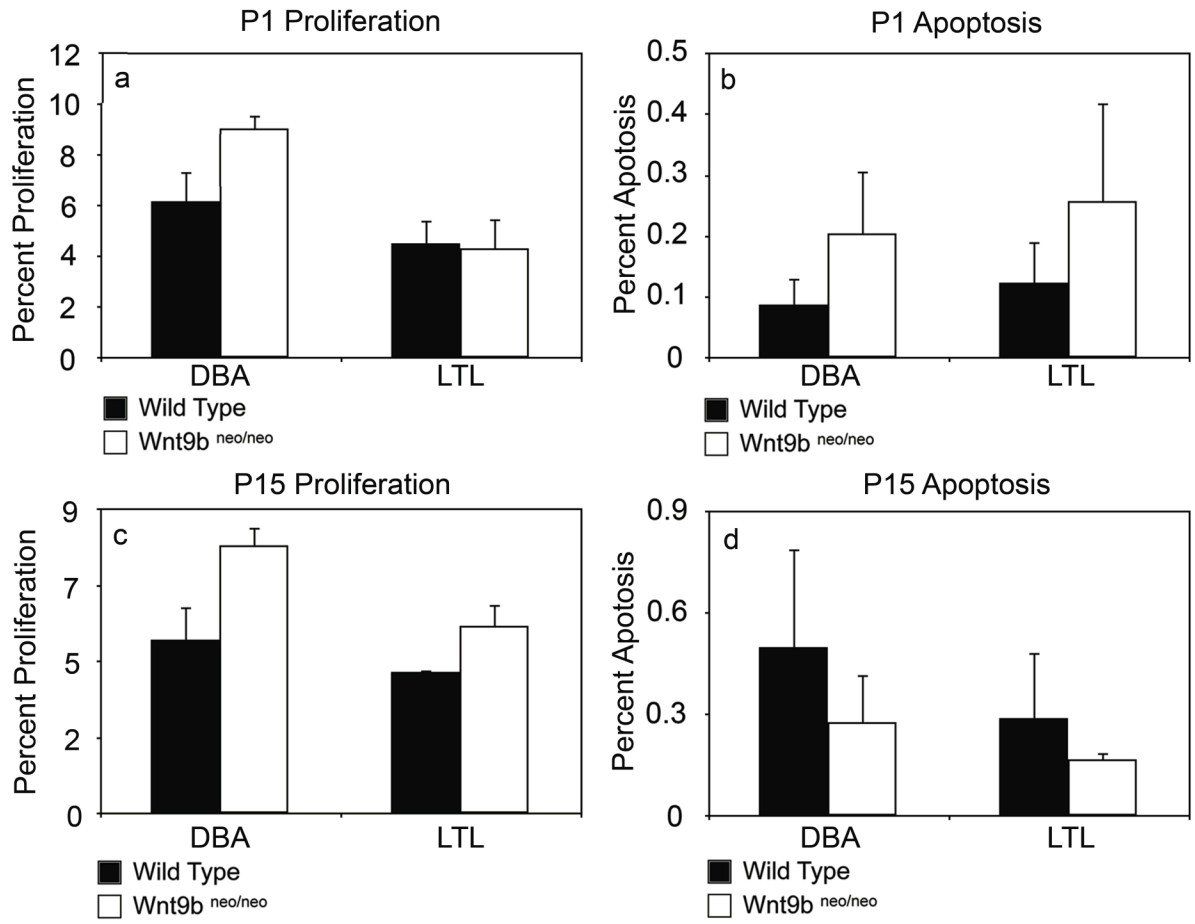
Supplementary figure 3: Loss of Wnt9b target gene expression at E17.5.
Section in situ hybridization with an antisense probe for the Wnt9b target gene Wnt4 (a-d) and Wnt11 (e-h) at E15.5 (a, b and e, f) and 17.5 (c, d and g, h) in wild type (a, c, e, g) and *KspCre;Wnt9b*$^{-/flox}$ (b, d, f, h) kidneys. Wnt4 is expressed normally in *KspCre,Wnt9b*$^{-/flox}$ kidneys

at E15.5 (compare a to b) but is absent from *KspCre;Wnt9b* $^{-/flox}$ kidneys at E17.5 (Compare c to d). There is no change in the expression of Wnt11 at E15.5 (e and f) or E17.5 (g and h).



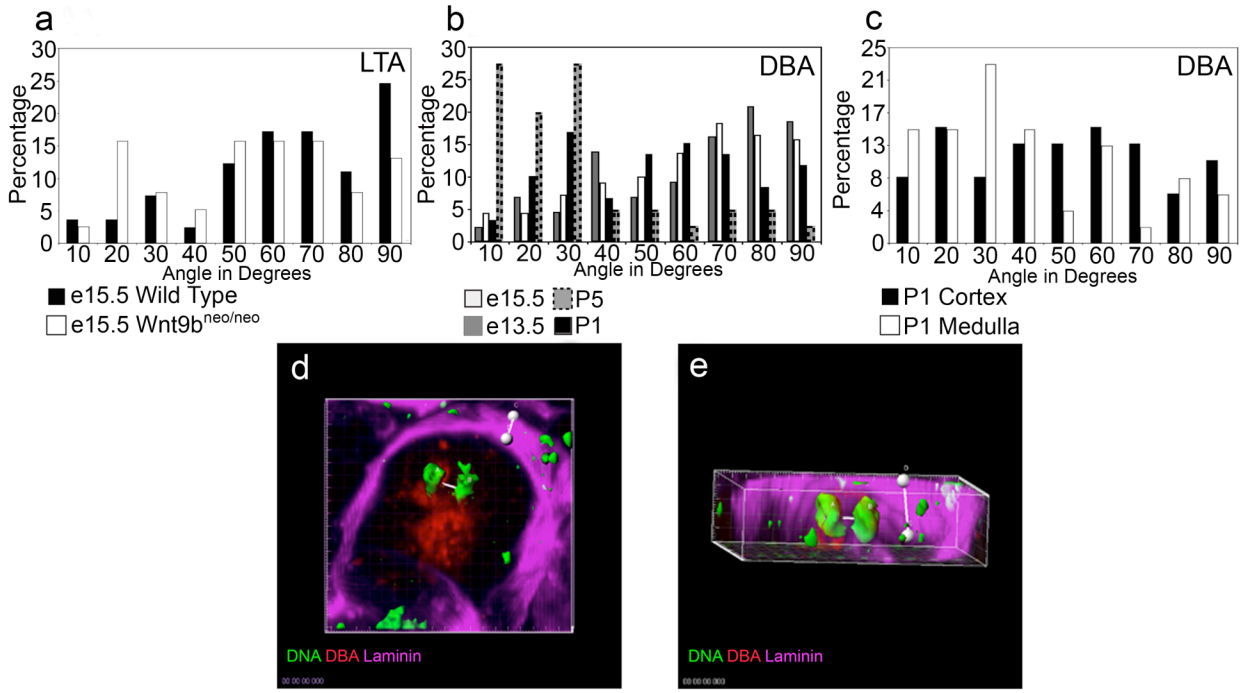
Supplementary figure 4: Ubiquitous removal of Wnt9b after E15.5 results in cyst formation.

H&E stained sections of P90 wild type (a) or *CaggCreErTm;Wnt9b*$^{-/flox}$ (b) kidneys. Insets show high magnification images of cortical epithelia. Mutant kidneys are significantly reduced in size and have cysts present.



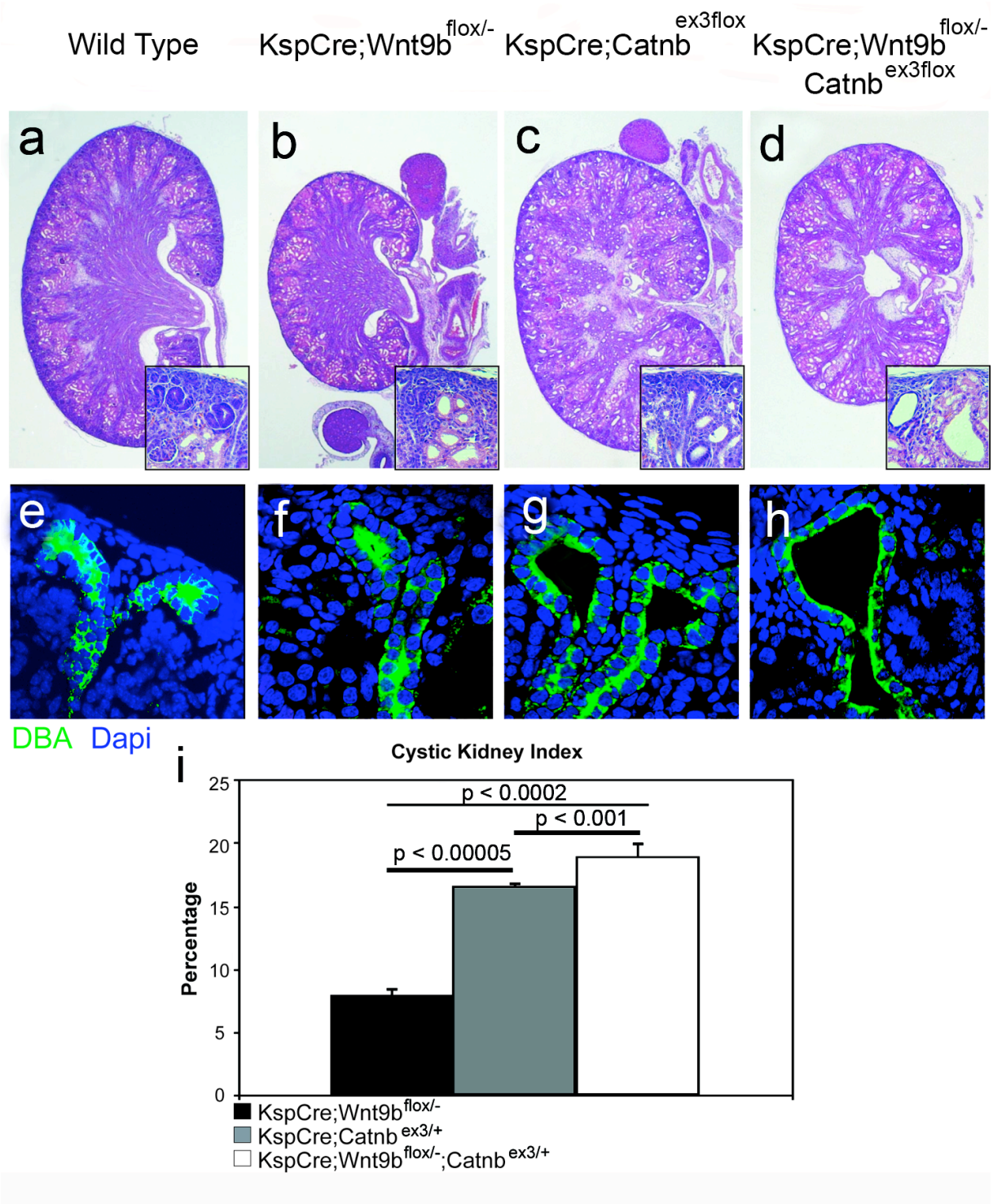
Supplementary figure 5: No change in rates of proliferation or apoptosis in Wnt9b mutant kidneys.

Graphical depiction of the rates of proliferation (a and c) or apoptosis (b and d) in both DBA and LTL positive tubules in P1 (a and b) or P15 (c and d) wild type (black bars) and *Wnt9b*$^{neo/neo}$ (white bars) kidneys. There was no significant difference in the rates of proliferation at P1 (P=.0.9 for DBA and 0.9 for LTL) or P15 (p=0.3 for DBA and 0.7 for LTL) or apoptosis at P1 (p=0.3 for DBA and 0.4 for LTL) or P15 (p=0.4 for DBA and 0.7 for LTL). N=3 kidneys (1-1.2 thousand cells), per stage, tubule segment, genotype and assay. Error bars represent the standard error for each sample.



Supplementary figure 6: Cell division becomes oriented after birth.

(a) Graphical representation of the angle between the mitotic spindles and the longitudinal axis of LTL-positive tubules at E15.5 indicates that cell division in both wild type (black bars) and *Wnt9b*$^{neo/neo}$ tubules (white bars) is randomly oriented at E15.5 when compared to the expected random distribution by the Kolmogorov-Smirnov (KS) test. $P > 0.50$ for both wild type (n=81) and mutant (n=48). (b) Graphical representation of the angle between the mitotic spindles and the longitudinal axis of wild-type, DBA-positive tubules at E13.5 (gray bars), E15.5 (white bars), P1 (black bars) and P5 (hatched gray bars). Although cell division appears random at both E13.5 and E15.5 ($p>.5$, n=43, and 50 for E13.5 and E15.5), it is no longer random at P1 ($0.05>p>0.02$, n=159) or P5 ($p<0.001$, n=81) indicating that cell division becomes oriented around the time of birth. (c) Quantitation of the angle between mitotic spindles and the longitudinal axis of DBA-positive tubules in the cortex (black bars) or medulla (white bars) at P1 suggests that there are no spatial differences in orientation of cell division (n=47 for cortex and 53 for medulla). (d and e) Representative image of E15.5 wild type dividing cell used to ascertain orientation of cell division. The image in e is the same as d rotated 90 degrees. The actual vectors for the tubule basal lamina and the dividing cell are shown.



Supplementary figure 7: Activation of β-catenin in a Wnt9b mutant background enhances cystogenesis.

H&E sections of P1 wild type (a), *KspCreWnt9b*$^{-/flox}$ (b), *KspCre;catnb1*^{ex3flox} (c), and *KspCre;Wnt9b*$^{-/flox}$*;catnb1*^{ex3flox} (d). Insets in a, b, c and d show high magnification images of cortical epithelia. Confocal images of P1 wild-type (e), *KspCre;Wnt9b*$^{-/flox}$ (f), *KspCre;catnb1*^{ex3flox} (g) and *KspCre;Wnt9b*$^{-/flox}$ *;catnb1*^{ex3flox} (h) kidneys stained with DBA lectin.

Quantitation of the cystic index for each genotype (i) shows that *KspCre;Wnt9b*$^{-/flox}$ *;catnb1*^{ex3flox} kidneys are more severely cystic than either *KspCre;Wnt9b*$^{-/flox}$ or *KspCre;catnb1*^{ex3flox} kidneys. N=3 kidneys per genotype and 4 sections per kidney. Error bars represent the standard error for each sample (Mann-Whitney U test).

Supplementary References:

1. Carroll, T.J., Park, J.S., Hayashi, S., Majumdar, A. & McMahon, A.P. Wnt9b plays a central role in the regulation of mesenchymal to epithelial transitions underlying organogenesis of the mammalian urogenital system. *Dev Cell* **9**, 283-92 (2005).
2. Hartman, H.A., Lai, H.L. & Patterson, L.T. Cessation of renal morphogenesis in mice. *Dev Biol* **310**, 379-87 (2007).
3. Ibraghimov-Beskrovnaya, O. Targeting dysregulated cell cycle and apoptosis for polycystic kidney disease therapy. *Cell Cycle* **6**, 776-9 (2007).
4. Marose, T.D., Merkel, C.E., McMahon, A.P. & Carroll, T.J. Beta-catenin is necessary to keep cells of ureteric bud/Wolffian duct epithelium in a precursor state. *Dev Biol* **314**, 112-26 (2008).
5. Lustig, B. et al. Negative feedback loop of Wnt signaling through upregulation of conductin/axin2 in colorectal and liver tumors. *Mol Cell Biol* **22**, 1184-93 (2002).
6. Leung, J.Y. et al. Activation of AXIN2 expression by beta-catenin-T cell factor. A feedback repressor pathway regulating Wnt signaling. *J Biol Chem* **277**, 21657-65 (2002).
7. Jho, E.H. et al. Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. *Mol Cell Biol* **22**, 1172-83 (2002).
8. Harada, N. et al. Intestinal polyposis in mice with a dominant stable mutation of the beta-catenin gene. *Embo J* **18**, 5931-42 (1999).