WNT9B SIGNALS THROUGH TWO DISTINCT PATHWAYS TO REGULATE PROGENITOR MAINTENANCE, DIFFERENTIATION AND MORPHOGENESIS DURING KIDNEY DEVELOPMENT

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by

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DEDICATION

This thesis is dedicated to the memory of Dr. Miles Allen Karner.

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ABSTRACT

The development of the kidney is dependent upon reciprocal interactions between the ureteric bud epithelium and a mesenchymal progenitor population known as the metanephric mesenchyme. Signals from the ureteric bud cause a subset of the progenitors to differentiate into tubules while the remainder of the mesenchyme undergoes proliferation/ self renewal. This process of progenitor expansion and differentiation reiterates itself during the course of development, ultimately resulting in the formation of approximately 20,000 tubules per mouse kidney. Establishment of the correct number of tubules depends on a careful balance between the induction of new tubules and the expansion of the existing tubule progenitor cells. The molecular mechanisms by which this balance is achieved are poorly understood. Previous studies suggested that Wnt/β-catenin signaling was necessary and sufficient to induce tubule formation. Further, it was hypothesized that the pathway must be repressed in the progenitors to keep them in an undifferentiated state. However, using a combination of microarray analysis and genetics, I have found that Wnt/β-catenin signaling is active in the progenitor compartments. My data indicate that rather than inhibiting β-catenin activity, factors in the progenitor cells (primarily the transcription factor

Six2) alter the transcriptional output of β-catenin signaling. Wnt/β-catenin signaling is required for stem cell renewal/proliferation of the progenitor cells. These data reveal a mechanism through which cells receiving the same Wnt9b signal can respond in distinct ways (differentiation vs. proliferation). Utilizing this simple mechanism, the kidney balances the induction of new tubules and the maintenance of tubule progenitors, thereby insuring formation of the proper number of tubules.

While the mechanisms regulating tubule formation have been well studied, little is known of the mechanisms that establish the length or diameter of these tubules. In the kidney, defects in the establishment or maintenance of tubule diameter are associated with one of the most common inherited human disorders, polycystic kidney disease. Here, I show that attenuation of Wnt9b signaling during kidney morphogenesis affects the planar cell polarity of the epithelium and leads to tubules with significantly increased diameter. Although previous studies showed that polarized cell divisions played an important role in the maintenance of postnatal kidney tubule diameter, we find that during early embryonic development, cell divisions are randomly oriented. Therefore the orientation of cell division plays little role in kidney tubule diameter establishment. Instead, our data suggest that diameter is established during early morphogenetic stages by convergent extension processes

and maintained by polarized cell divisions. Wnt9b, signaling through the non-canonical Rho/Jnk branch of the Wnt pathway, is necessary for both of these processes. These findings provide novel insights into the molecular mechanisms that regulate the kidney tubule development and morphogenesis and greatly enhance our ability to generate kidney stem cells and engineer tubules for damaged organs.

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LIST OF ABBREVIATIONS

ADPKD - Autosomal dominant polycystic kidney disease

AJ - Adherens junction

APC - adenomatous polyposis coli

ARPKD - Autosomal recessive polycystic kidney disease

Atro - Atrophin

BBS - Bardet-Biedl syndrome

BIO - 6-bromoindirubin-3'-oxime

Catnb - β-catenin

CE - Convergent extension

CK1α - Casein Kinase 1α

Crym - Mu crystallin

Cthrc1 - Collagen triple helix repeat containing 1

DBA - Dolichos bifloris agglutinin

Dgo - Diego

DMEM - Dulbecco's modified eagle's medium

DMSO - Dimethyl sulfoxide

Ds - Dachsous

Dsh - Dishevelled

E - Embryonic day

Fgf - Fibroblast growth factor

FJ - Four jointed

Fmi - Flamingo

Ft - Fat

Fzd - Frizzled receptor

GDNF - Glial derived neurotrophic factor

GSK3β - glycogen synthase kinase 3-β

HMG - high mobility group-type transcription factors

IFT - Intraflagellar transport

IM - Intermediate mesoderm

Inv - Inversin

IWP - Inhibitor of Wnt production

IWR - Inhibitor of Wnt response

Jnk - Jun Kinase

KS - Kolmogorov-Smirnov test

Lef/Tcf - Lymphoid-enhancing factor/T-cell factor

LRP - Low-density lipoprotein

LTL - Lotus tetragonolobus lectin

MDCK - Madin-Darby canine kidney cells

MET - Mesenchymal to epithelial transition

MM - Metanephric mesenchyme

OCD - Orientation of cell division

P - Postnatal day

PCP - Planar cell polarity

Pk - Prickle

PKC - Protein Kinase C

PKD - Polycystic kidney disease

Pla2g7 - Phospholipase A2 group 7

PTA - Pre-tubular aggregates

RV - Renal vesicle

Rspo - Rspondin

SB - S-shaped body

s.e.m - Standard error of the means

SHIVR - Surface heterochronic *in vitro* recombinations

SFRP - Secreted frizzled related protein

Stbm/Vang - Strabisimus/Van Gogh

Tafa5 - expressed sequence AW049604

THP - Hamm-Horsfall protein

UB - Ureteric bud

WD - Wolffian duct

Wdb - Widerborst

CHAPTER ONE

Introduction

Understanding the cellular and molecular events that govern kidney development is imperative due to the fact that many pathological events in the kidney recapitulate developmental processes. The crux of kidney development centers on the generation and development of vascularized epithelial tubules known as nephrons. In mice, each kidney contains up to 20,000 nephrons while in humans the adult kidney contains approximately 1 million nephrons. These nephrons regulate the fluid volume, acid/base homeostasis and mineral composition of blood by either excreting or reabsorbing water and inorganic electrolytes. Thus the normal function and health of all other organs are intimately associated with the proper development and function of the kidneys.

Kidney development is an intricate process that is dependent upon the integration of multiple signaling and morphological events. These events must not only be balanced at the sub-cellular and cellular levels but must also be translated into morphological events and cellular movements at the tissue level. As such, defects in the development of the kidney and urogenital tract constitute some of the most common congenital birth defects. Misregulation of the Wnt signal transduction pathway has been implicated in multiple congenital birth defects and kidney diseases including renal hypoplasia and aplasia, autosomal recessive polycystic kidney disease (ARPKD), autosomal dominant polycystic kidney disease

(ADPKD) [1-7], Nephronophthisis [8], Wilms Tumor [9, 10], and SERKAL syndrome [11]. A better understanding of the role that Wnt signaling plays during kidney development is essential to further our ability to diagnose and treat kidney pathologies that occur later in life. This thesis explores the role of Wnt9b signaling during kidney development and provide clues about its role in regulating progenitor cell maintenance, mesenchymal-to-epithelial transition (MET), and cellular morphogenesis.

General overview of kidney development

During mammalian embryonic development, three sets of kidneys form sequentially in an rostro-caudal manner within the intermediate mesoderm (IM): the pronephros, mesonephros and metanephros [12] (Figure 1-1). Signals from the surface ectoderm and the somites induce cells in the IM to undergo a MET to form the Wolffian duct (WD) and the pronephros [12-14]. In mammals, unlike other vertebrates such as *Xenopus* and zebrafish, the pronephros is a transient organ and appears to be non-functional physiologically. However, pronephros formation is essential for the development of both the meso- and metanephros [15]. After formation, the WD migrates caudally, inducing cells of the adjacent mesenchyme to aggregate and form the mesonephric tubules. Unlike the pronephros, the mesonephric tubules are functional. In amphibians and fish, the mesonephros functions as the adult excretory organ. In humans, the mesonephric kidney maintains the homeostasis of

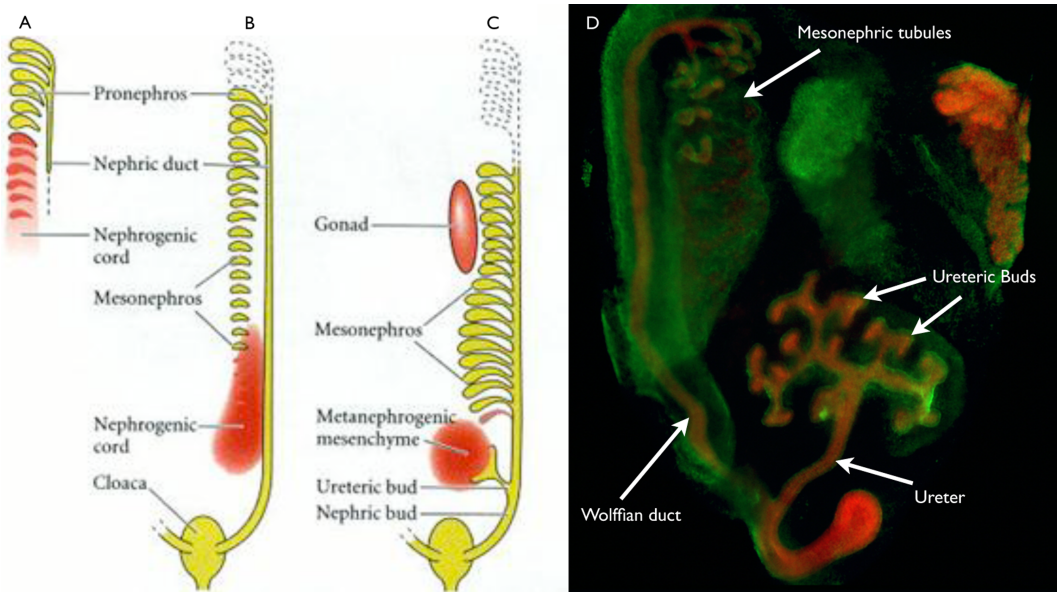

Figure 1-1: Kidney development.
Schematic representation of the developing kidneys. (A) After induction, the nephric duct (wolffian duct) migrates caudally and induces the formation of tubules and a single glomerulus to form the pronephros. (B) As the pronephros degenerates, the mesonephric tubules are induced by the migrating Wolffian duct. (C) Upon reaching the metanephric mesenchyme, the Wolffian duct branches to form the ureteric bud. The ureteric bud invades the metanephric mesenchyme and begins to induce nephrons and undergo branching morphogenesis to form the metanephric kidney. (D) An E11.5 urogenital system that has been cultured for 48 hours and stained with E-cadherin (red) and Laminin (green). Both the mesonephric and metanephric kidney are apparent. At this stage the ureteric bud has invaded the metanephric mesenchyme and undergone multiple branching events forming many bud tips. A-C is modified from *[12]*

the amniotic fluid, a function necessary for the development of some organs including the lungs.

The third and final kidney to form is the metanephros. The WD continues its caudal migration until it reaches the metanephric mesenchyme (MM) at the level of the hind limb buds. Here signals produced from the MM induce an outgrowth of the WD known as the ureteric bud (UB) which invades the MM and induces the aggregation and formation of the nephron progenitors, known as the renal vesicles (RV) [16-18]. The UB undergoes branching morphogenesis to form the collecting duct system of the kidney while continually inducing the formation of many RVs (Figure 1-1) [19, 20]. These RVs become vascularized and fuse to the collecting duct to form the nephron. This final kidney, the metanephros, is the functional adult kidney in mammals. In the mouse, the metanephric kidney will ultimately become about 1 cm in length and contain approximately 10-20,000 nephrons. For comparison, the adult human kidney will become 10-12 cm in length and contain up to 1,000,000 nephrons [21, 22].

General overview of the Wnt gene family

The Wnt signal transduction pathway plays multiple important roles during kidney development [16-18]. The Wnts encode a family of secreted glycolipoproteins that function in multiple biological processes including embryonic development and disease pathogenesis [23]. Mouse and invertebrate homologues were originally described independently as the

segment polarity gene *wingless* (Wg) in flies, and the oncogene, *Int-1* in mice [24-26]. The moniker “Wnt” results from a combination of the original two names **W**g and I**nt**. Wnt signals are transduced by a family of seven-pass transmembrane G-protein coupled receptors of the frizzled (Fzd) family. The potential for complexity in the Wnt pathway is great, as mammals express 19 distinct Wnt ligands, 10 Fzd receptors and at least 4 co-receptors in unique and dynamic patterns during embryogenesis. In the kidney, all 10 Fzd receptors and 8 Wnts are expressed during development [16-18, 27-32] . The signal transduction cascade that is activated downstream of each receptor/co-receptor complex adds to this complexity. Reception of a Wnt signal can activate one of multiple downstream pathways in the receiving cell (Figure 1-2). In the best characterized pathway, termed the canonical/β-dependent pathway, Wnt regulates β-catenin dependent transcriptional activity downstream of both Fzd and Dsh. The second pathway, known as the non-canonical/planar cell polarity (PCP) pathway, utilizes Fzd and Dsh, but instead of regulating β-catenin, these molecules activate Rho GTPases and polarize the actin cytoskeleton [33]. The third pathway is known as the Wnt/Calcium pathway. In this pathway, a Wnt activates protein kinase C (PKC), NFat, and calmodulin-dependent protein kinase II (CamKII) and induces an influx of Ca2+ into the cell [34]. The Wnt/Calcium pathway may work in a manner more analogous to the canonical pathway by affecting gene transcription by activating calcium-responsive transcription factors [34-38].

The pathway that a Wnt signals through may be dependent upon specific ligand/receptor or ligand/ligand interactions, receptor/co-receptor combinations, as well as the intracellular environment in the cell receiving the Wnt signal.

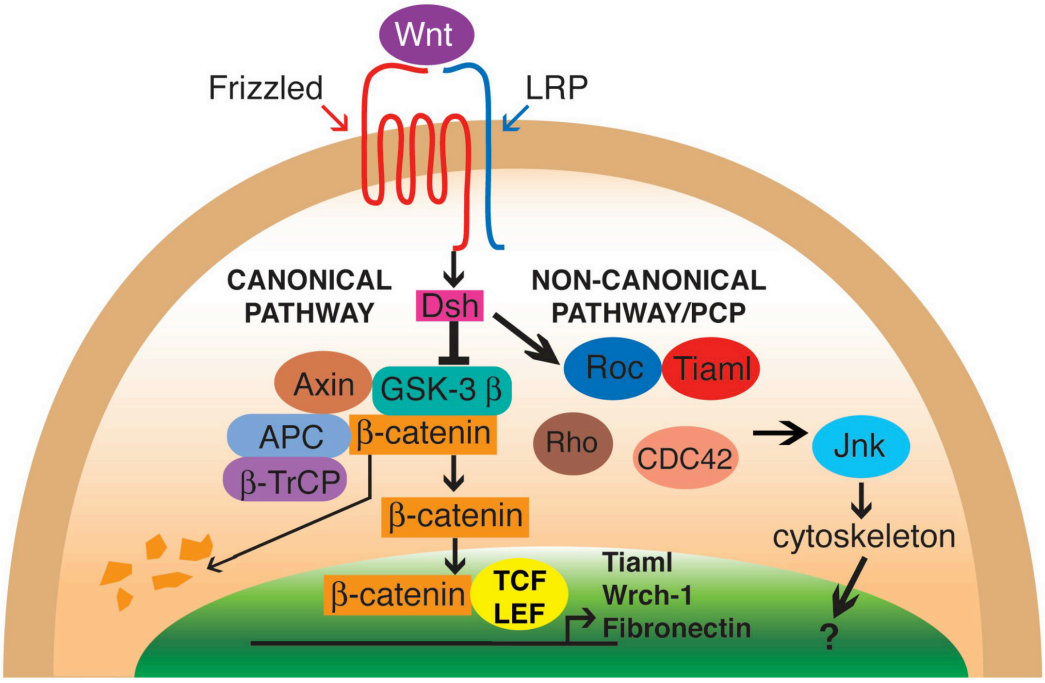

Figure 1-2: Wnt signal transduction pathways.
Schematic representation of the canonical and non-canonical PCP/CE Wnt pathways. In the canonical pathway, binding of a Wnt ligand to its receptors results in the repression of the β-catenin destruction complex (GSK-3β, Axin, APC and β-TrCP) which results in the accumulation of β-catenin. β-catenin then translocates into the nucleus where it interacts with the Lef/Tcf transcription factors and activates the expression of canonical Wnt target genes. The canonical and non-canonical pathways diverge after Dsh. The non-canonical/PCP branch signals through Rho, Cdc42 and JNK to polarize the actin cytoskeleton and regulate cell cell movements. Adapted from [39].

Canonical/β-catenin dependent Wnt Signaling

The canonical or β-catenin dependent Wnt pathway is the best characterized Wnt pathway. Binding of a Wnt ligand to Fzd receptor and

LRP co-receptor activates an intracellular signaling cascade that impinges on the stability of the bifunctional transcriptional co-activator, β-catenin. Normally, β-catenin interacts with E-cadherin and localizes to the adherens junctions (AJ). In a cell not exposed to a Wnt ligand, cytoplasmic levels of β-catenin are kept low through interactions with the β-catenin destruction complex. This complex is composed of the two scaffolding proteins adenomatous polyposis coli (APC) and Axin, and the serine/threonine kinases glycogen synthase kinase 3-β (GSK3B) and Casein Kinase 1a (CK1a) (Figure 1-2). Any β-catenin not associated with the AJs is phosphorylated sequentially at serine 45 by CK1a and then at threonine 41, serine 37 and serine 33 by GSK3 [40, 41]. These phosphorylations create a binding site for the E3 ubiquitin ligase β-Trcp, which binds to and ubiquitylates β-catenin leading to its destruction by the proteasome [42, 43].

The reception of a Wnt signal activates and recruits the cytoplasmic protein Dishevelled (Dsh) to the cell membrane. Dsh recruits Axin to the cell membrane thus disrupting the β-catenin destruction complex [41, 44]. Disruption of the destruction complex results in the stabilization and accumulation of cytoplasmic β-catenin. Stabilized β-catenin translocates into the nucleus and interacts with the Lymphoid-enhancing factor/T-cell factor (Lef/Tcf) family of the high mobility group (HMG)-type transcription factors to regulate canonical Wnt target gene transcription (Figure 1-2) [45-47]. Normally Lef/Tcf transcription factors interact with groucho,

CtBP1 and other co-factors which are thought to have a repressive effect on transcription [48-50]. β-catenin is thought to either replace these co-repressors or recruit co-activators and convert the Lef/Tcf complex into transcriptional activators. However, certain isoforms of the Lef/Tcfs may always function as transcriptional repressors even in the presence of β-catenin [51].

Non-canonical Wnt/Planar Cell Polarity Signaling

Wnt ligands also signal in a manner that is independent of β-catenin activity. These so-called non-canonical Wnt pathways can be further subdivided into the Wnt/Ca^{2+} pathway and the Planar Cell Polarity/ Convergent Extension (PCP/CE) pathway. PCP describes the polarization or organization of cells within the plane of a tissue, while CE describes the intercalation of cells in an epithelial sheet that results in the narrowing of that tissue with a concomitant increase in length perpendicular to the direction of the narrowing of the tissue, hence a convergence and an extension of the tissue. CE movements are active during gastrulation, neurulation and organ formation in many species [52-61]. PCP is most readily apparent in the uniform orientation of the bristles on a fly cuticle, feathers on a bird, or the hair on a mouse [39]. On a microscopic level, PCP is evident in numerous tissues of diverse phyla including the orientation of photoreceptor cells in the fly eye, stereocilia orientation in the mouse cochlea, muscle fiber orientation in the mouse somite, or cellular orientation in neural tube of both zebrafish and frogs [53, 62-66].

Many of the genes that regulate PCP were originally identified in *Drosophila* and affected the orientation of wing hairs, cuticular bristles or eye ommatidia. Some of the molecules involved, such as Fzd and Dvl, were previously implicated in canonical Wnt signaling, leading to the inference that PCP is a Wnt dependent process [67, 68]. However, in flies there is no evidence of any role for a Wnt ligand in PCP signaling [69]. In flies, PCP is thought to be set up by a long-range signal that establishes the direction of the polarization. This long-range signal is interpreted by two groups of genes. The first group is denoted as the "upstream group" and include the genes *four-jointed (Fj), Dachsous (Ds), Widerborst (Wdb), Fat (Ft), and Atrophin (Atro)* [70-76]. This group is hypothesized to interpret the long-range signal and establish the direction of polarity. This signal biases the asymmetric sub-cellular localization of the second set of proteins known as the "core group." This group includes the genes *Frizzled (Fzd), Flamingo (Fmi), prickle, (Pk), Diego (Dgo), Strabisimus/Van Gogh (Stbm/Vang)* and *Disheveled (Dsh),* and is involved in establishing the planar polarity of the individual cells. In flies, the primary molecular readout of PCP is the sub-cellular localization of the proteins *Stbm/Vang* and *Pk* to the proximal side of the cell and *Dgo, Fzd*, and *Dvl* on the distal side [33, 64, 65, 77, 78]. The core group acts primarily in a cell-autonomous fashion but also has a non-autonomous effect on the polarity of adjacent wild type cells. This non-autonomous effect is based on the location of the wild type cell relative to the mutant cell and is dependent on

the mutant gene, a concept known as “domineering non-autonomy” [70, 79]. For example, *fzd* mutant clones show non-autonomous defects in wild type epithelial cells distal to the mutant clone while *vang* mutants induce polarity defects only on the proximal side of the mutant clones. The polarized localization of these PCP components is necessary for the polarized activation of cell type-specific effectors, such as the RhoGTPases Cdc42 or Rac1 or the actin assembly proteins Fuzzy and Inturned. This ensures the two tiers of upstream signals are converted into proper polarization of the cytoskeleton [80-85].

Wnt ligand involvement in PCP

In flies, both Fzd and Dsh are active in the PCP pathway [67, 68], however there is no evidence that either a Wnt ligand, LRP co-receptor, or β-catenin play any role in these processes. In other systems Wnt ligands play a significant role in the PCP pathway. Loss of function mutations in some Wnts have strikingly similar phenotypes to mutations of core PCP genes. For example, in Zebrafish, null mutations of both *Pipetail/Wnt5a* and *Silberblick/Wnt11* result in defects in convergent extension movements similar to mutations in the PCP component, *Trilobite/Stbm* [86-88]. Likewise, in mice, Wnt5a mutations result in PCP defects and genetically interact with Vangl2. Selectively inhibiting Wnt signaling using secreted Wnt inhibitors disrupts the orientation of cochlear hair cells, another PCP defect [89-91]. Wnt5a may signal through Fzd3 and Fzd6 to

regulate PCP in the cochlea as *Fzd3*$^{-/-}$;*Fzd6*$^{-/-}$ double mutant mice also have disrupted hair cell orientation [92].

It appears that Wnts are active in PCP signaling, however, most experimental data indicates they act as permissive rather than instructive cues. Recent data in vertebrates indicate a Wnt can act instructively during chick myogenesis. Wnt11 from the neural tube activates Rok2 and Jnk downstream of Fzd7 to polarize the elongation of myocytes located in the somites [62]. Recent data from *C. elegans* support the hypothesis that Wnts act instructively to set up PCP. In worms, β-catenin independent Wnt signaling regulates mitotic spindle orientation and endodermal induction of the EMS cell [93-96]. The Wnt involved, *MOM2*, is sufficient to reorient the mitotic spindle in the EMS cell relative to the MOM2 producing cell. In addition to the mitotic spindle, the Frizzled receptor, LIN-17, becomes enriched on the membrane adjacent to the MOM-2 producing cell [97]. This is not an EMS cell phenomenon as a separate Wnt, LIN-44, is necessary and sufficient for the asymmetric localization of both Fzd/Lin-17 and Dsh/MIG-5 during B cell division [98, 99]. Similarly the Wnts, *MOM-2*, LIN-44 and EGL-20, all affect the polarized localization of Fzd/LIN-17 and Ryk/LIN-18 in the vulval epithelium and are necessary for the proper polarization of the vulva [100, 101]. These data indicate that Wnts are instructive during PCP signaling in some contexts.

Canonical/Non-canonical pathway specificity

Historically, Wnts have been classified as canonical or non-canonical based on their ability to induce a secondary dorsal-ventral axis when injected into *Xenopus* embryos or transform C57MG mouse mammary epithelial cells. According to these criteria, Wnts such as Wnt1 and Wnt3a were classified as canonical Wnts, while Wnt5a and Wnt11 were classified as non-canonical Wnts. These classifications are not absolute as some Wnts transform cells but may be unable to induce a secondary axis in *Xenopus* embryos or *vice versa*.

Individual Wnt ligands may be able to activate both pathways depending on the cellular context. Pathway specificity may be provided by the Fzd receptor or the intracellular composition of the receiving cell. For example, Wnt5a, the quintessential non-canonical Wnt signal canonically in some contexts. However, in cell culture Wnt5a can activate a β-catenin reporter system only in cells that express Fzd4 and Lrp5. In *Xenopus,* the co-injection of Wnt5a and Fzd5 generates an ectopic axis while the injection of Wnt5a alone does not [102, 103] leading to the inference that the receptor complex provides signal specificity. However, Wnt11, another so-called non-canonical Wnt, was recently described as the long sought after canonical Wnt that activates β-catenin to set up the dorsal-ventral axis in the *Xenopus* embryo [104].

The gene *Wnt4* interacts with *Fzd6* and activates a β-catenin reporter gene in MDCK cells [105]. However, the functional relevance of

this interaction is not clear. Unlike Wnt5a and Fzd5, co-injection of Wnt4 and Fzd6 into *Xenopus* embryos was insufficient to generate an ectopic dorsal axis [105]. The take home message from these studies is that Wnt ligands activate multiple pathways, but the mechanisms that determine pathway specificity are not known. Collectively, these results indicate that other factors, independent of both the ligand and the receptor, determine Wnt pathway specificity.

In addition to the Fzd receptor, a co-receptor of either the arrow/ low-density lipoprotein (LRP) family, the transmembrane atypical tyrosine kinase Ryk, or the single-pass receptor tyrosine kinase Ror2 are necessary in some contexts to transduce Wnt signals and may provide some pathway specificity [106-110]. Wnt ligand binding to Fzd and either LRP5 or LRP6 activates the canonical pathway. Similarly, Ryk also transduces canonical Wnt signals in both flies and mice. The Ror co-receptor appears to mediate non-canonical Wnt signaling as *Ror1*$^{-/-}$ and *Ror2*$^{-/-}$ mutant phenotypes are very similar to *Wnt5a*$^{-/-}$ phenotypes. *Ror2* also interacts genetically with *Wnt5a* and is necessary for Wnt5a directed cell migrations [89, 109-111]. However, Ror2 may also transduce canonical Wnt signals as it has been recently shown that Wnt5a/Ror2 can both activate and inhibit the canonical pathway [109]. Pathway specificity may be dependent upon specific Fzd/co-receptor combinations or the presence of other cofactors such as the secreted glycoprotein collagen triple helix repeat containing 1 (Cthrc1). Cthrc1 binds to and stabilizes the

Wnt/Fzd/Ror2 receptor complex to activate non-canonical/PCP signaling [112]. Cthrc1 may activate PCP signaling by stabilizing either PCP specific Wnt/Fzd complexes or by changing the nature (from canonical to non-canonical) of the complex in specific cells.

Pathway specificity appears to also be influenced by the intracellular make up of the cell receiving the Wnt signal. Intracellular effectors have been identified that bias the nature of the Wnt signal. Many of these effectors, such as Daple, Idax and Inversin, act at the level of Dsh. Both Daple and Idax interact with Dsh and inhibit β-catenin signaling [113-115]. However, some Dsh interacting proteins, like Inversin (Inv) and Daam1, act as molecular switches to inhibit β-catenin signaling and promote non-canonical signaling. These activate non-canonical signaling uniquely as Inversin functions by promoting degradation of Dsh while Daam1 binds Dsh and promotes Rho activation [8, 116]. Many Dsh regulators are expressed in the kidney during development and may regulate the strength, duration or even the pathway specificity of Wnt signaling in the kidney (www.genepaint.org). In fact, *Inv* mutations results in kidney cyst formation due to defects in non-canonical Wnt signaling [8].

Wnt signaling during kidney development

Kidney development is dependent on reciprocal interactions that regulate the formation of approximately 20,000 nephrons in the mouse. Many different processes, including branching morphogenesis, tubule induction, progenitor maintenance, and tubule morphogenesis, act in

concert during kidney development to induce the correct number of nephrons. Wnt signaling plays indispensable roles in many of the processes regulating kidney development. Defects in any of these processes results in a decrease in nephron endowment. Significant decreases in nephron endowment have serious health risks in humans ranging from kidney failure to chronic hypertension [22]. Here I will give a brief overview of the processes regulating kidney development and the role that the Wnt pathway plays during development.

Nephron induction

The crux of kidney development is the induction of nephrons. Signals from the UB induce the MM to aggregate and form the pre-tubular aggregates (PTA) which subsequently undergoes a mesenchymal-to-epithelial transition to form the renal vesicles (RV). The ureteric bud is necessary for both nephron induction and the survival of the MM. Mechanical removal of the ureteric bud or a delay in invasion of the MM both result in rapid apoptosis and clearance of the MM. After UB invasion, nephron induction is a highly patterned process. Normally, the PTA are found in the MM ventral to the UB. This pattern in maintained until post-natal day 5 in the mouse, when induction markers are found on the dorsal aspect of the UB and kidney development ceases [117]. The signals that regulate nephron induction have been well studied. Originally it was shown that in addition to the ureteric bud, a number of other tissues can act as heterologous inducers of tubulogenesis *in vitro*, including embryonic

spinal cord. [12, 118]. Subsequent studies determined that Wnt1 from the spinal chord was sufficient to induce tubules when exposed to MM [119]. Multiple Wnts, including *Wnt6*, *7b*, *9b* and *11*, are expressed in the UB and could potentially regulate nephron induction. However, only Wnt6, 7b and 9b are able to induce tubule formation in isolated MM. Despite the potential of these Wnts to induce RV formation, genetic data indicate that nephron induction is dependent on the sequential activation of only two Wnts, Wnt9b and Wnt4 [16, 17].

Wnt9b is expressed in the ureteric epithelium and is necessary for the kidney to form. Mice lacking Wnt9b form aplastic kidneys and die at birth. The primary defect in *Wnt9b* mutants is a failure to induce the expression of tubule markers such as Pax8 and Wnt4 [16] Like Wnt9b, Wnt4 is also necessary for RV formation. However, Wnt4 is expressed in the cells of the MM receiving the Wnt9b signal. Mice lacking Wnt4 form hypo-plastic kidneys and die within 24 hours of birth [17]. The primary defect in Wnt4 mutant embryos appears to be a failure to convert the mesenchyme to epithelial structures at E12.5. However, by later stages there is some conversion occurring in these mutants, indicating another Wnt may be able to compensate for Wnt4 in this role. Wnt6 is up-regulated in the UB of Wnt4 mutants and is most likely the Wnt that partially rescues the *Wnt4*$^{-/-}$ phenotype as Wnt6 can compensate for Wnt4 *in vitro* [120].

While Wnt9b is necessary to induce Wnt4 expression, the exact role of Wnt9b is unclear. Functional studies show that Wnt4, like Wnt9b, is sufficient to induce RV in the absence of the UB. Wnt4 can functionally replace Wnt9b and induce tubule formation in *Wnt9b*$^{-/-}$ MM. However, Wnt9b is unable to induce MET in *Wnt4*$^{-/-}$ MM [16] implying that the main function of Wnt9b is to induce the expression of Wnt4 leading to tubule induction. These data also indicate that while both Wnt9b and Wnt4 are necessary and sufficient for RV induction, they may signal through separate cells, pathways or receptors. However, determining the pathway through which both Wnt9b and Wnt4 are signaling is challenging due to the epistatic nature of these two Wnts. Activation of the canonical Wnt/β-catenin pathway is sufficient to induce RVs [121-124]. Thus it seems obvious then that either one or both Wnt9b and Wnt4 must be signaling via the canonical pathway. Removal of β-catenin from the MM results in a loss of tubule induction and a loss of both Wnt9b and Wnt4 target genes. In the same study, the activation of β-catenin was sufficient to rescue the expression of the Wnt4 targets Fgf8 and Lhx1, in a *Wnt4* mutant background [122]. It appears that Wnt4 signals canonically to regulate tubule induction; however, the molecular nature of the Wnt9b signal remains elusive.

Tubule morphogenesis

After the mesenchyme is induced to form the RV, many more events must occur to form a functional nephron. After formation, the RV

invaginates first at one pole to form the comma-shaped body and then the S-shaped body. The proximal cleft of the S-shaped body is invaded by a capillary, forming the glomerulus. The distal cleft of the S-shaped body fuses with the collecting duct and forms a common lumen. After fusion to the collecting duct the S-shaped body undergoes extensive growth and elongation to form the nephron (Figure 1-3). During development the nephron normally maintains a constant diameter. Increases in the diameter of kidney tubules lead to decreased kidney function and are characteristic of cystic kidney diseases that are common in humans. Tight regulation of Wnt signaling is essential for proper development of the kidney tubules.

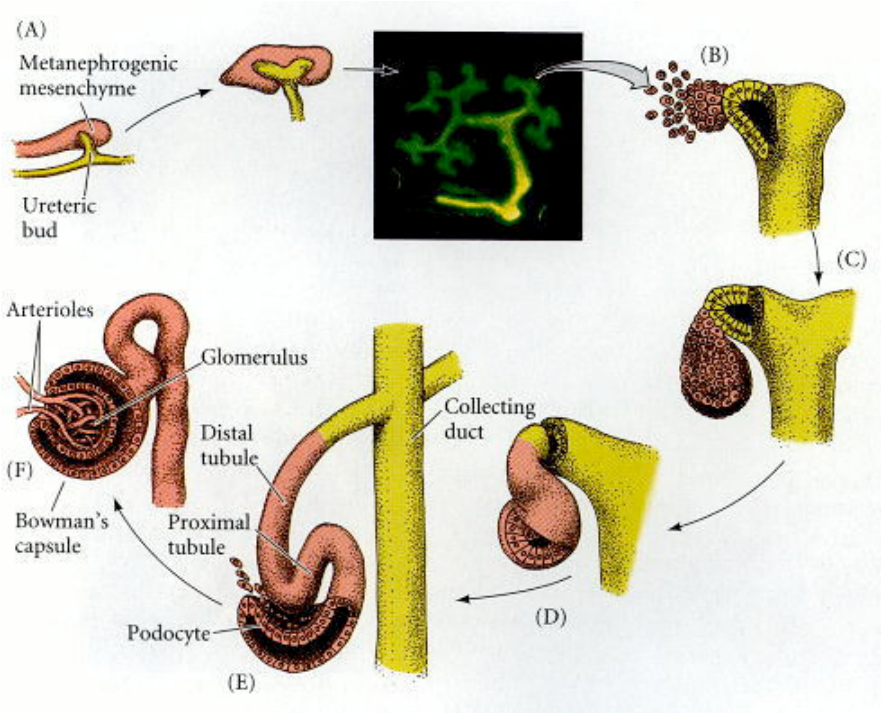

Figure 1-3: Nephron development in the kidney.
(A) After invasion of the ureteric bud, the metanephric mesenchyme induces the bud to branch. (B) The ureteric bud induces the mesenchyme to aggregate and undergo a mesenchymal-to-epithelial transition to form the renal vesicles. (C) The RV fuses to the collecting duct at the distal end

while the proximal end is invaded by a capillary (E) forming the glomerulus (F). The developing nephron continues to grow and undergo morphogenesis between these two poles to form the mature nephron. Adapted from *[12]*

In mice, the loss of canonical Wnt signaling prevents formation of the tubules, while inappropriate activation of the Wnt pathway in these developing tubules leads to cyst formation [1, 16, 122]. In fact, improper stimulation of the canonical Wnt pathway is a hallmark of various types of human cystic kidney diseases including ADPKD, ARPKD and nephronophthisis [125]. Activation of canonical Wnt signaling may have a mitogenic effect that contributes to cyst progression [126].

It is unclear how tubule diameter in the kidney is established or maintained. However, recent studies have suggested that defects in PCP signaling contribute to cystogenesis. PCP regulates the orientation of cell division during many diverse processes [97, 127, 128]. In adult mouse kidney tubules, cell division is oriented parallel to the proximal-distal or long axis of the kidney tubule. This orientation appears to be necessary to maintain the normal luminal diameter as mutations in the transcription factor HNF1B randomize the mitotic spindle leading to an increased luminal diameter and cystogenesis [129]. Additional studies indicate that PCP may regulate the orientation of cell division (OCD) in the kidney since the deletion of the gene *Fat4,* a PCP determinant, results in changes in OCD and cystogenesis [130].

Recent studies indicate that the cilium is necessary for these oriented cell divisions. The cilium is an organelle found on all vertebrate cells that functions both as an extracellular sensor and as an intracellular signaling organizer. Mutations affecting the primary cilium lead to many distinct phenotypes including renal cyst formation in both humans and in animal models [131-134]. In mice, kidney specific removal of the intraflagellar transport proteins *Kif3a*, *Ift20* or *Ift88* result in the loss of cilia due to defects in intra-flagellar transport. Interestingly the loss of kidney cilia results in the formation of tubular cysts due to randomized OCD [126, 135-137]. This phenomenon is not restricted to mice as human mutations in *IFT80* are associated with renal cystic disease in patients with Jeune asphyxiating thoracic dystrophy [138]. The cilium appears to convert cues from the extracellular environment into intracellular signaling events. Loss of the cilium leads to increased canonical β-catenin signaling [126, 136, 139]. Inversin, which regulates Dsh stability and the switch between canonical and non-canonical Wnt signaling, localizes to the cilium and may be responsible for this effect [8].

The PCP effectors Dsh and Inturned are essential for ciliogenesis in *Xenopus* [84]. Interestingly, cilia appear to be necessary for PCP signaling. In Zebrafish, mutations in the basal body proteins BBS 1, 4 or 6 result in convergence and extension defects and genetically interact with both Wnt5b and Wnt11 [140]. How the cilium regulates or refines PCP is unknown. In the kidney, sensing urine flow or forming a molecular scaffold

for Dsh regulators like Inv might be mechanisms by which the cilium regulates PCP. Nevertheless it is apparent that PCP signaling is necessary for normal tubular morphogenesis. However, the mechanism by which PCP is established in these epithelial tubules is not known.

Mesenchymal progenitor maintenance

The maintenance and expansion of nephron progenitor cells throughout development is necessary for kidney development. Any defects in progenitor cell maintenance results in a decrease in tubule progenitors and a reduction in nephron endowment. Similar to the induced mesenchyme, the progenitor compartment is highly patterned during kidney development. The progenitor compartment is located dorsal to the UB and is demarcated by the expression of many factors, including the transcription factors *Six2* and *Cited1* [141-143]. This *Six2*+ *Cited1*+ progenitor population is self renewable and capable of generating all lineages of the nephron throughout kidney development [142, 143]. However, maintenance of the progenitor population only requires Six2 activity but not Cited1 [141, 144, 145]. Normally, there is precise patterning in the formation of renal vesicles with respect to the *Six2* positive progenitor cells. The progenitors are found on the dorsal, or cortical, side of the UB, while the induced mesenchyme and RV are found on the ventral, or medullary side. This patterning is maintained throughout development of the kidney ending abruptly at post natal day 5 in the mouse. At this time there is an “explosion” of tubulogenesis resulting in a

loss of progenitor markers and the appearance of RV's on the dorsal aspect of the UB for the first time [117]. This explosion of nephrogenesis results in a depletion of the progenitor cells and an end of kidney development. The molecular nature of this switch is unknown.

The maintenance of the progenitor domain and the cortico-medullary patterning of RV induction is perturbed in mice that lack *Six2* activity. In *Six2*$^{-/-}$ animals, there is precocious and ectopic formation of RVs dorsal to the UB [141]. Activation of the canonical Wnt pathway is both necessary and sufficient for RV induction [121, 123]. Genetic activation of β-catenin in the progenitor cells results in ectopic RV formation, a phenotype similar to that observed in *Six2*-/- mutants [122], leading to the hypothesis that Six2 maintains progenitor identity in part by inhibiting β-catenin activity. However, a recent study showed that β-catenin regulates survival and proliferation of the nephron progenitors [124]. In *Six2*-/- animals there is ectopic expression of the Wnt9b targets Pax8 and Wnt4 in the progenitor domain indicating the progenitor cells, not just the induced MM, are responding to Wnt9b. Wnt9b is necessary for this effect as *Wnt9b*$^{-/-}$ *Six2*$^{-/-}$ mutants fail to form kidneys, similar to loss of Wnt9b alone [16, 143]. Collectively these data indicate that Six2 functions to maintain renal progenitors by inhibiting both Wnt9b and β-catenin activity.

Non-canonical Wnt signaling may be important for the maintenance of the progenitor compartment. The PCP pathway downstream of Wnt4

was recently shown to regulate expansion of isolated progenitor cells. Inhibition of either Rho or Jnk resulted in decreased colony size, while their activation resulted in hyperexpansion [146]. Interestingly, small molecule mediated and genetic activation of the canonical Wnt pathway resulted in a significant decrease in colony size, presumably due to increased differentiation of the progenitor cells. Wnt4 from the renal vesicle may actively signal to and regulate maintenance of the progenitor compartment. Whether other Wnts, presumably from the UB, fulfill this role *in vivo* is unknown.

Branching morphogenesis

UB growth and branching is critical for normal nephron endowment and urogenital development, and a failure in these processes leads to birth defects such as renal agenesis, hypoplasia, or congenital obstructive uropathy [19, 20, 147-149]. After invading the MM, the UB begins a cyclical pattern of RV induction, growth and branching. This cycle repeats itself throughout the embryonic and into the post-natal period, when simultaneously, branching and tubule induction end [117]. The most well studied regulator of UB outgrowth and branching morphogenesis is glial derived neurotrophic factor (GDNF). *GDNF* is expressed by the MM and signals to the WD and UB through the RET receptor tyrosine kinase and GFRa1 co-receptor which are both expressed at the branching tips of the ureteric bud [150-153]. In mice, the UB undergoes extensive branching morphogenesis until 5 days after birth to form the collecting duct system

and the extra-renal ureter in the adult kidney. In addition to the GDNF/Ret pathway, Wnt signaling has been shown to regulate branching morphogenesis. Kidneys cultured in the presence of the Wnt antagonist DKK1 showed a significant decrease in UB branching [32]. Interestingly activation of β-catenin by either LiCl or 6-bromoindirubin-3'-oxime (BIO) also inhibits UB branching [123].

Genetic data support a role for both Wnt9b and Wnt11 in the regulation of branching morphogenesis, although the pathway they are signaling through is unclear. Wnt11 is expressed in the tips of the UB similar to Ret and GFRa1 [16, 18, 154, 155]. In addition to the UB tips, *Wnt11* is also expressed in the medullary stroma beginning at approximately E14.5. Mice lacking Wnt11 have reduced branching morphogenesis and form smaller kidneys when compared to wild type. Wnt11 appears to act in parallel with *GDNF*/*Ret* to regulate branching. Loss of Wnt11 leads to a loss of GDNF in the MM while loss of *Ret* leads to a loss of Wnt11. Wnt11 appears to interact genetically with Ret supporting this hypothesis [18]. Wnt11 most likely signals to the MM to regulate UB branching, as evidenced by loss of GDNF in Wnt11 mutants. But it is also possible that Wnt11 signals to the UB as well.

Wnt9b mutants also have branching morphogenesis defects as the secondary branching of the UB is perturbed. The UB forms and invades the MM normally but fails to branch after the primary bifurcation event [16]. These defects do not appear to be the result of a defect in RV induction as

branching occurs normally in Wnt4$^{-/-}$ animals. The secondary branching defect in *Wnt9b* mutants appears to be due to a down-regulation of both *GDNF* and *Wnt11* by E12.5. These data indicate that while the initial outgrowth of the UB is Wnt9b independent, the later secondary branching is dependent on Wnt9b signaling out to the MM. The pathway through which Wnt9b and Wnt11 are signaling to regulate UB branching is unknown. Specific ablation of β-catenin in the progenitor cells phenocopies the *Wnt9b*$^{-/-}$ mutant phenotype indicating either *Wnt9b* or *Wnt11* signals to the MM through β-catenin to regulate branching [122]. However, Park et al showed in the same study that activation of β-catenin in the progenitor cells was unable to rescue branching in *Wnt9b*$^{-/-}$ mutants, arguing against this hypothesis [122].

Whether Wnt signaling from the MM to the UB is necessary for branching morphogenesis is unknown. Many Wnts, including *Wnt2b*, *Wnt4*, *Wnt5a*, and *Wnt11* are expressed in tissues other than the UB. *Wnt4* is expressed in the induced mesenchyme and the inter-medullary stroma. *Wnt2b* is expressed in the perinephric cells and the mesenchyme surrounding the ureter, while *Wnt5a* and *Wnt11* are expressed in the inter-medullary stromal cells along with *Wnt4*. These Wnts may signal to the UB to regulate survival, growth or branching. In support of this, treatment of isolated UBs with β-catenin agonist LiCl leads to increased UB survival and branching. In the same study, Lin et al showed that Wnt2b, but not Wnt4, promoted survival and growth similar to LiCl treatment [156].

Genetic removal of β-catenin from the UB resulted in decreased branching morphogenesis and renal agenesis in multiple studies [157, 158]. Interestingly, activation of β-catenin in the UB also inhibits UB branching leading to renal hypoplasia, indicating that tight regulation of β-catenin is necessary for normal branching [157]. UB derived Wnt7b does not appear to affect branching morphogenesis but is indirectly necessary for renal pelvis formation by regulating the expression of *Wnt11* and *Wnt4* in the stroma. Wnt7b signals to the surrounding medullary stroma through β-catenin to induce the expression of both *Wnt4* and *Wnt11*. These two Wnts are thought to then signal back to the UB and regulate PCP and oriented cell divisions leading to medullary formation [31].

CHAPTER TWO

Canonical Wnt9b signaling regulates progenitor cell expansion during renal development.

Abstract

Kidney development is dependent upon interactions between the ureteric bud epithelium and a mesenchymal progenitor population known as the metanephric mesenchyme. Signals from the ureteric bud cause a subset of the progenitors to differentiate into tubules while the remainder proliferate/self renew. This process of progenitor expansion and differentiation reiterates itself during the course of development, ultimately resulting in the formation of approximately 20,000 tubules per mouse kidney. Significant decreases in tubule number have serious health risks in humans ranging from kidney failure to chronic hypertension [22]. Establishment of the correct number of tubules depends on a careful balance between the induction of new tubules and the expansion of the existing tubule progenitor cells. The molecular mechanisms by which this balance is achieved are poorly understood. Previous studies suggested that Wnt/β-catenin signaling was necessary and sufficient to induce tubule formation. Further, it was hypothesized that the pathway must be repressed in the progenitors to keep them in an undifferentiated state. However, using a combination of microarray analysis and genetics, we have found that in fact Wnt/β-catenin signaling is active in the progenitor compartments. Our data indicate that rather than inhibiting β-catenin activity, factors in the progenitor cells (primarily the transcription factor Six2) alter the transcriptional output of β-catenin signaling. In the progenitor population, we find that Wnt/β-catenin signaling is required for

stem cell renewal/proliferation. We have uncovered a mechanism through which cells receiving the same Wnt signal can respond in distinct ways (differentiation vs. proliferation). Utilizing this simple mechanism, the kidney is able to balance tubule induction and stem cell maintenance, thereby insuring formation of the proper number of tubules. These findings provide novel insights into the molecular mechanisms that regulate the kidney development and enhance our ability to generate kidney stem cells and engineer tubules for damaged organs.

Introduction

During organ formation, a balance is struck between stem cell proliferation/renewal and differentiation to ensure proper organ size. The precarious nature of this balance is particularly evident in the developing metanephric kidney. Kidney formation is dependent on inductive interactions that result in the differentiation of a loosely associated population of mesenchymal progenitor cells into epithelial nephrons. The nephron is a vascularized tubule that maintains acid base homeostasis and filters metabolic wastes and inorganic ions from the blood. During mouse development, each kidney will form up to 20,000 nephrons (approximately 1,000,000 in an adult human) all derived from an initial progenitor population of approximately 12,000 cells at E11.5 [143]. The crux of kidney development is obtaining a balance between the maintenance/expansion and differentiation of the nephron progenitor population. Tipping this balance in favor of one or the other results in a reduction in nephron endowment and can have dire consequences including renal hypoplasia, chronic hypertension and even kidney failure [22]. Although factors regulating progenitor cell expansion and differentiation have been well identified, how the balance between these two events is maintained is not understood.

Nephron induction requires the sequential activation of two Wnts, Wnt9b and Wnt4 [16, 17]. Wnt9b secreted from the ureteric bud induces the renal progenitor cells to aggregate and express Wnt4 in the newly

formed pre-tubular aggregates (PTA). Wnt4 signals through the canonical Wnt/β-catenin pathway to regulate gene expression and epithelialization of the induced cells into RVs [122]. Although the molecular nature of the Wnt4 signal has been determined, the pathway Wnt9b activates is still uncertain. Further, it is unclear why Wnt9b is capable of activating Wnt4 expression only in a small proportion of the metanephric mesenchyme. Six2, a transcription factor that is required cell autonomously to keep the progenitor population in a undifferentiated state, has been proposed to function to block canonical Wnt activity in the progenitor cells [141, 143]. Loss of Six2 results in a hypersensitivity to Wnt9b and Wnt4 resulting in precocious and ectopic tubule formation/differentiation [143]. However, the manner in which Six2 performs this function remains undetermined.

During normal development, there is precise spatial relationship between the forming PTAs, the ureteric bud, and the mesenchymal progenitor cells. The progenitors are found on the cortical or capsular side of the UB, while the PTAs are found below the bud tips, adjacent to the ureteric bud stalk. In the mouse this pattern is maintained until post natal day 5 when the progenitors are exhausted and kidney development ceases [117].

We sought to identify novel molecular targets of Wnt9b using microarray analysis of E11.5 wild type and mutant mesenchyme. We found that contrary to current models, Wnt9b signals through the β-catenin pathway to the progenitor cells. Our data indicate that rather than

inhibiting β-catenin activity, Six2 is necessary to alter the transcriptional output of β-catenin signaling resulting in expansion of the nephron progenitors. Based on our results, we suggest a model whereby Wnt9b is the key factor that regulates the balance between stem cell proliferation and differentiation. In the progenitor cells, β-catenin, stabilized by Wnt9b cooperates with Six2 to elicit progenitor cell expansion. In pre-tubular aggregate cells that do not express Six2, β-catenin promotes differentiation triggering a mesenchymal to epithelial transition. These results expand our knowledge of the molecular mechanisms that regulate progenitor cell expansion and differentiation during kidney development.

Results

Identification of novel Wnt9b target genes

We have previously shown that Wnt9b secreted from the ureteric bud is necessary and sufficient for the induction of renal vesicles [16]. To identify novel molecular targets of Wnt9b we performed microarray analysis comparing mRNA profiles from isolated metanephric mesenchymes of E11.5 wild type and *Wnt9b*$^{-/-}$ animals. Analysis of these data using the gene set analysis toolkit (http://bioinfo.vanderbilt.edu/webgestalt) resulted in the identification of 33 putative Wnt9b targets that were down regulated at least 2 fold in *Wnt9b*$^{-/-}$ MM (Table 2-1).

We utilized *in situ* hybridization to validate the microarray in wild type E11.5 kidneys. Of the 31 down-regulated genes analyzed, all showed expression in the metanephric mesenchyme at e11.5. Based on spatial differences in the expression patterns, we grouped the genes into two distinct classes. The first group represents genes expressed in the pre-tubular aggregates. This classification includes the previously identified targets Pax8 and Wnt4 and several newly identified targets including Cadherin 4, C1qdc2, Daple etc (Figure 2-1A-D, Table 2-1 and [16]). The second group is characterized by expression in the mesenchymal progenitor cells (Table 2-1). This group includes the representative genes Btbd11, Cited1, Mu crystallin (Crym), Phospholipase A2 group 7 (Pla2g7), expressed sequence AW049604 (Tafa5), and Uncx4.1. (Figure 2-2A-F and Table 2-1).

Gene Description	Gene Name	Fold Change	Domain	Expression in Wnt9b mutants
Ets variant gene 5	Etv5	.496, .476	Prog	--
Phospholipase C-like 3	Plcl3	.496	PTA	---
Glial cell line derived neurotrophic factor	Gdnf	.495	Prog	--
Riken cDNA 0610010D24	Rik0610010D24	.494	PTA	---
Amphiphysin	Amph	.492	Prog/PTA	--
Paired box gene 8	Pax8	.49	PTA	---
Musashi homolog 2	Msi2	.486	PTA	-
Transferrin receptor	Tfrc	.483	Prog	--
C1q domain containing 2	C1qdc2	.481	PTA	---
BTB (POZ) domain containing 11	Btbd11	.481, .257, .209	Prog	---
Solute carrier family 12, member 2	Slc12a2	.479	Prog	---
Claudin 9	Cldn9	.47	Prog	---
Crystallin, mu	Crym	.46	Prog	+/-
Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 1	Cited1	.453	Prog	---
Expressed sequence AW049604	Tafa5	.448	Prog	---
Phospholipase A2, group VII	Pla2g7	.441	Prog	---
Lymphoid enhancer binding factor 1	Lef1	.44	PTA	---
Expressed sequence AL022943	AL022943	.44	ND	ND
Sorbin and SH3 domain containing 2	Sorbs2	.434	PTA	---
Riken cDNA 9430071P14	Rik9430071P14	.418	ND	ND
Riken cDNA 5930427L02	Rik5930427L02	.411	Prog	---
Solute carrier family 45, member 3	Slc45a3	.399	PTA	---
Fibroblast growth factor 9	Fgf9	.399	PTA	---
Winless-related MMTV integration site 4	Wnt4	.38, .347	PTA	---
Cadherin 4	Cdh4	.362, .328	PTA	---
Unc4.1 homeobox	Uncx4.1	.36	Prog/PTA	--
Integrin alpha 8	Itga7	.347	ND	--
Riken cDNA 2310045A20	Rik 2310045A20	.305	Prog	--
Chemokine (C-X-C motif) receptor 4	Cxcr4	.248	Prog	---
Glutathione peroxidase 6	Gpx6	.203	PTA	---

Table 2-1: **Genes significantly down-regulated 2 fold in *Wnt9b* mutant metanephric mesenchyme.** Expression domain was determined by *in situ* hybridization. Prog denotes genes expressed in the mesenchymal progenitor domain. PTA denotes genes expressed in the pre-tubular aggregates. Expression patterns for some genes were not determined (ND). Comparison of gene expression in wild type and *Wnt9b* mutants indicated that some genes that were completely absent from *Wnt9b* mutant MM (---), significantly reduced (--), or minimally reduced or not changed (-).

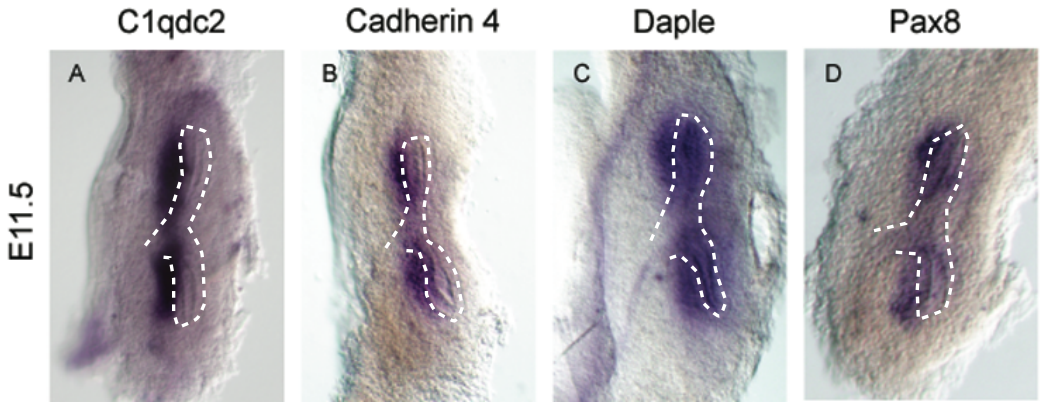

Figure 2-1: Expression of genes representative of the pre-tubular aggregate group.
In situ hybridization evaluating the expression of C1qdc2 (A), Cadherin 4 (B), Daple (C) and Pax8 (D). These genes are expressed in the pre-tubular aggregates ventral to the ureteric bud and are representative of genes classified as PTA Wnt9b targets (Table 3-1). The dashed lines outline the ureteric bud epithelium. In each image, the ventral pre-tubular aggregates are located on the left of the ureteric bud while the dorsal progenitors are located on the right side of the ureteric bud.

As several previous studies suggested that canonical Wnt signaling was blocked in the mesenchymal progenitor population, the identification of potential Wnt9b targets in the progenitor population was unexpected. Section in situ hybridization on adjacent sections revealed that the Wnt9b targets Pla2g7, Cldn9, and Rik2310045a20 overlapped with the bona fide progenitor marker Six2 [141] in wild type MM at e11.5 (Figure 2-3A, B and not shown). Further, one Wnt9b target, Cited1, is also a marker of self-renewing progenitor cell in the mesenchyme [142].

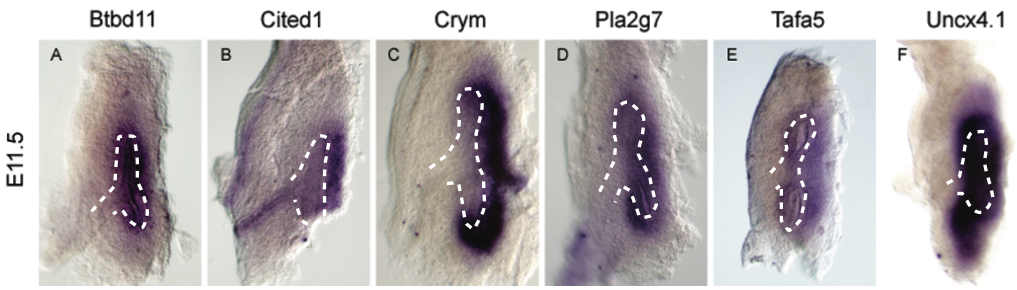

Figure 2-2: Expression of genes representative of the mesenchymal progenitor group.
In situ hybridization evaluating the expression of Btbd11 (A), Cited1 (B), mu-Crystallin (C), Pla2g7 (D), Tafa5 (E) and Uncx4.1 (F). These genes are expressed in the mesenchymal progenitors dorsal to the ureteric bud and represent the expression patterns of genes classified as progenitor Wnt9b targets (Table 3-2). The dotted line outlines the ureteric bud.

To verify that the identified genes were indeed are targets of Wnt9b we examined their expression in Wnt9b mutant mesenchyme at e11.5 using *in situ* hybridization (Figure 2-4). Irrespective of their expression domain, all genes examined were significantly reduced or completely undetectable in Wnt9b mutants at E11.5 (Figures 2-4 compare A to B for C1qdc2, D to E for Btbd11, G to H for Cited1, J to K for Pla2g7, M to N for Tafa5 and P to Q for Uncx4.1).

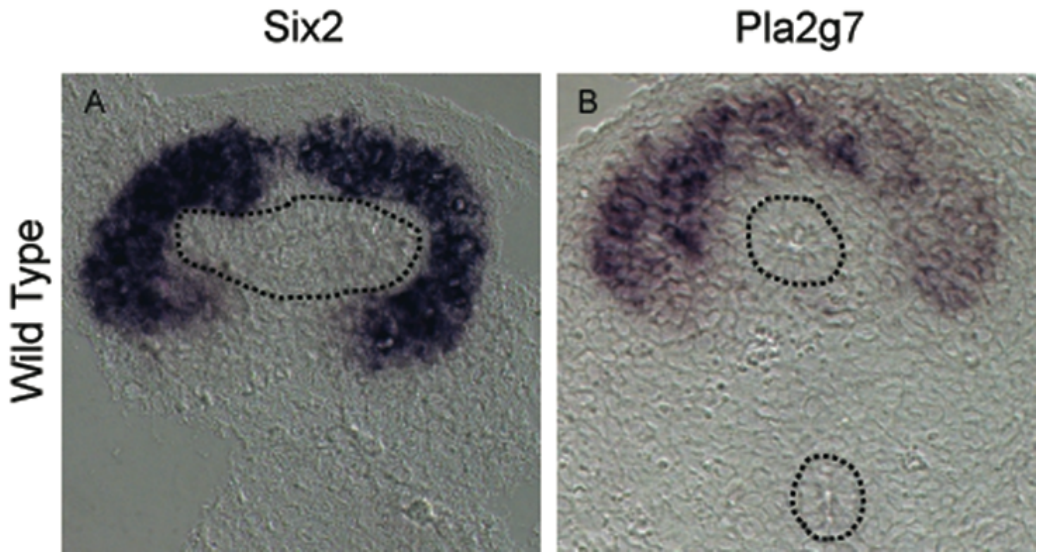

Figure 2-3: Progenitor gene Pla2g7 overlaps with the mesenchymal progenitor marker gene Six2.
Section *in situ* hybridization evaluating sections of e11.5 wild type kidney shows that the progenitor gene Pla2g7 (B) is expressed in cells expressing the progenitor marker gene, Six2 (A). The dotted lines in A and B highlight the ureteric bud epithelium.

To determine whether the target genes were primary targets of Wnt9b or secondary targets due to a loss of Wnt4 expression and a failure to induce pre-tubular aggregate formation, we evaluated gene expression in *Wnt4*$^{-/-}$ animals at e11.5. The majority of target genes, including the PTA marker C1qdc2 and progenitor markers Tafa5, Pla2g7, Cited1 and Uncx4.1, were normally expressed in *Wnt4*$^{-/-}$ MM at e11.5 (Figures 2-4 compare A to C for C1qdc2, D to F for Btbd11, G to I for Cited1, J to L for Pla2g7, M to O for Tafa5 and P to R for Uncx4.1) suggesting they were indeed direct targets of Wnt9b. Not surprisingly, 5/15 class I genes (PTA markers) were also completely absent from *Wnt4*$^{-/-}$ MM (Figure 2-5, A-C for Daple and D-F for Plcl3 and not shown) suggesting that they were

indirect targets identified due to the loss of Wnt4 in Wnt9b mutants. The patterned expression of Wnt9b targets at the earliest stages of metanephric development suggests that Wnt9b directly signals to multiple distinct cell types within the metanephric mesenchyme with distinct molecular responses.

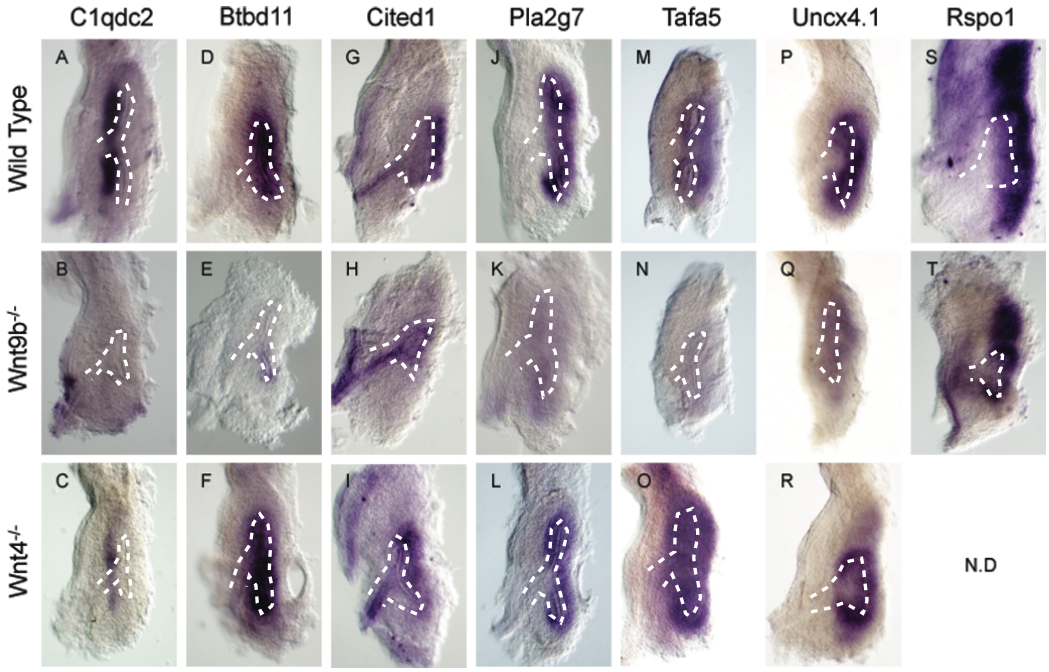

Figure 2-4: Wnt9b signals to both the pre-tubular aggregates and the mesenchymal progenitors.
In situ hybridization evaluating the expression of C1qdc2 (A-C), Btbd11 (D-F), Cited1 (G-I), Pla2g7 (J-L), Tafa5 (M-O), Uncx4.1 (P-R), and Rspondin 1 (S and T) in wild type (A, D, G, J, M, P and S), Wnt9b$^{-/-}$ (B, E, H, K, N, Q and T) and Wnt4$^{-/-}$ (C, F, I, L, O and R) at e11.5. The pre-tubular aggregate gene C1qdc2 is lost in Wnt9b mutants but unaffected in Wnt4 mutant animals (compare B and C to A). Progenitor genes are absent or significantly reduced in Wnt9b mutants and unaffected in Wnt4 mutants (compare E and F to D, H and I to G, K and L to J, N and O to M, and Q and R to P for Btbd11, Cited1 , Pla2g7, Tafa5 and Uncx4.1 respectively). The canonical Wnt agonist Rspondin 1 (Rspo1) is expressed in the progenitor cells (S) and is reduced in Wnt9b mutants (T).

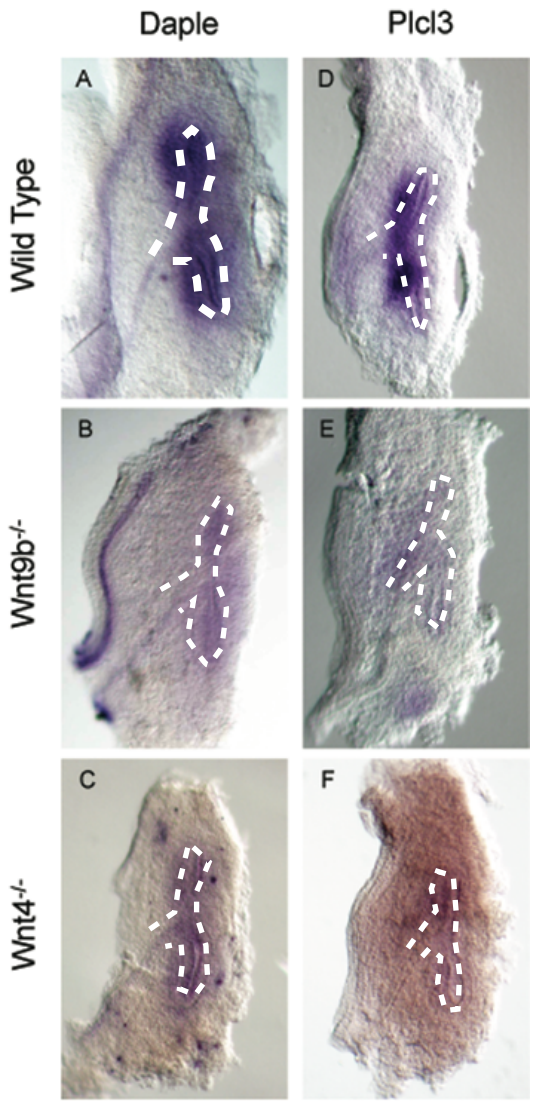

Figure 2-5: Identification of novel Wnt4 pre-tubular aggregate target genes.

In situ hybridization evaluating the expression of *Daple* (A-C), and *Phospholipase C like 3* (*plcl3*) (D-F) in wild type (A and D), *Wnt9b*$^{-/-}$ (B and D) and *Wnt4*$^{-/-}$ (C and F) at e11.5. The pre-tubular aggregate genes *Daple* and *Plcl3* are lost in both *Wnt9b* and *Wnt4* mutants (compare B and C to A and E and F to D respectively). The dotted lines outline the ureteric bud epithelium.

Wnt9b activates the canonical/β-catenin pathway.

Although it is known that the Wnt9b is necessary and sufficient for renal vesicle formation, it is unclear whether this is the result of canonical or non-canonical pathway activity. Further, as the canonical pathway was thought to play an inhibitory role in progenitor cell expansion/maintenance, determination of Wnt9b pathway usage in this cell population was also of great interest. The identification of genes that appear to be direct targets of Wnt9b allows us to unambiguously determine pathway usage within the distinct cell populations.

To determine the molecular nature of the Wnt9b signal we utilized a combination of *ex vivo* organ culture and genetics. Recently described small molecule inhibitors of the Wnt pathway represent useful molecular tools to study the role of Wnt signaling in kidney organ culture [159, 160]. In this study, we utilized two individual inhibitors, IWP2 and IWR1, that function at distinct points in the Wnt pathway. IWP2 inhibits porcupine function thus blocking secretion of Wnt ligands. Application of this molecule would be predicted to block both canonical and non-canonical Wnt signaling. IWR1 blocks the activity of Tankyrase1 and 2, resulting in the stabilization of Axin1 and 2 [159, 160]. Application of this compound should specifically inhibit the canonical/β-catenin dependent pathway. E11.5 wild type urogenital systems were cultured for 48 hours in the presence of either DMSO, IWP2 or IWR1 and assayed for the expression of genes that were shown previously to be a beta-catenin target gene

(Wnt11) or genes that are not regulated by beta-catenin (Wnt9b and Six2). As expected all of these genes are expressed in kidneys cultured in DMSO (Figure 2-6 A-D). Treatment of kidneys with either IWP2 or IWR1 inhibited the expression of both Wnt11 and Pax8 but did not affect either Wnt9b or Six2 expression (Figure 2-6). These data indicate that IW treatment is specifically inhibiting the Wnt pathway.

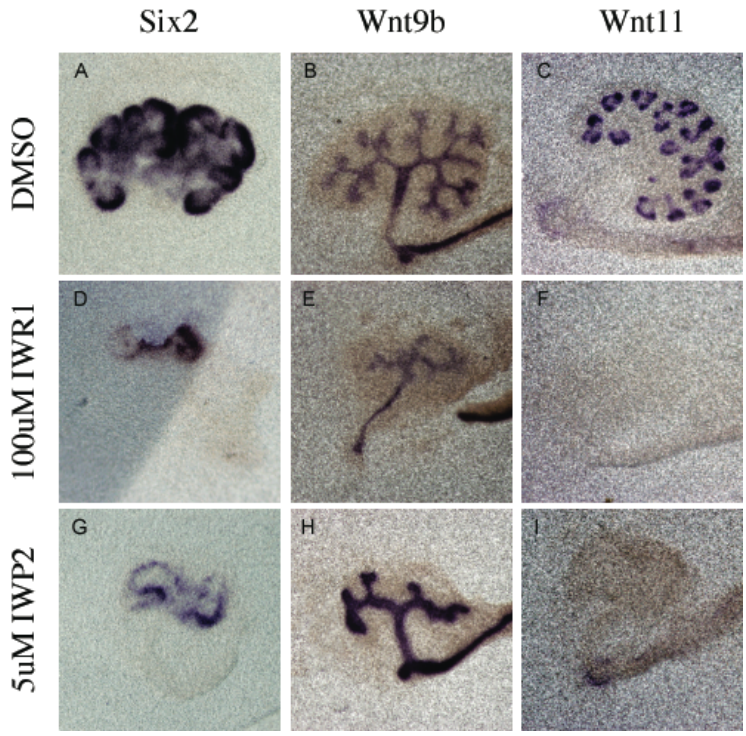

Figure 2-6: IW treatment specifically inhibits Wnt/β-catenin target gene expression.
In situ hybridization evaluating the expression of genes that are lost in Wnt mutants (Wnt11) or not affected by loss of Wnt signaling Six2 and Wnt9b in e11.5 cultured in the presence of either DMSO (A and D), IWP2 (B and E) or IWR1 (C and F). Neither Six2 nor Wnt9b is affected by either IWP2 or IWR1 treatment (Compare A to D and G and B to E and H for Six2 and Wnt9b respectively). However, Wnt11 is lost in both IWP2 and IWR1 treated kidneys (compare C to F and I). These data indicates the effect of IW treatment is due to inhibition of the Wnt pathway and not due to toxicity of the compounds.

I next cultured E11.5 wild type urogenital systems for 48 hours in the presence of either DMSO or IWP2 and assayed for the expression of Wnt9b targets. As expected, the PTA targets, C1qdc2 and Pax8 and the progenitor markers Btbd11, Pla2g7, Tafa5 and Uncx4.1 were all expressed in kidneys cultured in DMSO (Figure 2-7 A, D, G, J, M, and P). However, inhibition of Wnt ligand secretion using IWP2 resulted in a complete loss of all of these genes within 24 hours of drug administration (data not shown). This was not a delay in kidney development, as markers were still undetectable after 72 hours of culture (Figure 2-7 and data not shown). This is also not a result of toxicity as several non Wnt targets including Six2 and Wnt9b were expressed in treated kidneys (Figure 2-6). These data indicate that the secretion of Wnt ligands is necessary for the expression of both progenitor and PTA Wnt9b target genes.

To test if the loss of Wnt9b target genes could be specifically attributed to inhibition of the canonical Wnt pathway, we cultured kidneys in the presence of IWR1. As expected, IWR1 administration led to the complete inhibition of PTA genes, C1qdc2 and Pax8 (Figure 2-7). Similarly, culture in IWR1 inhibited the expression of progenitor target genes Pla2g7, Tafa5 and Uncx4.1 (Figure 2-7). Interestingly, Btbd11, a progenitor gene that requires Wnt9b activity and is inhibited by IWP2 treatment is minimally affected by IWR1 treatment (Figure 2-7 G,H and I), indicating that Btbd11 is a novel non-canonical Wnt9b target gene.

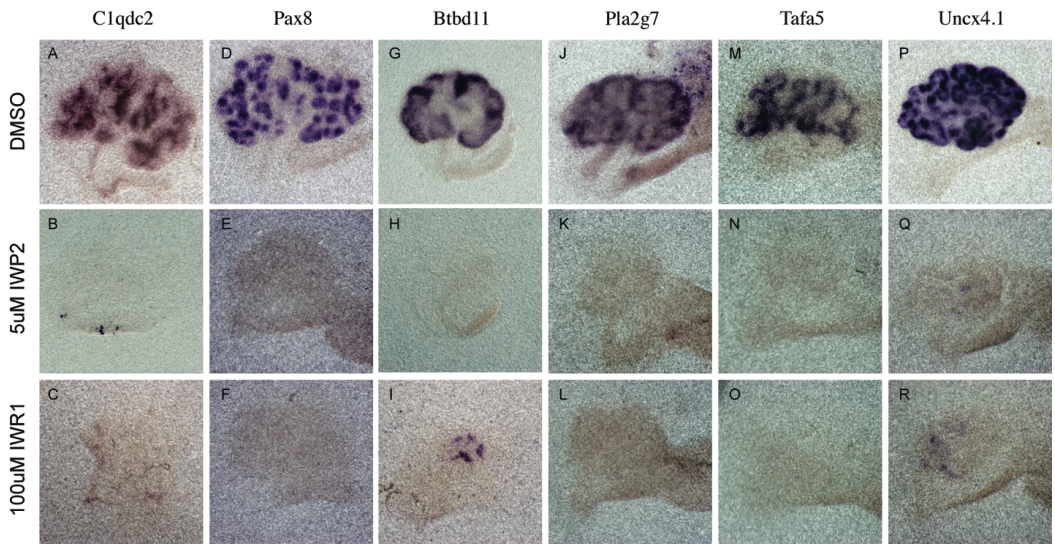

Figure 2-7: Wnt9b target gene expression is affected by modulation of Wnt signaling.
In situ hybridization evaluating the expression of C1qdc2 (A-C), Pax8 (D-F) Btbd11 (G-I), Pla2g7 (J-L), Tafa5 (M-O) and Uncx4.1 (P-R) in e11.5 cultured in the presence of either DMSO (A, D, G, J, M and P), IWP2 (B, E, H, K, N and Q) or IWR1 (C, F, I, L, O and R). Both pre-tubular aggregate genes and progenitor genes are expressed in cultured kidneys (A, D, G, J, M and P respectively). IWP2 culture results in a significant reduction in the expression of PTA genes (compare B to A and E to D) and progenitor genes (compare H to G, K to J, N to M and Q to P). IWR1 culture leads to a significant reduction in the PTA genes C1qdc2 and Pax8 (compare C to a and F to D respectively) and the progenitor genes Pla2g7, Tafa5 and Uncx4.1 (compare L to J, O to M and R to P respectively). IWR1 treatment has a minimal effect on the expression of the progenitor gene Btbd11 compared to IWP2 treatment suggesting it is a β-catenin independent (non-canonical) target of Wnt9b (compare I to G and H). These data indicate that both canonical and non-canonical Wnt signals are active in the mesenchymal progenitor cells.

To verify the above results, we ablated β-catenin from the progenitor population utilizing the Rarb2Cre deleter strain and a floxed allele of β-catenin (catnb^{flox}). Although the phenotype of the Rarb2Cre;catnb$^{-/flox}$ pups was extremely variable, the majority (8/10) formed hypoplastic kidneys and died within 2 days of birth (not shown). Morphologically the Rarb2Cre;Catnb$^{-/flox}$ kidneys were indistinguishable

from wild type until e12.5 (not shown). This appears to be due to a failure to ablate β-catenin protein from the mesenchyme prior to this time. However, *in situ* hybridization showed both Pla2g7 and Tafa5 were significantly down-regulated in the MM of Rarb2Cre;Catnb$^{flox/-}$ animals at e12.5 (Figure 2-8). Collectively, these data indicate that β-catenin is necessary for the expression of Wnt9b progenitor target genes.

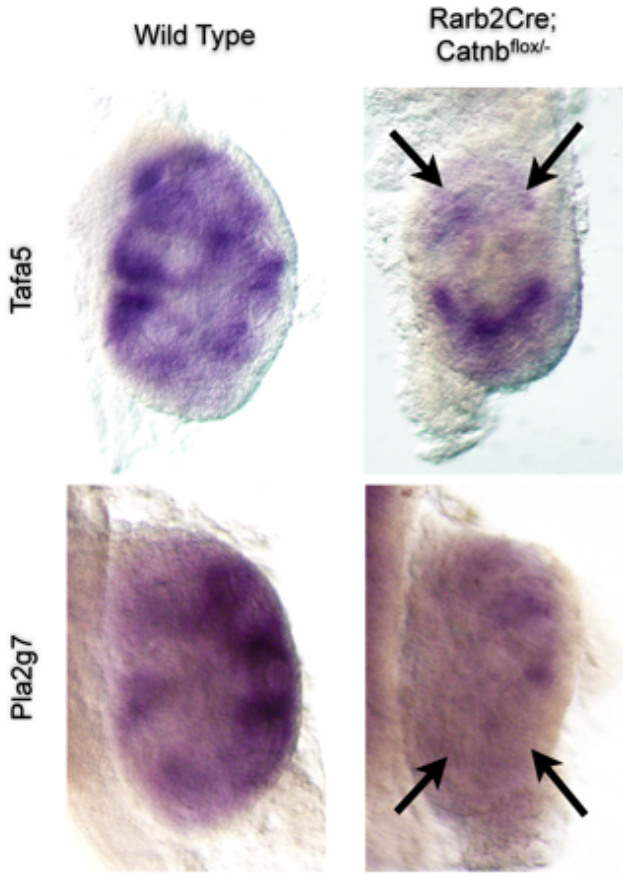

Figure 2-8: β-catenin is necessary for the expression of Wnt9b progenitor target genes.
In situ hybridization evaluating the expression of Tafa5 (A, B) and Pla2g7 (C, D) in wild type (A, C) or Rarb2Cre;Catnb$^{flox/-}$ (B, D) e12.5 kidneys. Both Tafa5 and Pla2g7 are significantly reduced in Rarb2Cre;Catnb$^{flox/-}$ (compare A to B and C to D respectively). Arrows denote progenitor cells that do not express either Tafa5 (B) or Pla2g7 (D).

β-catenin signaling induces the expression of Wnt9b targets

To test if β-catenin activation was sufficient to activate target gene expression, I isolated e11.5 metanephric mesenchyme and cultured them in the presence or absence of the β-catenin agonist, LiCl, for 48 hours and assayed for gene expression [161]. 15mM LiCl was sufficient to induce the expression of the PTA targets C1qdc2 and Pax8, while DMEM alone was not (Figure 2-9 A, B, C and D). Next we evaluated the expression of Wnt9b progenitor targets in cultured mesenchyme. As expected, isolated

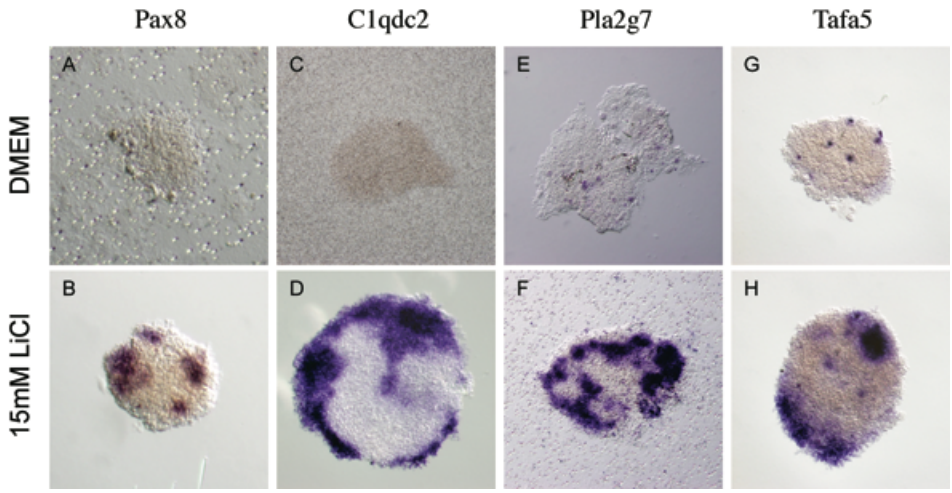

Figure 2-9: Activation of β-catenin is sufficient to induce the expression of Wnt9b target genes.
In situ hybridization evaluating the expression of Pax8 (A, B), C1qdc2 (C, D), Pla2g7 (E, F), and Tafa5 (G, H) in isolated metanephric mesenchyme cultured for 48 hours in the presence of DMEM (A, C, E and G) or 15mM LiCl (B, D, F and H). Mesenchyme cultured in the presence of media alone do not express either the pre-tubular aggregate genes Pax8 and C1qdc2 (See A and C respectively) or the progenitor target genes Pla2g7 and Tafa5 (See E and G respectively). Culturing mesenchyme in 15mM LiCl induces the expression of the pre-tubular aggregate targets Pax8 and C1qdc2 (compare B to A, and D to C respectively). LiCl also induces the expression of the progenitor target genes Pla2g7 and Tafa5 (compare F to E and H to G respectively). These data indicate that canonical Wnt signaling is sufficient to induce the expression of Wnt9b progenitor target genes.

mesenchyme cultured in DMEM alone for 48 hours was devoid of any progenitor marker gene expression (Figure 2-9 E and G). However, mesenchymes cultured with LiCl expressed the Wnt9b progenitor genes Pla2g7 and Tafa5 (Figure 2-9 F and H respectively).

Next, we tested whether activation of β-catenin in the progenitor cells of a $Wnt9b^{-/-}$ mutant was sufficient to rescue the expression of Wnt9b target genes. To accomplish this, we expressed an activated allele of β-catenin ($catnb1^{exon3flox}$) in the progenitor cells of Wnt9b mutants using the Rarb2Cre transgene. Activation of β-catenin in the progenitor cells of $Wnt9b^{+/-}$ embryos results in an anterior expansion of both Tafa5 and Pla2g7 relative to wild type (Figure 2-10 compare A to B and E to F). This expansion is probably reflective of the expression of this cre line outside of the metanephric mesenchyme in the mesenchyme surrounding the nephric duct and the mesonephric tubules [162]. Consistent with this hypothesis, we also observed ectopic expression of Tafa5 and Pla2g7 in the mesonephric tubules in Rarb2Cre;$catnb1^{exon3flox}$ animals (Data not shown). Activation of β-catenin in the renal progenitors of $Wnt9b^{-/-}$ mutants also rescues the expression of Tafa5 and Pla2g7, although the expression was somewhat mosaic (Figure 2-10 compare C to D and G to H respectively). Collectively, our data indicate that Wnt9b signals through β-catenin to directly regulate gene expression in the progenitor cells. In support of this hypothesis, the promoters of Pla2g7, Tafa5 and Uncx4.1 all

contain multiple consensus β-catenin binding sites (our unpublished observation).

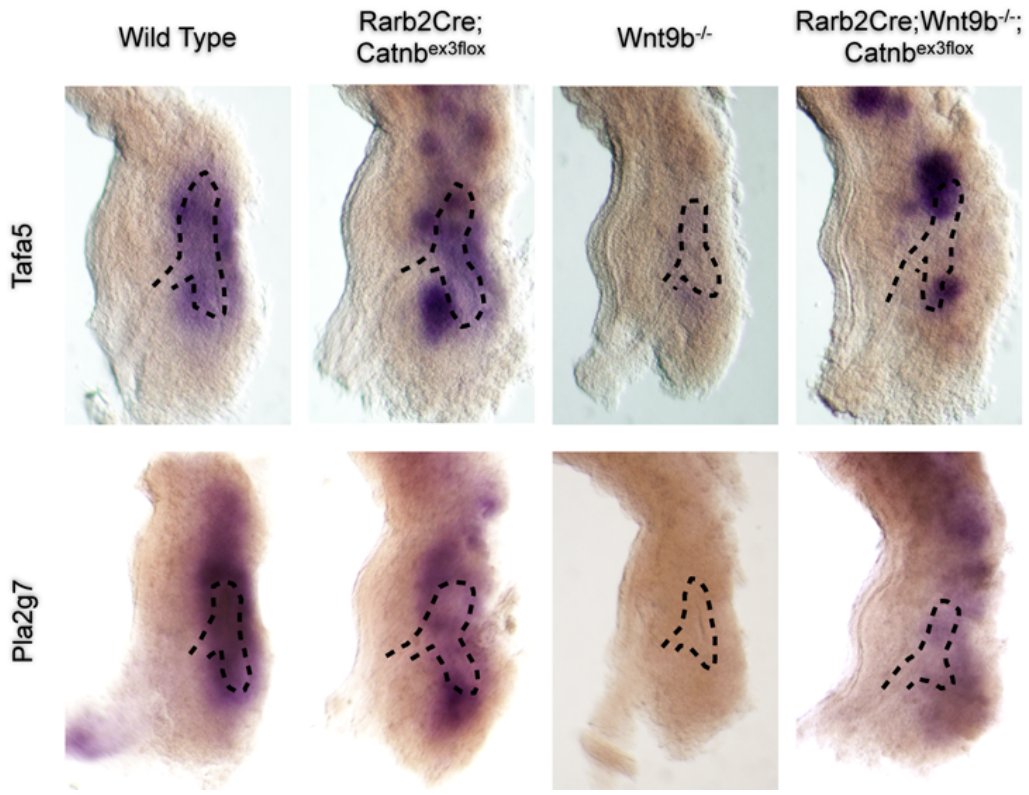

Figure 2-10: Activation of β-catenin rescues Wnt9b progenitor target gene expression *in vivo*.
In situ hybridization evaluating the expression of Tafa5 (A-D) and Pla2g7 (E-H) in wild type (A, E), Rarb2Cre;Catnb^{exon3flox} (B, F), *Wnt9b*$^{-/-}$ (C, G) and Rarb2Cre;*Wnt9b*$^{-/-}$;Catnb^{exon3flox} (D, H) e11.5 kidneys. Both Tafa5 and Pla2g7 are expressed normally in wild type kidneys and are significantly reduced in *Wnt9b*$^{-/-}$ kidneys (See A, E and C, G). Activation of beta-catenin (Rarb2Cre;Catnb^{exon3flox}) induces an anterior expansion and a more punctate expression of both *Tafa5* and *Pla2g7* (compare A to B and E to F respectively). Both *Pla2g7* and *Tafa5* are rescued by the activation of beta-catenin in Wnt9b mutant kidneys (Rarb2Cre;Wnt9b$^{-/-}$;Catnb^{exon3flox}) (compare A to B and E to F respectively). These data indicate Wnt9b signals through β-catenin to regulate target gene expression in the progenitor domain.

Interestingly, our microarray analysis also indicated that the canonical Wnt agonist Rspondin 1 (Rspo1) is significantly down-regulated

1.27 fold in Wnt9b mutant MM. We analyzed the expression of Rspo1 in the developing kidney using *in situ* hybridization. Rspo1 is expressed throughout the progenitor domain at e11.5 and is slightly reduced in the MM of Wnt9b mutants (Figure 2-4 S, T). In addition to Rspo1 another canonical agonist Rspo3 is also expressed in the progenitor domain (Figure 2-11 B). Further, our study, along with several previous studies, indicates that Wnt signaling activates the expression of several canonical pathway antagonists, like Daple and Sfrp2, in the PTA (Figure 2-11C and [141]). The presence of canonical agonists in the progenitor domain coupled with the expression of canonical antagonists in the PTA (and the secreted antagonist Dkk1 from the stalk of the ureteric bud (Figure 2-11D) indicates that β-catenin signaling is active in the progenitor cells and attenuated in the renal vesicles. Although previous studies suggested that Wnt9b and β-catenin signaling must be inhibited in the progenitor cells [122, 124, 141, 143, 163], these data raise the possibility that the progenitor cells are actually receiving the highest levels of β-catenin signaling.

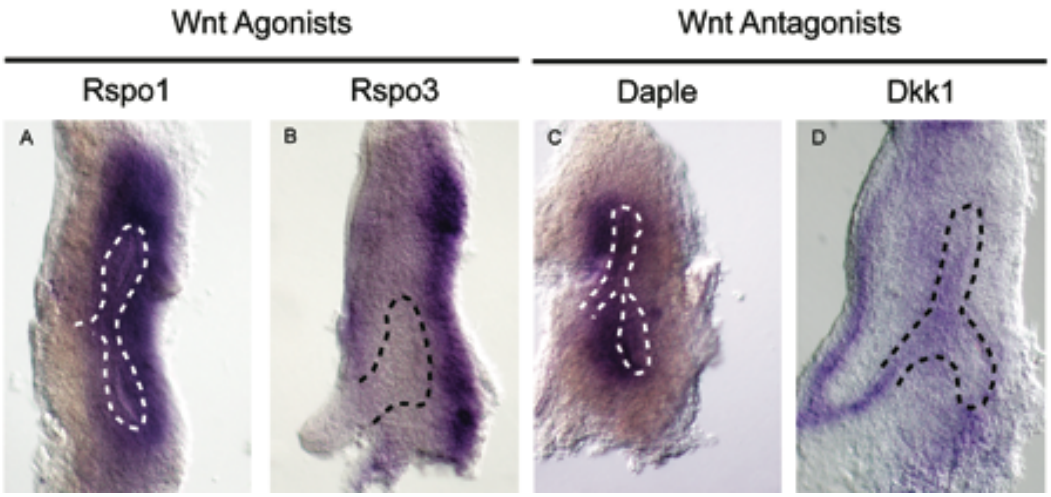

Figure 2-11: Expression of canonical Wnt agonists and antagonists in the developing kidney.
In situ hybridization evaluating the expression of *Rspo1* (A), *Rspo3* (B), *Daple* (C) and *Dkk1* (D) in wild type E11.5 kidneys. The canonical Wnt agonists *Rspo1* and *Rspo3* are both expressed in the progenitor cells at E11.5 (A and B respectively). The Dsh interacting protein *Daple* is expressed in the pre-tubular aggregates (C) while the secreted Wnt antagonist *Dkk1* is expressed in the stalk of the ureteric bud (D) and would be predicted to be secreted to the cells of the pre-tubular aggregates. These data indicate the progenitor cells may receive the highest levels of β-catenin signaling.

Six2 is necessary for the expression of Wnt9b progenitor target genes.

Previous studies suggested that Six2 inhibited Wnt9b and β-catenin signaling within the progenitor cells [143]. However, our data indicate that Wnt9b is actively signals through β-catenin to all cells of the MM but that the transcriptional response to Wnt9b varies depending on the position of the cell receiving the Wnt9b signal. We wondered if the disparate response to Wnt9b signaling in the progenitor cells was dependent upon Six2 activity. To determine the effect of loss of Six2 on Wnt9b target genes, we evaluated their expression in $Six2^{-/-}$ mutants at e11.5. As

expected, *in situ* hybridization revealed that C1qdc2 is expanded into the progenitor domain in *Six2*$^{-/-}$ mesenchyme at e11.5 (Figure 2-12 A and B).

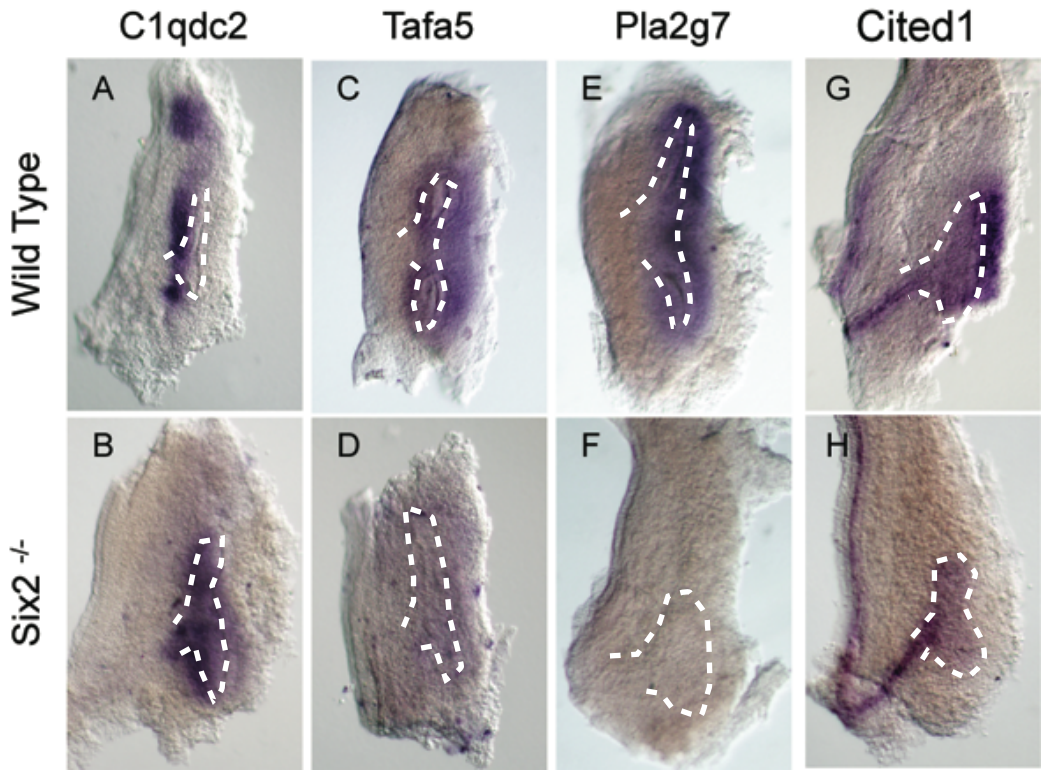

Figure 2-12: Six2 is necessary for the expression of Wnt9b progenitor target genes.
In situ hybridization evaluating the expression of the pre-tubular aggregate target gene *C1qdc2* (A, B) and the progenitor targets *Tafa5*, *Pla2g7*, and *Cited1* (C--H) in wild type (A, C, E and G) and *Six2*$^{-/-}$ e11.5 kidneys (B, D, F and H). C1qdc2 is expanded into the progenitor domain in *Six2*$^{-/-}$ (compare B to A). The progenitor targets *Tafa5*, *Pla2g7* and *Cited1* are significantly reduced in *Six2*$^{-/-}$ kidneys (compare D to C, F to E and H to G respectively). These data indicate Six2 is necessary to mediate the progenitor response to Wnt9b signaling.

These results are similar to those observed for Pax8, Wnt4 and Sfrp2 and likely explain the precocious and ectopic tubule induction that characterizes the *Six2*$^{-/-}$ mutants [141, 143]. We next evaluated the expression of Wnt9b progenitor targets in *Six2*$^{-/-}$ mutants. *In situ*

hybridization revealed that the Wnt9b targets Cited1, Pla2g7 and Tafa5 were absent from *Six2*$^{-/-}$ mesenchyme at e11.5 while non-Wnt9b targets such as Eya1, GDNF and Pax2 were still present (Figure 2-12 C-H and [141]). These results suggest that both Wnt9b and Six2 are necessary for the expression of Wnt9b progenitor targets. To rule out the possibility that premature differentiation of the mesenchyme is responsible for the loss of Wnt9b targets in Six2 mutant kidneys (even though other progenitor markers are still present), we evaluated the expression of Wnt9b target genes in Six2 mutants at e10.5, 24 hours prior to any signs of premature differentiation. *In situ* hybridization indicates that Tafa5 is not expressed in the MM of either *Wnt9b*$^{-/-}$ or *Six2*$^{-/-}$ embryos at e10.5 (Figure 2-13).

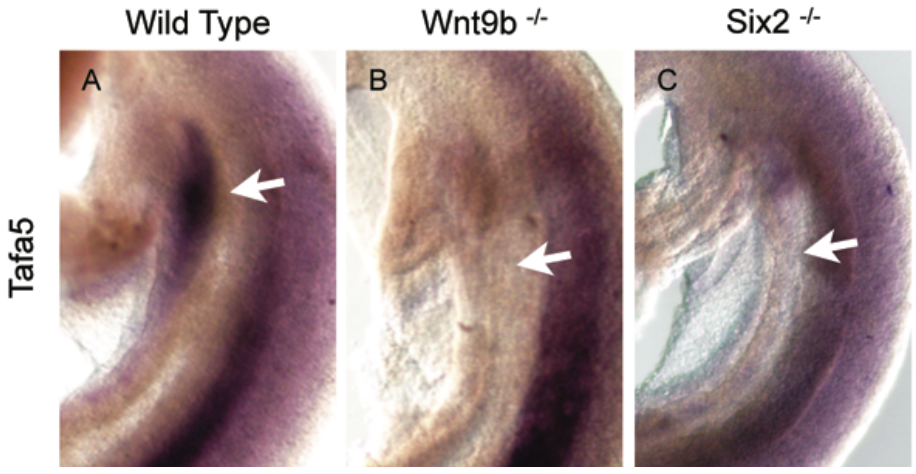

Figure 2-13: Expression of Tafa5 requires both Six2 and Wnt9b activity.
In situ hybridization evaluating the expression of the progenitor target gene *Tafa5* in wild type (A), *Wnt9b*$^{-/-}$ (B) and *Six2*$^{-/-}$ (C) E10.5 embryos. Tafa5 expression is lost from the metanephric mesenchyme (arrows) in both *Wnt9b*$^{-/-}$ and *Six2*$^{-/-}$ animals (Compare B and C to A respectively). These data indicate that Wnt9b and Six2 cooperate to regulate the expression of progenitor Wnt9b target genes.

Wnt9b regulates progenitor cell proliferation

The data presented above suggest that canonical Wnt9b signaling is active in the progenitor cell population. Previous studies have demonstrated roles for Wnt signaling in progenitor cell specification, survival/maintenance and renewal/proliferation [164-168] . To test a role for Wnt9b in specification, we examined the expression of several additional progenitor markers that were not significantly changed in Wnt9b mutants. Six2 and Pax2 are still expressed up to e13.5, although relative to wild type, at E13.5 the domain of expression is greatly reduced compared to wild type (Figure 2-14). To test whether this population was still competent to respond to the inductive signal, mutant mesenchyme was removed at E13.5 and recombined with a ureteric bud from a wild type e11.5 kidney. The mutant progenitors were competent to form tubules (Figure 2-15 E,F) and activate Wnt9b progenitor markers when recombined with a wild type E11.5 ureteric bud (Fig 2-15).

The reduced size of the progenitor population in Wnt9b mutants suggests that Wnt9b may be involved in cell proliferation and/or survival. To test a role for Wnt9b in survival, we examined rates of apoptosis in null mesenchyme at E11.5. This analysis revealed no significant difference in

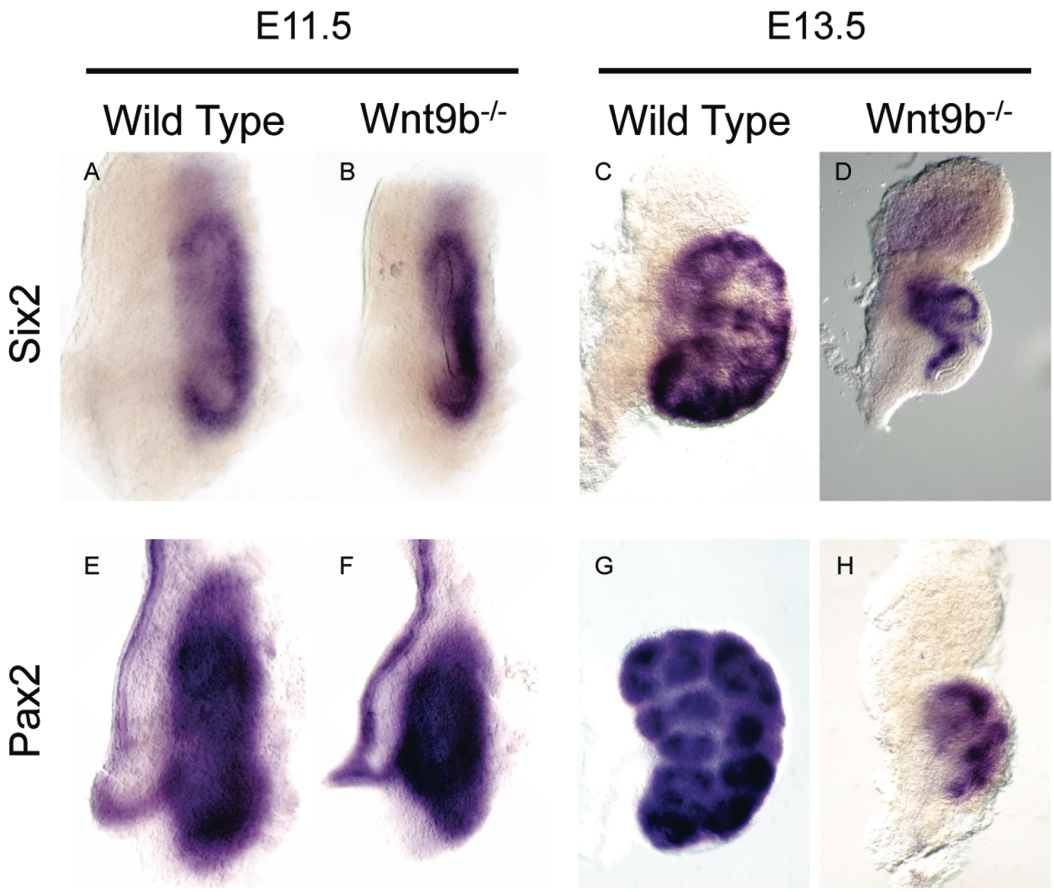

Figure 2-14: Wnt9b is not necessary for progenitor specification
In situ hybridization evaluating the expression of the progenitor cell markers Six2 (A-D) and Pax2 (E-H) in wild type or *Wnt9b*$^{-/-}$ kidneys at E11 and 13.5. Neither Six2 nor Pax2 is affected upon loss of Wnt9b at E11.5 (Compare A and B for Six2 and E and F for Pax2). Both Six2 and Pax2 continue to be expressed at E13.5 but do not appear to expand properly in Wnt9b$^{-/-}$ kidneys (compare C to D and G to H). These data indicate Wnt9b is not necessary to specify the progenitor population.

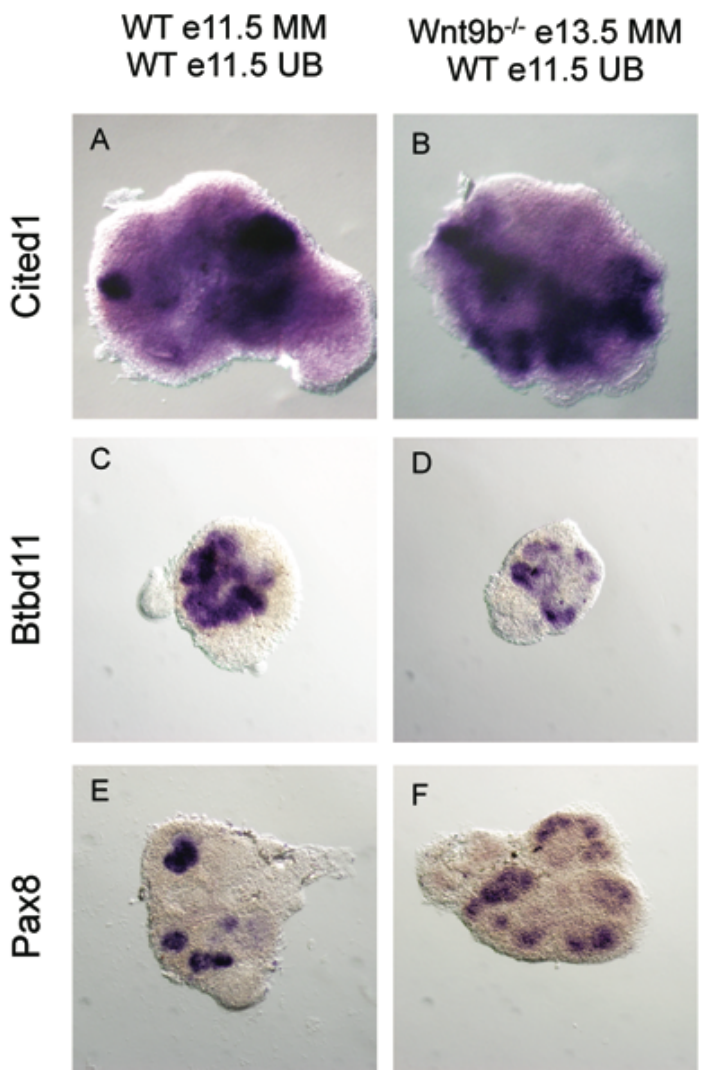

Figure 2-15: Wnt9b$^{-/-}$ progenitor cells are competent to respond to Wnt9b.

In situ hybridization evaluating the expression of the progenitor target genes *Cited1* and *Btbd11* (A-D) and the PTA gene *Pax8* (E, F) in recombined metanephric mesenchymes. Wild type E11.5 metanephric mesenchyme (MM) that has been recombined with a wild type E11.5 ureteric bud (UB) is competent to turn on both progenitor (A, C) and PTA Wnt9b target genes (E). Similarly MM isolated from an E13.5 *Wnt9b*-/- also turns on both progenitor and pre-tubular aggregate targets (B, D and F). These data indicate that the progenitor cells that form in *Wnt9b*$^{-/-}$ animals are competent to respond to the Wnt9b signal.

apoptosis indicating Wnt9b does not regulate cell survival (Data not shown).

Finally, we examined the rates of proliferation in Pax2 positive wild type and Wnt9b mutant mesenchyme at E11.5. Pax2 positive, wild type e11.5 MM had a rate of proliferation of 2.48% as assayed by the number of phospho-histone H3 positive cells per Pax2 positive mesenchyme. This rate was significantly affected by the loss of Wnt9b as mutants had a 0.48% rate of proliferation (Figure 2-16, n=3 individual animals and 2376 (wild type) or 2122 (*Wnt9b*$^{-/-}$) total cells. p=0.007 T-test). To ensure the effect on proliferation was due to the loss of Wnt9b and not due to the loss of Wnt4 from the MM we also evaluated proliferation in *Wnt4*$^{-/-}$ MM. *Wnt4*$^{+/-}$ MM averaged 3.12% proliferation. Although proliferation was also significantly decreased in *Wnt4*$^{-/-}$ MM (1.81% n=3 p=0.006 T-test), the rate in Wnt4 mutants was much higher than observed in Wnt9b mutants. Future studies need to determine whether the differences in proliferation rates can be attributed to restrictions in the spatial activities of the two Wnts (i.e. Wnt4 only signals to the pre-tubular aggregates while Wnt9b signals to the progenitors).

Late ablation of Wnt9b results in premature loss of the progenitor cells

We previously found that using the KspCre deleter mouse to remove Wnt9b from the kidney by E17.5 resulted in kidneys that were

significantly smaller than wild type (See Chapter 4). KspCre becomes active in the ureteric bud tips (adjacent to the progenitors) some time

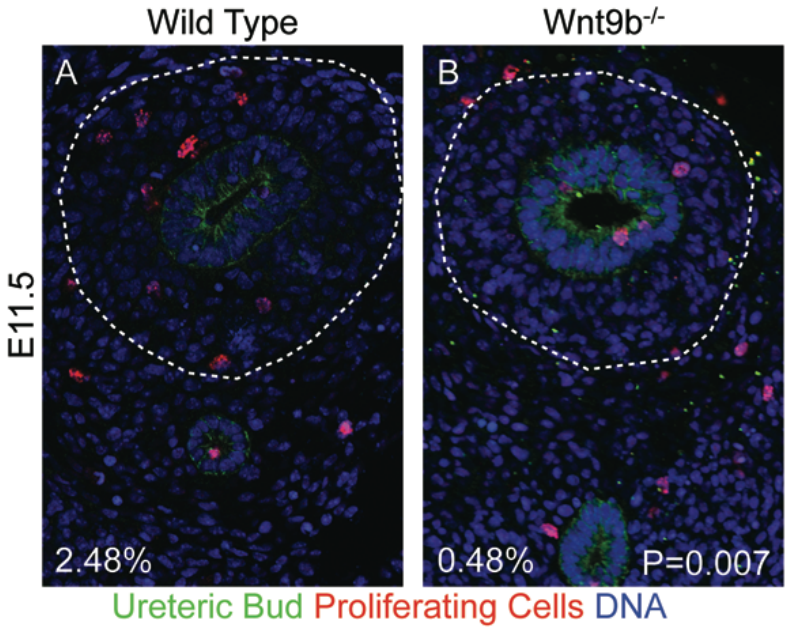

Figure 2-16: Wnt9b regulates proliferation of the metanephric mesenchyme.
E11.5 kidney sections stained with an antibody recognizing a marker of cell proliferation, phosphorylated Histone H3 (red), the ureteric bud marker E-cadherin (green) and the DNA marker Sytox Green (Blue). Serial sections were labeled with the metanephric mesenchyme marker Pax2 to for counting purposes (not shown). PH3 staining reveals a significant (p=0.007) decrease in cell proliferation in *Wnt9b*$^{-/-}$ kidneys (compare outlined MM in A to B). These data indicate that Wnt9b regulates proliferation in the MM.

between E15.5 and 17.5. We originally attributed this phenotype to a deficit in tubule induction [169]; however our new data suggest this may be a deficit in progenitor cell expansion. To test this hypothesis, we examined Wnt9b dependent and independent progenitor markers in KspCre;Wnt9b-/flox kidneys at P1 and found that they completely lacked progenitor cells (Figure 2-17 compare I to J). To determine the kinetics of progenitor cell loss in these mutants, we examined the mutant kidneys at

embryonic stages. Pax2, Six2 and the Wnt9b target genes, Pla2g7, Tafa5 and Uncx4.1 were all present and expressed at comparable levels to wild type

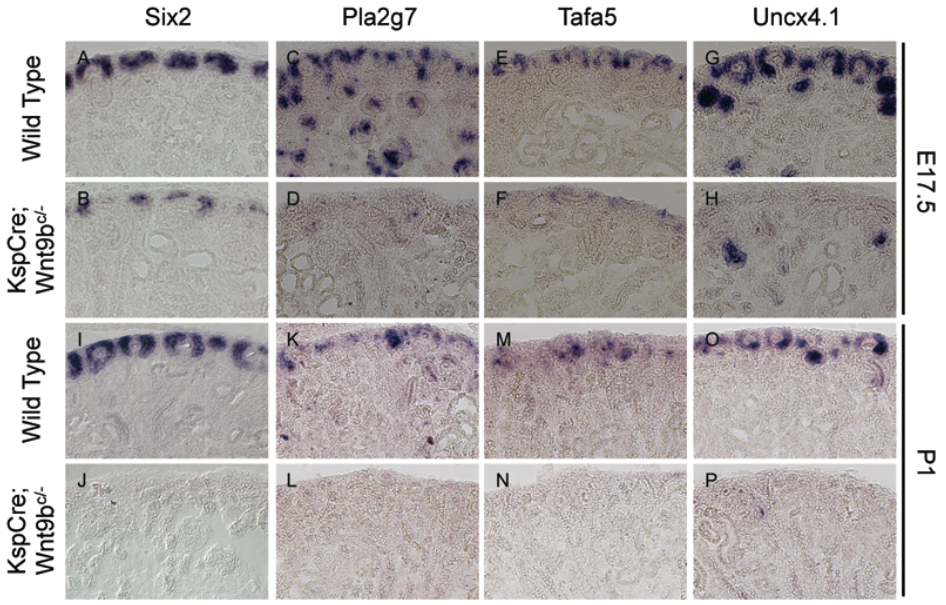

Figure 2-17: Wnt9b is necessary for expansion of progenitor cells. Section *in situ* hybridization evaluating the expression of the progenitor cell marker Six2 (A, B, I and J) and the Wnt9b target genes Pla2g7, Tafa5 and Uncx4.1 (C-H, and K-P) in wild type or KspCre;Wnt9b$^{c/-}$ kidneys at E17.5 and P1. Six2 positive progenitor cells are significantly reduced at e17.5 (compare A to B) and are completely absent by birth (P1 compare I to J) in KspCre;Wnt9b$^{c/-}$ kidneys. *Pla2g7* and *Uncx4.1* are absent from the progenitor population as early as e17.5 (compare C and D for Pla2g7 and G and H for Uncx4.1) indicating Wnt9b has been removed from the UB at this stage. Tafa5 expression is significantly reduced in the progenitor cells at E17.5 (Compare E to F). All progenitor target genes are not expressed at P1 (K-P). These data indicate Wnt9b signaling is necessary for expansion of the progenitor cells.

kidneys at E15.5 (Not shown). At E17.5, Pax2 and Six2 levels were reduced in the MM of KspCre;Wnt9b$^{-/c}$ animals (Figure 2-17 and not shown) while Pla2g7 and Uncx4.1 were completely lost (Fig 2-17 C,D and G,H). Tafa5 was highly reduced although still expressed at low levels in

the progenitor cells at E17.5 but absent at P1 (Figure 2-17 E,F,G and H). The loss of expression for these genes was specific to progenitor cells as Pla2g7 expression in the medullary stroma was maintained in mutants (not shown). From these data we conclude Wnt9b signaling is necessary for the expansion of the progenitor population.

Discussion

A tight balance between differentiation and stem cell expansion and maintenance is essential for forming a fully functioning kidney. Relatively minor decreases in tubule number are associated with congenital forms of hypertension [22]. How this balance is mediated is not well understood. Previous models suggested that part of this balance required the inhibition of canonical Wnt activity in the progenitor population [122, 143]. However, in this study we demonstrate that Wnt9b is actively signaling to the progenitor cells where it plays an essential role in expansion and/or self-renewal.

Multiple lines of evidence suggest that the role for Wnt9b in the progenitors is through the canonical/β-catenin dependent pathway. First, we show using small molecules specific to the canonical pathway as well as β-catenin loss of function mutants that canonical activity is required for the expression of the progenitor genes. Secondly, we show that activation of the canonical pathway is sufficient to induce expression of these genes in the absence of Wnt9b. That this activation is not mimicking signaling downstream of Wnt4 is supported by the observation that the progenitor markers are expressed normally at least until E14.5 in Wnt4 mutants. Further, two agonists of the canonical Wnt pathway, Rspo1 and 3, are expressed specifically in the progenitor cells. Finally, several of these targets contain highly-conserved, consensus Lef/Tcf binding sites in their non-coding regions.

Previous studies suggested that Wnt9b signaled through the canonical pathway directly to induce differentiation and mesenchymal to epithelial transition [122]. However, given our current findings, it is possible that Wnt9b plays no direct role regulating MET in the PTAs. It is possible that activation of PTA markers is an indirect result of Wnt9b's role in proliferation. Once the progenitor population (or PTAs) reach a certain size, a subset of the cells may undergo MET by default [124]. We think this scenario is unlikely for several reasons. First, several of the PTA targets of Wnt9b are expressed directly adjacent to and along the entire length of the Wolffian duct (including in a sub-population of the metanephric mesenchyme) at E10.5, 24 hours before any significant expansion of this cell population and 48 hours prior to signs of MET. Secondly, one target of Wnt9b is Cadherin 4, a protein that is directly involved in cell-cell adhesion. Therefore, we think it is most likely that Wnt9b signals canonically to both the progenitors and the PTA population with distinct molecular and cellular readouts in each.

How does Wnt9b induce such a disparate response to β-catenin signaling and what factors mediate the decision between differentiation and self-renewal? No doubt the molecule Six2 is an important player in this cell fate decision. In this study we show that Six2 is not only necessary for the maintenance of the progenitor compartment but also for response to the Wnt9b signal. The precise nature of the interaction between Six2 and β-catenin is still unclear. It is plausible that Six2

regulates the expression of progenitor specific factors that interact with and alter the specificity of β-catenin. However Six2 may also directly (or indirectly) interact with β-catenin to drive expression of individual genes.

A simple model that explains all of the genetic analysis in this and previous studies is that the combination of β-catenin and Six2 results in progenitor renewal while β-catenin alone results in differentiation. Cells that express Six2 without activated β-catenin do not differentiate or proliferate and are quiescent. Cells that express Six2 and receive a Wnt9b signal are induced to proliferate and maintain the progenitor pool. Cells that receive the Wnt9b/β-catenin signal but do not express Six2 are induced to differentiate. A question that remains is how Six2 is repressed in the pre-tubular aggregates. It is plausible that factors originating from the stalk of the ureteric bud or from the previous round of tubule formation signal back to inhibit Six2 or alternatively, that a signal(s) emanating from the cortical stroma is required to maintain Six2.

It is also possible that a gradient of β-catenin activity determines the decision between differentiation and proliferation. The expression of the potent β-catenin agonist Rspondin 1 within the progenitor population supports the hypothesis that β-catenin is extremely active in this cell type. This coupled with the expression of many canonical Wnt antagonists, including secreted Wnt inhibitors, in the PTAs may indicate that the progenitor domain “sees” the highest level of β-catenin signaling. This coupled with the presence of Six2 would result in proliferation and self

renewal. As these cells begin to traverse the UB the levels of Wnt ligand (and thus β-catenin) they are receiving would begin to be attenuated due to the expression of secreted antagonists in the PTA like Sfrp2 and Dkk1 from the stalk of the UB. Cells in this position, which receive lower levels of β-catenin signaling and have reduced or no Six2 present, differentiate and undergo MET under the guidance of Wnt4.

Finally, it is of note that at least one Wnt9b target, Btbd11, did not appear to be a target of the canonical pathway. These data indicates that it is likely that both canonical and non-canonical pathways are active in the progenitor cells. Although it is possible that the effects on progenitor cell expansion are solely the result of abrogation of the non-canonical pathway, we feel this is unlikely. Ablation of β-catenin from the mesenchyme or culture of embryonic kidneys with the canonical pathway antagonists has a similar effect on progenitor cell expansion to loss of Wnt9b so the canonical pathway is certainly playing an essential role in this pathway. However, conclusive evidence will require additional characterization of the non-canonical pathway in this process.

In this summary, we have identified a novel mechanism by which progenitor cells respond to the same signal with distinct results, namely self-renewal vs. differentiation. By using this relatively simple mechanism, the kidney is able to balance tubule induction and stem cell maintenance insuring the proper number of tubules form. These findings provide novel insights not only into the molecular mechanisms that regulate kidney

development but also will enhance our ability to generate renal stem cells to repair or replace missing or damaged organs.

CHAPTER THREE

Results

Wnt9b signaling regulates planar cell polarity and kidney tubule morphogenesis

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Abstract

Although many vertebrate organs, such as kidneys, lungs and liver, are composed of epithelial tubules, little is known of the mechanisms that establish the length or diameter of these tubules. In the kidney, defects in the establishment or maintenance of tubule diameter are associated with one of the most common inherited human disorders, polycystic kidney disease. Here, we show that attenuation of Wnt9b signaling during kidney morphogenesis affects the planar cell polarity of the epithelium and leads to tubules with significantly increased diameter. Although previous studies showed that polarized cell divisions maintain the diameter of postnatal kidney tubules, we find that cell divisions are randomly oriented during embryonic development. Our data suggest that diameter is established during early morphogenetic stages by convergent extension processes and maintained by polarized cell divisions. Wnt9b, signaling through the non-canonical Rho/Jnk branch of the Wnt pathway, is necessary for both of these processes.

Introduction

Epithelial and endothelial tubules are some of the most common structures in the vertebrate body plan. Alterations in the shape of these structures have significant impact on their function. For instance, the functional unit of the kidney, the nephron, is a vascularized epithelial tubule whose proper three-dimensional structure is essential for its function in maintaining body fluid composition [148]. Defects in the establishment or maintenance of nephron diameter play causal roles in one of the most common genetic maladies in humans, polycystic kidney disease [170]. Studies in mice and humans have suggested that increased rates of cell proliferation are associated with, and may directly cause, cyst formation [170, 171]. However, examination of the developing epithelial tubules of worms and flies indicate that cellular processes that are independent of changes in cell number (e.g. cell size, membrane biosynthesis, cell polarity and cell movements) have significant impact on the establishment and maintenance of tubular diameter [172-183].

The Wnts encode a family of secreted glycolipoproteins that function in multiple biological processes including embryonic development and disease pathogenesis [23, 108, 184]. The Wnt signal is transduced through a Frizzled (*Fzd*) receptor and the cytoplasmic protein Dishevelled (*Dsh*). At the level of Dsh, the pathway can branch down either the canonical pathway that utilizes β−catenin as a transcription factor, or one of the non-canonical pathways that are β -catenin independent.

Previous studies have indicated that tight regulation of Wnt signaling is essential for proper development of the kidney tubules. Loss of canonical Wnt signaling in mice prevents formation of the tubules, while inappropriate activation of the Wnt signal transduction pathway leads to cyst formation [1, 16, 122]. In fact, improper stimulation of the canonical Wnt pathway is a hallmark of various types of human cystic kidney diseases including autosomal dominant polycystic kidney disease, autosomal recessive polycystic kidney disease and nephronophthisis [125]. Current models suggest that inappropriate activation of the canonical (β-catenin dependent) branch of the Wnt signal transduction pathway has a mitogenic effect that contributes to cyst progression [126]. However, recent studies have suggested that defects in planar cell polarity (PCP), a process that may be regulated by non-canonical (β-catenin independent) Wnt signaling may also contribute to cystogenesis [129].

PCP describes the polarization of cells perpendicular to their apical/ basal axis [39]. Genetic screens in Drosophila have identified multiple factors that are required for the establishment of PCP including two components of the Wnt pathway, Fz and Dsh [67, 68]. Whether Wnt ligands play a direct role in establishing PCP is somewhat controversial. Studies in Drosophila suggest that Wnts are not involved in PCP, while studies in vertebrates suggest they may play a permissive role and studies in worms suggest they play an instructive role [39, 86, 97, 185, 186]. There is currently no evidence suggesting that Wnt signaling is involved in

the establishment or maintenance of PCP in any epithelial tubules or that Wnts are involved in regulating epithelial tubule diameter.

We previously showed that Wnt9b was necessary for the earliest events in the induction of the kidney tubules [16]. Here, we demonstrate that Wnt9b is also required for morphogenesis of the nephron. Wnt9b produced by the ureteric bud and collecting ducts is required autonomously and non-autonomously for proper planar cell polarity within the collecting ducts and the adjacent proximal tubules. Specifically, we show that these tubules develop in two distinct phases. During the first phase, cell division is not oriented but the diameter of the epithelium decreases. We propose that convergent extension-like processes drive the lengthening and thinning of the tubules and establish diameter. In the second phase, polarized cell divisions predominate and maintain tubule diameter.

We have found that Wnt9b regulates both phases of development, perhaps through a role in regulating cell orientation. In contrast to its proposed canonical role in tubule induction, Wnt9b's role in tubule morphogenesis is mediated by the non-canonical/planar cell polarity signal transduction branch. This study is the first demonstration that loss of non-canonical Wnt signaling can contribute to cystogenesis as well as the first indication that convergent extension processes regulate tubule diameter in a vertebrate.

Results

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 [16], 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 (Figure 3-1A and C). Normally, renal vesicle induction is confined to the outermost (cortical) region of the kidney, as indicated by Pax8 positive cells (Figure 3-1B) and induction is reported to cease by P5 in mice [117]. However, Wnt9b is expressed in the distal (medullary) P1 collecting ducts (arrowhead in Figure 3-1A) 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.

Attenuation of Wnt9b signaling leads to dysplastic/cystic kidneys

Embryos completely lacking functional Wnt9b fail to form kidneys, resulting in death on P1. Mice that are homozygous for a hypomorphic allele of *Wnt9b* (*Wnt9b*$^{neo/neo}$) survive for several days to weeks post partum although 100% (N>30) of *Wnt9b*$^{neo/neo}$ animals die within 1 month

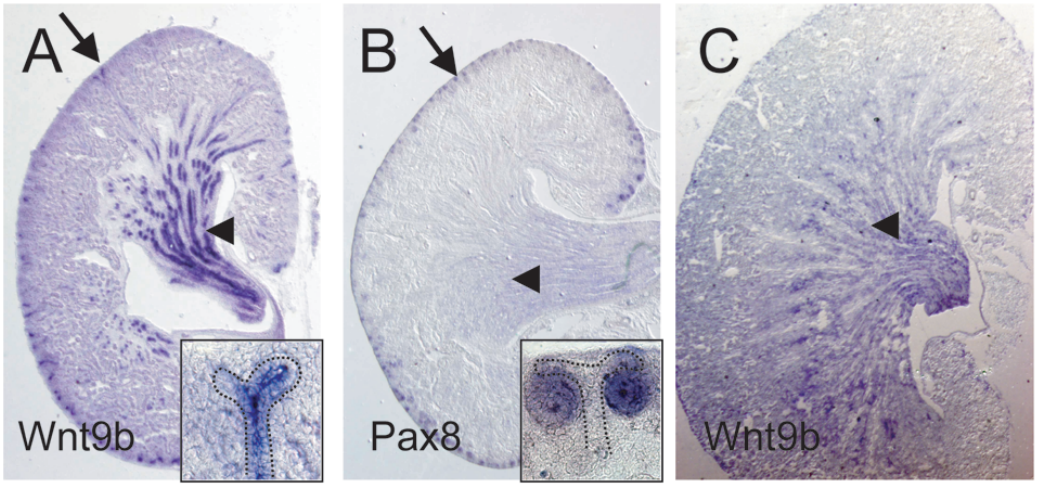

Figure 3-1: Expression of Wnt9b in the kidney.
Sections of P1 (a and b) and adult (7 month) kidneys hybridized with riboprobes to Wnt9b (a and c) and Pax8 (b). (a) Wnt9b is expressed in both the tips of the ureteric buds (arrows and inset) and in the distal collecting ducts in the kidney medulla (arrowhead) 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) In adult kidneys, Wnt9b continues to be expressed in the distal collecting duct (arrowhead) after nephrogenesis has ceased.

To test if the *Wnt9b*$^{neo/neo}$ cystic phenotype was the result of a direct role for Wnt9b in tubule diameter regulation rather than a secondary effect caused by deficits in renal vesicle induction, we attempted specifically to ablate Wnt9b from the collecting duct stalks, the cells we hypothesized were the source of Wnt9b during tubule maturation/morphogenesis. To accomplish this, we crossed mice with a conditionally inactive (floxed) allele of *Wnt9b* with mice carrying *KspCre* [187]. We found that, similar to what

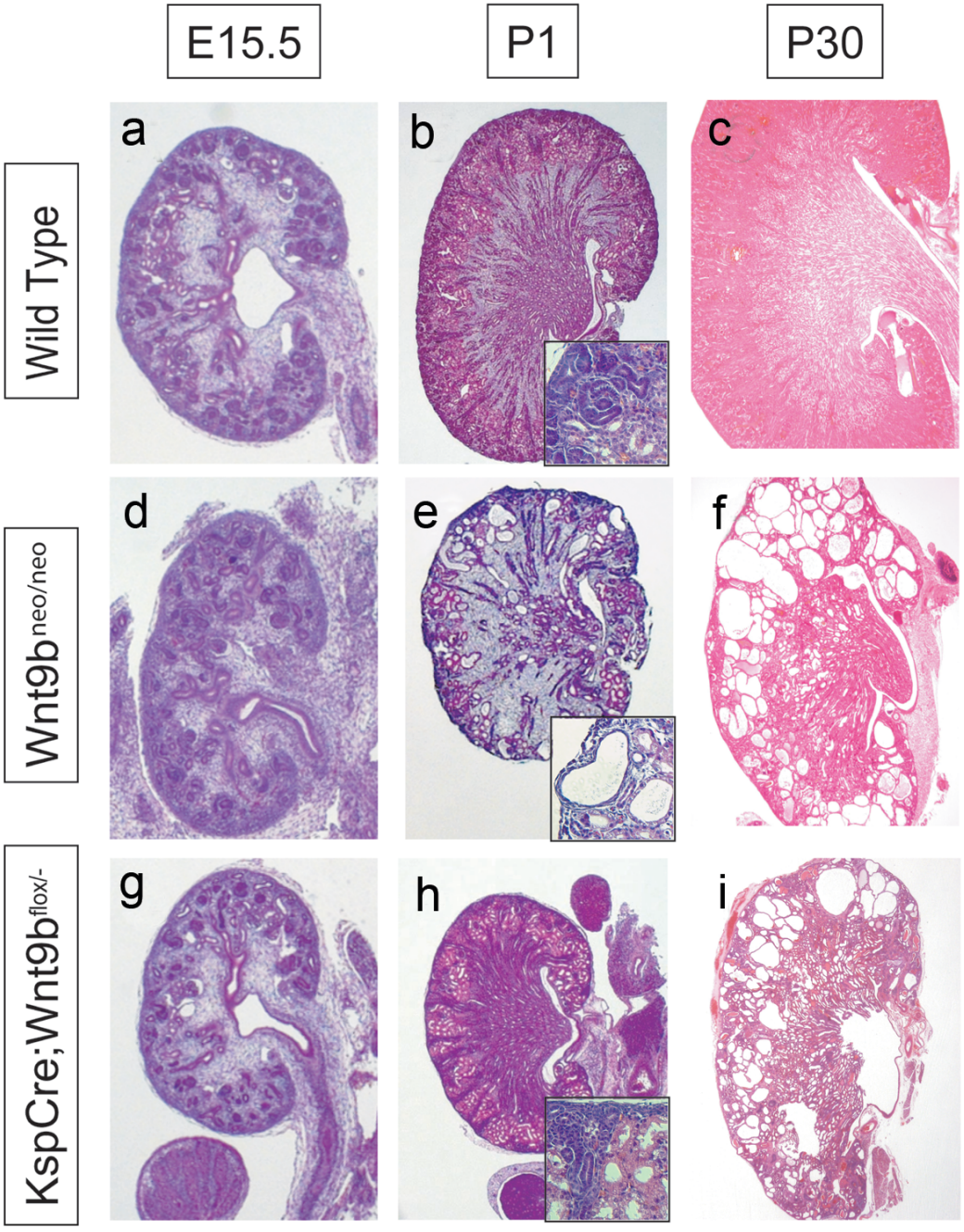

Figure 3-2: Defects in Wnt9b signaling result in cyst formation.
Hematoxylin and eosin-stained sections of wild-type (A-C), Wnt9b$^{neo/neo}$ (D-F) and KspCre;Wnt9b$^{flox/-}$ (G-I) at E15.5 (A,D,G), P1 (B,E,H) or P30 (C,F,I). Wnt9b mutant kidneys appear normal at E15.5 (compare D and G to A) but are smaller at birth (compare E and H to B). Wnt9b$^{neo/neo}$ kidneys also show signs of cystic dysplasia at P1 (E). At one month of age, mutant kidneys are slightly smaller and severely cystic compared to wild-type kidneys (compare F and I to C). Insets in B, E and H show high-magnification images of cortical epithelia.

has been described for expression of Ksp-cadherin protein (*cadh16*) in the rabbit [188], the Ksp promoter drives expression of Cre recombinase in the collecting duct stalks but not in the ureteric bud tips at least through E15.5 (Figure 3-3A, B, E and G and data not shown). KspCre appears to be active throughout the collecting ducts and ureteric bud tips at E17.5 (Figure 3-3F 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 (Figure 3-3). 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 wild type litter mates (Figures 3-2A and G, Figure 3-4 A, 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 (Figure 3-3 C, D, G and H and not shown). At P1, a time point 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 litter mates, 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

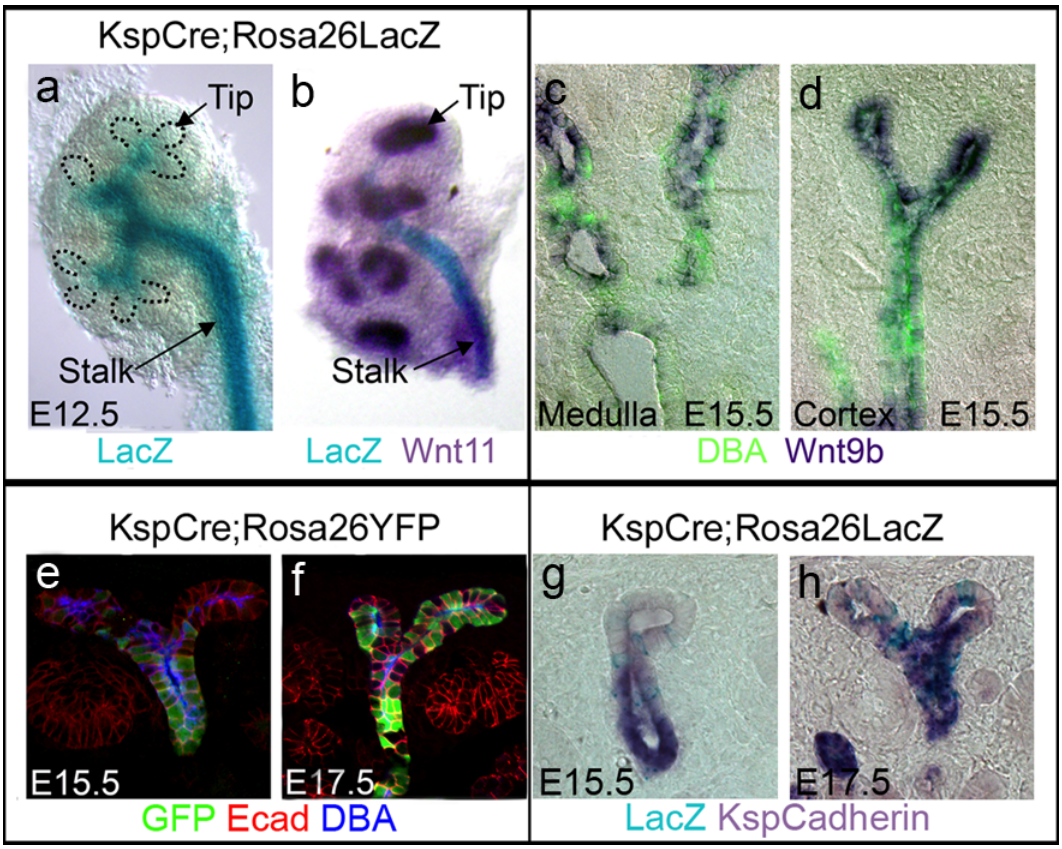

Figure 3-3: *KspCre* is expressed only in the ureteric stalk during early stages of kidney development.
E12.5 *KspCre*;*R26LacZ* kidneys stained with X-gal (light blue in a) or X-gal and a Wnt11 antisense riboprobe (light blue and purple, respectively in b). LacZ staining is apparent in the ureteric 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 colocalizes 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.

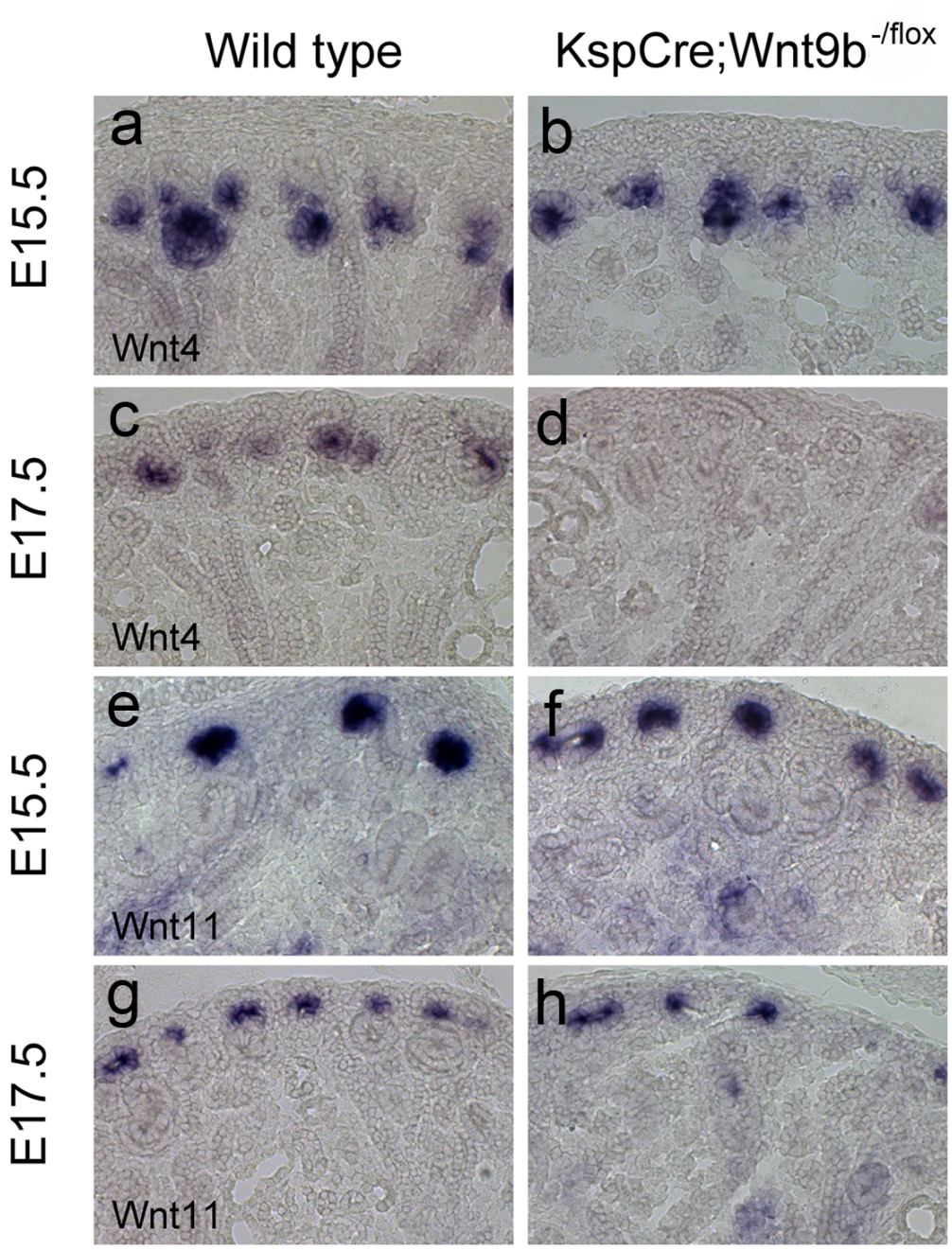

Figure 3-4: Loss of Wnt9b target gene expression at E17.5.
Section *in situ* hybridization with an antisense probe for the Wnt9b target genes *Wnt4* (a-d) and *Wnt11* (e-h) at E15.5 (a, b and e, f) and 17.5 (c, d and g, h) in wild type (a, c, e, g) and *KspCre;Wnt9b*$^{-/flox}$ (b, d, f, h) kidneys. Wnt4 is expressed normally in *KspCre,Wnt9b*$^{-/flox}$ kidneys at E15.5 (compare a to b) but is absent from *KspCre;Wnt9b* $^{f-/flox}$ 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).

did not display any overt external differences compared to wild-type litter mates. 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 litter mates (*KspCre;Wnt9b*$^{+/flox}$) showed no defects in kidney histology or function (not shown).

The *KspCre;Wnt9b*$^{-/flox}$ mice developed cystic kidneys similar to the *Wnt9b*$^{neo/neo}$ mice, although the onset of cystogenesis appeared to be slightly delayed. While *Wnt9b*$^{neo/neo}$ mice showed signs of tubule dilation as early as E15.5 (not shown) and had pronounced cysts by P1 (Figure 3-2E and data not shown), few cysts were visible in *KspCre;Wnt9b*$^{-/flox}$ kidneys at P1 (Figure 3-2H). However, cysts were prevalent in *KspCre;Wnt9b*$^{-/flox}$ kidneys at P10 and by P30 little normal epithelium remained in mutants (Figure 3-2I and data not shown). To further support the hypothesis that Wnt9b had an additional role in kidney tubule morphogenesis, we performed a temporal knockout of this gene using a ubiquitously expressed, tamoxifen inducible form of Cre (CaggCreErTm). 15.5 days post-conception, tamoxifen was administered to *Wnt9b*$^{flox/flox}$ dams that had been bred to *CaggCreERTm;Wnt9b*$^{+/-}$ males. When tamoxifen was administered at this time point, the *CaggCreErTm;Wnt9b*$^{-/flox}$ offspring developed cysts and no mutant animals survived past P90.

(Figure 3-5 and data not shown). However, ablation of Wnt9b after P10 (by administration of tamoxifen to 10 day old pups) did

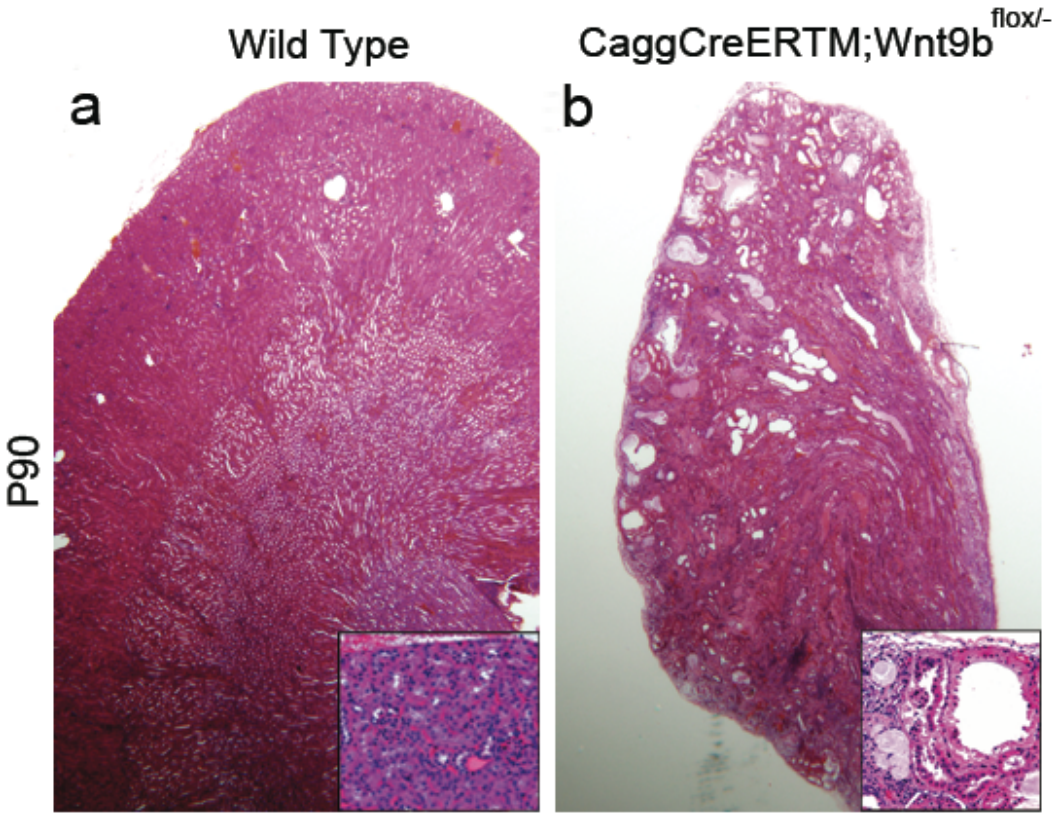

Figure 3-5: Ubiquitous removal of Wnt9b after E15.5 results in cyst formation.
H&E stained sections of P90 wild type (a) or *CaggCreErTm;Wnt9b*$^{-/flox}$ (b) kidneys. Insets show high magnification images of cortical epithelia. Mutant kidneys are significantly reduced in size and have cysts present.

not have a discernible effect on kidney morphology or function when assessed up to one year later (data not shown). These data refute the hypothesis that cyst formation is due to a defect in tubule induction or tubule maintenance. Instead, Wnt9b appears to have an additional, essential function in tubule morphogenesis.

Wnt9b acts non-autonomously to regulate tubule diameter.

Wnt9b is expressed in the collecting ducts throughout embryonic development and into adult stages (Figure 3-1A and C and [16]). To determine if Wnt9b is acting to regulate morphogenesis of the collecting ducts or if it is affecting morphogenesis of the adjacent renal vesicle derived epithelia (or both), we determined the origins of the *Wnt9b* mutant cysts. *Wnt9b*$^{neo/neo}$ and wild-type littermate kidneys were examined with markers of the proximal tubules (Lotus tetragonolobus lectin, LTL), collecting ducts (Dolichos bifloris agglutinin, DBA) and thick ascending limb of the loop of Henle (Tamm-Horsfall protein, THP) at E15.5, 18.5, P15 and P30.

Marker analysis suggested that at E15.5 and P1, cysts were present predominantly in proximal tubules (a tissue that does not express Wnt9b) and, to a lesser extent in the Wnt9b-expressing collecting ducts (Figure 3-6B and J and data not shown). No cysts were found in the loop of Henle or in the glomeruli at or prior to birth (Figure 3-6F and data not shown). However, by P15 cysts were present in all nephron segments examined (glomerulus, proximal tubule, loop of Henle and collecting duct)

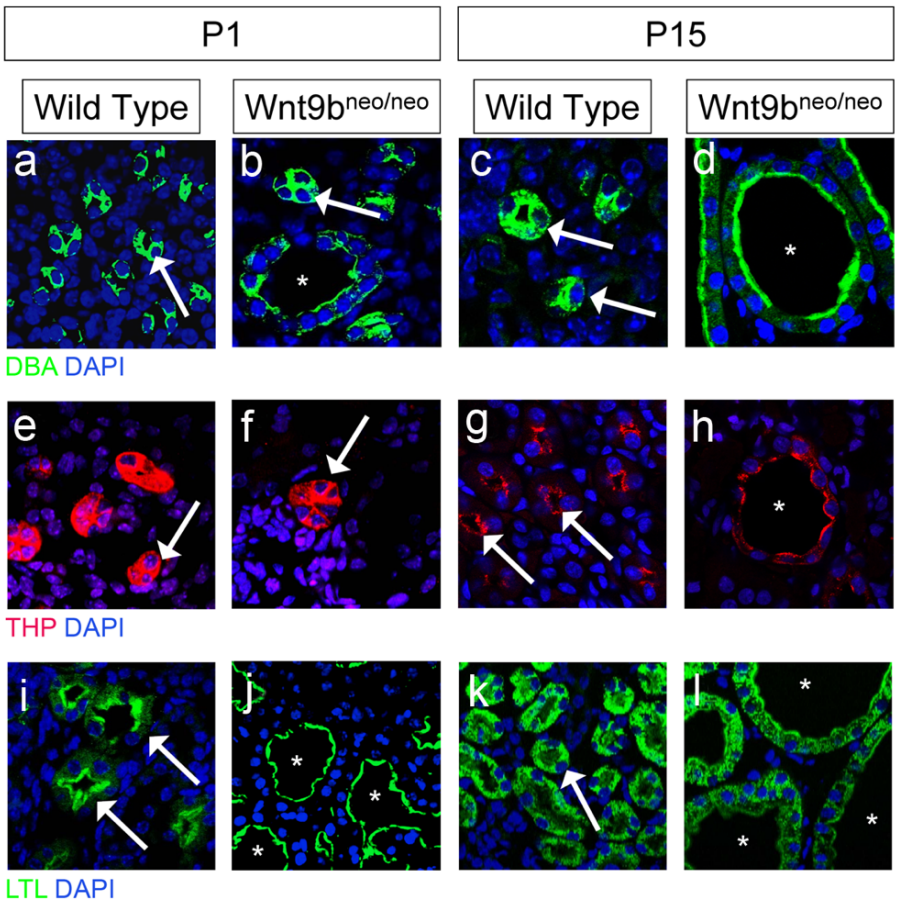

Figure 3-6: Characterization of cyst origin in Wnt9bneo/neo kidneys. (a–l) Sections of P1 (a,b,e,f,i,j) and P15 (c,d,g,h,k,l) kidneys stained with the collecting duct-specific marker Dolichos bifloris agglutinin (DBA) (a–d), the loop of Henle marker Tamm Horsfall protein (THP) (e–h) and the proximal tubule marker Lotus tetragolonobus lectin (LTL) (i–l). In all panels, arrows denote normal tubules and asterisks denote cystic tubules. At birth, cysts are found primarily in the proximal tubules (compare i to j). Cysts are also found in the collecting ducts, although the majority of DBA-positive epithelia appear normal (see arrows in b). Cysts were not observed in the loop of Henle at birth (compare e to f). By P15, cysts are present in all segments of the nephron (compare c to b, g to h and k to l). Nuclei were counterstained with DAPI (blue).

in approximately equal ratios (Figure 3-6D, H and L and data not shown). Similar results were seen in P15 *KspCre;Wnt9b*$^{-/flox}$ kidneys (Data not shown). These data demonstrate that after its initial role in tubule induction, Wnt9b functions non-autonomously (and possibly autonomously) to regulate the diameter of the kidney tubules.

Wnt9b is required for polarized cell division in the postnatal kidneys

To gain insights into the mechanism underlying cyst formation, Wnt9b mutant kidneys were characterized at the cellular and molecular level. In most mouse models of cystic kidney disease, cystogenesis is associated with increased rates of cell proliferation and/or apoptosis [1]. 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 (Figure 3-7). These data suggested that the mechanism for cyst formation in *Wnt9b* mutants is independent of changes in cell number.

Recent studies have suggested that cell division is oriented within the plane of the tubular epithelium in postnatal kidneys and defects in orientation occur in at least five distinct models of PKD [129, 130, 135, 136]. The non-canonical, or planar cell polarity, branch of the Wnt pathway has been implicated in oriented cell division in gastrulating

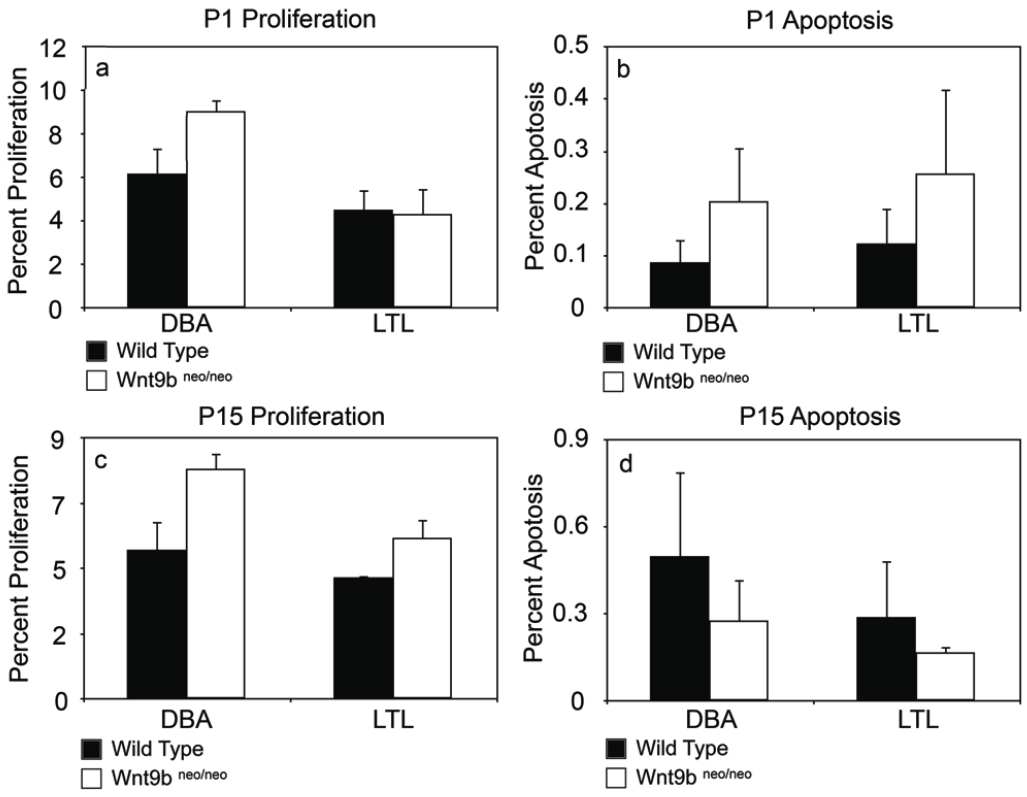

Figure 3-7: No change in rates of proliferation or apoptosis in Wnt9b mutant kidneys.

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.

zebrafish and in worms [97, 127]. However, there are several examples, such as the extending Drosophila germband and the developing mouse vasculature endothelium, where oriented cell division appears to be independent of Wnt signaling [189, 190]. The mechanism that establishes planar polarity in the kidney epithelium remains unknown.

To assess whether Wnt9b regulated the orientation of cell division, we measured the orientation of mitotic spindles in the collecting ducts of post-natal kidneys (for example see Figure 3-9 D and E). To avoid complications from examining epithelia that already were cystic, we initially examined kidneys from the *KspCre;Wnt9b*$^{-/flox}$ line that develops cysts post-natally. We found that in pre-cystic, P5 *KspCre*;*Wnt9b*$^{-/flox}$ collecting ducts, cell division was not oriented within the plane of the epithelium compared to wild type controls (Figure 3-8) suggesting that Wnt9b is necessary for the oriented cell divisions that occur in the post-natal kidney. The convoluted nature of the P5 proximal tubule prevented us from collecting accurate data on that segment at that stage of development.

<u>Cell division is not oriented within the proximal tubule and collecting duct epithelium of prenatal kidneys.</u>

As cysts are present in *Wnt9b*$^{neo/neo}$ kidneys before birth, the mechanism for establishing tubule diameter must be active during

embryogenesis. To test whether orientation of cell division played a mechanistic role in the establishment of wild type tubule diameter, we also

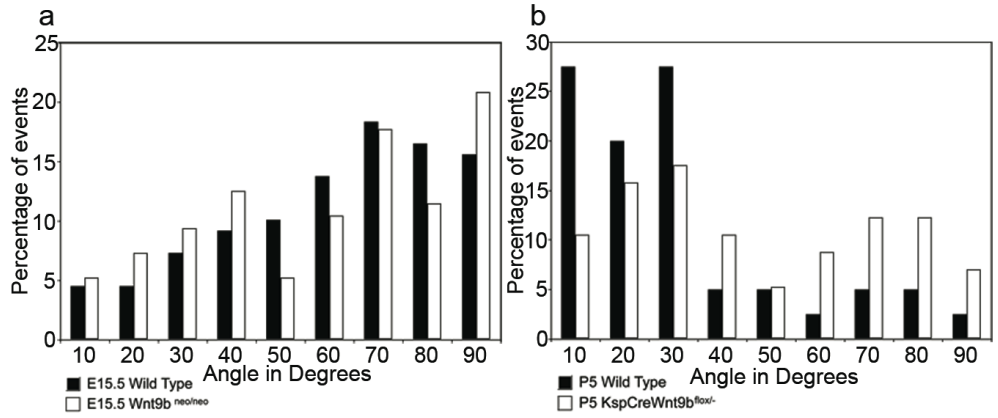

Figure 3-8: Cell division becomes oriented after birth in a Wnt9b-dependent process.
(a) Graphical representation of the angle between the mitotic spindles and the longitudinal axis of DBA-positive tubules at E15.5 indicates that cell division in both wild-type (black bars) and Wnt9bneo/neo tubules (white bars) is randomly oriented at E15.5 when compared to the expected random distribution by the Kolmogorov-Smirnov (KS) test. P>0.55 for both wild-type (n=109) and mutant (n=96). (b) At P5, the orientation of dividing cells in KspCre;Wnt9b$^{-/flox}$ DBA-positive cells (white bars, n=50) is significantly different (P=0.01, Mann-Whitney U test) from wild-type (black bars, n=45), indicating that Wnt9b is necessary for orientation of cell division that occurs postnatally.

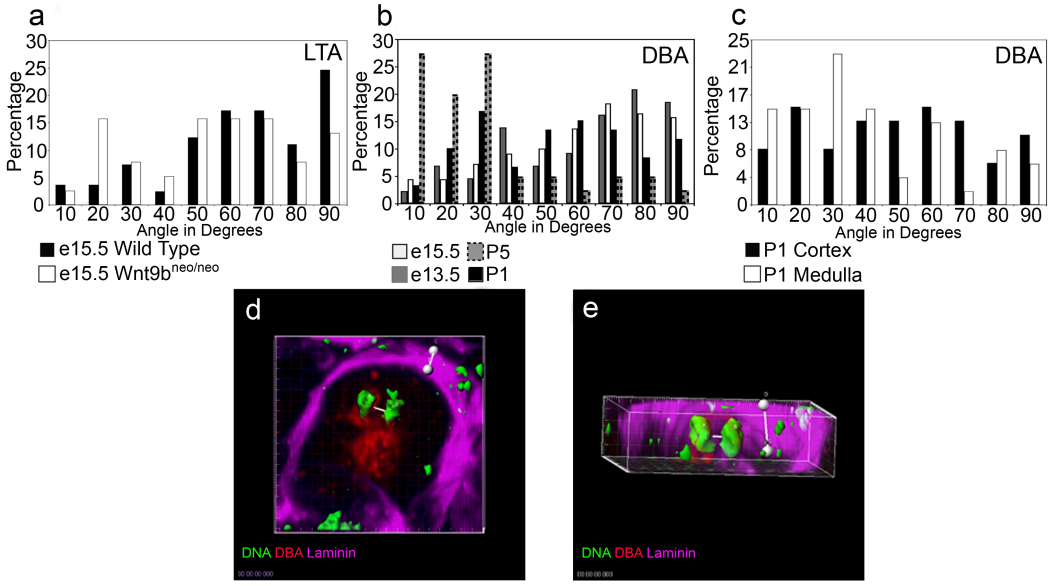

Figure 3-9: Cell division becomes oriented after birth.
(a) Graphical representation of the angle between the mitotic spindles and the longitudinal axis of LTL-positive tubules at E15.5 indicates that cell division in both wild type (black bars) and *Wnt9b*$^{neo/neo}$ tubules (white bars) is randomly 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) Graphical representation 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. Cortex n=47. Medulla n=53. (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.

measured the orientation of mitotic spindles in straight segments of proximal tubules and collecting ducts at E13.5 and E15.5. Surprisingly, we found that cell division was not oriented within the plane of the tubular epithelium in wild-type collecting duct or proximal tubule at these times (Figure 3-8A and Figure 3-9A, B and data not shown). In fact, the distribution of cell divisions was not significantly different from that predicted for a completely random distribution (Figure 3-8A and Figure 3-9A, B and data not shown).

To determine when cell division becomes oriented, we examined mitotic spindles in proximal tubules and collecting ducts at early post-natal stages. We found that at P1, orientation was no longer random (from a statistical perspective) but also was not tightly oriented within the plane of the epithelium as compared to later post natal stages (Figure 3-9B). The distribution of mitotic angles in P1 kidneys is biphasic with peaks at 30 and 60 degrees respectively (Figure 3-9B). Two possible explanations exist for this biphasic distribution: either cell division becomes oriented centrifugally (that is from the medulla outward as development proceeds), or there is a general shift towards oriented cell divisions that occurs around the time of birth. To determine if cell divisions become oriented first in the oldest kidney tubules, we compared mitotic angles between cortical and medullary DBA positive tubules. The distribution of mitotic angles showed a similar bi-phasic distribution in both domains supporting the idea that cell division is becoming oriented throughout the kidney at P1 (Figure 3-9C).

As mentioned, at P5 the majority of cell divisions within the collecting duct are well-oriented, with 75% of mitotic spindles being oriented within 30 degrees of the longitudinal axis of the tubule (Figure 3-8B and Figure 3-9B). Due to the convoluted structure of the P5 proximal tubule, we were not able to accurately measure orientation of cell division in this segment. However, similar to the collecting ducts, orientation of cell division in the P1 proximal tubules is no longer random indicating a trend towards oriented (Data not shown).

These data suggest that during embryonic stages, cell divisions are not oriented in the proximal tubules or collecting ducts but that they become oriented, at least within a subset of cells, around the time of birth. Therefore, oriented cell divisions cannot be playing a role in establishment of tubule diameter or in the defects seen in prenatal *Wnt9b* mutant kidneys. In support of this hypothesis, the orientation of cell division of *Wnt9b* mutant collecting ducts and proximal tubules was not significantly different from wild type (i.e. it was random) prior to birth (Figure 3-8A and Figure 3-9A).

<u>The number of cells that make up the circumference of the kidney tubule decreases during embryogenesis.</u>

In the absence of cell loss, cell division that is not oriented within the plane of the tubular epithelium would be predicted to lead to an increase in the number of cells within the tubule wall and, in the absence

of changes in cell shape or size, a concomitant increase in cross-sectional tubular diameter. To test if wild type tubules increased the number of cells in their walls during the embryonic period, the average number of cells that make up the circumference of both proximal tubules and collecting ducts was calculated (Figure 3-10A-D and data not shown).

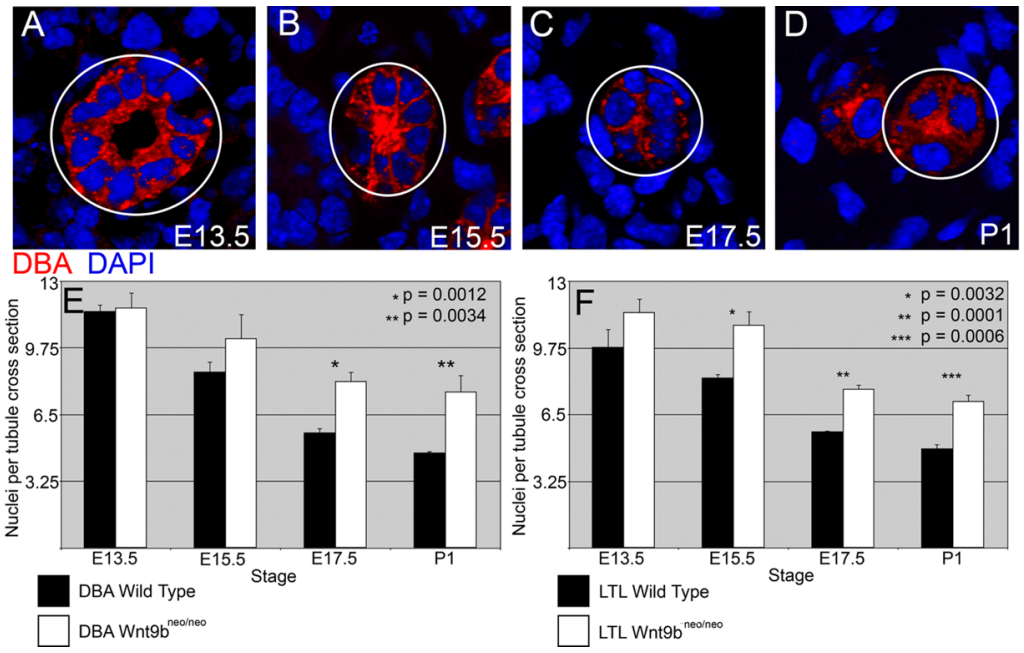

Figure 3-10: Wnt9b is required for the elongation and narrowing of kidney tubules.
(a–d) Representative sections through wild type DBA-positive tubules from E13.5 (a), E15.5 (b), E17.5 (c) and P1 mice (d) showing the number of nuclei composing the wall of the tubule. Outlined tubules represent transverse sections. Quantitation reveals that the number of cells within the wall of wild-type collecting duct (black bars in e; n 1/4 563, 606, 844 and 692 for E13.5, 15.5, 17.5 and P1, respectively) and proximal tubules (black bars in f; n 1/4 425, 1030, 791 and 778 for E13.5, 15.5, 17.5 and P1, respectively) significantly decreases during the embryonic period. The number of cells within the tubule wall is significantly increased in
Wnt9b mutant kidneys (white bars in e and f; n1/4 384 or 412, 521 or 424, 915 or 902, and 665 or 635 for DBA or LTL at E13.5, E15.5, E17.5 or P1, respectively). n 1/4 3 kidneys for each stage, tubular segment and genotype. Error bars, s.e.m.

Counts were taken from E13.5 (the earliest stage at which we could find both LTL and DBA positive tubules) to P1 (prior to oriented cell divisions). Contrary to the expectation, we found that the number of cells that make up the tubular circumference decreases by more than half from E13.5 to P1 in both collecting ducts and proximal tubules (Figure 3-10E and F). Importantly, the rate of cell loss during this period cannot account for this decrease (Figure 3-7B and D and data not shown), suggesting that some unidentified process must be driving the decrease in the number of cells making up the tubular circumference during the embryonic period.

Wnt9b mutants show defects in planar cell polarity.

One process that could lead to a decrease in the number of cells within the circumference of the tubule, without affecting total cell number in the tubule, is convergent extension. Convergent extension describes the directed integration/intercalation of cells within an epithelium that makes the epithelium longer and narrower [53, 86, 191-195].

Convergent extension movements rely on dynamic cell shape changes and cell intercalations that are the results of reorganization of the cytoskeleton. Mediolateral elongation of cells perpendicular to the axis of extension is correlated with, and appears necessary for, intercalation of cells during convergent extension in multiple tissues [66, 196-198]. Examination of frontal sections of developing wild-type kidney tubules indicated that the majority of collecting duct cells showed polarized

elongation (Figure 3-11A-C and data not shown) and that greater than 70% of elongated cells were oriented between 45 and 90 degrees (perpendicular) of the longitudinal axis of the tubule (Figure 3-11G). Moreover, 41.3% of elongated cells were oriented within 70-90 degrees (Figure 3-11A, B and G). In contrast, collecting duct cells in *Wnt9b*$^{neo/neo}$ mutants showed a randomized elongation (Figure 3-11D-F and data not shown). Only 38% of cells in *Wnt9b*$^{neo/neo}$ mutants were elongated within 45-90 degrees and only 14% within 70-90 degrees (Figure 3-11D, E and G). These defects suggest that Wnt9b plays a role in establishing planar polarity of the kidney epithelium. Similar results were found in the *KspCre;Wnt9b*$^{-/flox}$ mutants (Figure 3-11H). These data suggest that Wnt9b mutant epithelia have defects in planar cell polarity that affect both cell movements and oriented cell divisions.

If defects in polarized cell orientation lead to defects in convergent extension movements, one would predict that the mutant tubules would possess a greater number of cells in their cross sectional circumference. Indeed, this was the case. *Wnt9b*$^{neo/neo}$ mutants had a significantly increased number of cells per tubule wall in the proximal tubules and

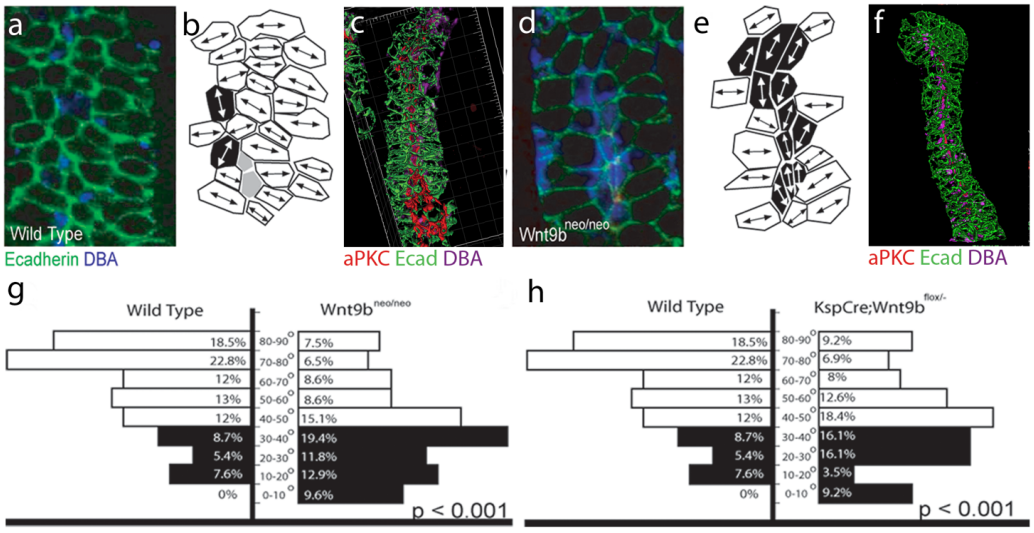

Figure 3-11: Wnt9b is necessary for the orientation of polarized cells perpendicular to the axis of extension.

(a–f) Confocal images (a,d), cell outlines (b,e) and three-dimensional reconstructions (c,f) of frontal sections through E15.5 wild-type (a–c) and Wnt9bneo/neo (d–f) kidneys stained with antibody to E-cadherin (green), antibody to aPKC (red) and DBA (blue). In all cases, proximal is up and distal is down. Images in a and d represent sections just basal to the apical membrane as marked by antibody to aPKC (red, not seen). Mediolaterally elongated cells are marked in white, proximal-distally elongated cells in black and unelongated cells in gray in b and e. The majority of wild-type cells are seen to be mediolaterally elongated perpendicular to the axis of extension (white in b). Wnt9bneo/neo cells are still elongated but the direction of elongation appears to be random (note increased number of black, proximal-distally elongated cells in e relative to b). (c,f) Three-dimensional reconstructions of wild-type (c) or Wnt9bneo/neo (f) E15.5 tubules to allow for visualization of cell orientation. Arrows indicate angle of orientation for marked cells. (g,h) Quantitation of the angle of cellular elongation relative to the proximal distal axis of the tubule for wild-type (left in g and h) and Wnt9bneo/neo mutant (right in g) or KspCre;Wnt9b–/flox (right in h) cells. White bars indicate cells that are perpendicular to the axis of elongation (45–90°), whereas black bars represent cells that are parallel (0–45°). The percentage of cells within each 10° increment is indicated. There is a significant change in the orientation of the elongated cells between wild-type and mutants (P o 0.001, KS test). The data were gathered from at least three different animals. The total number of oriented cells analyzed is 92, 86 or 93 for wild-type, KspCre;Wnt9bflox/– or Wnt9bneo/neo, respectively. Wild-type cells are from littermate controls.

collecting ducts at E13.5, 15.5, 17.5 and P1 (Figure 3-10E and F and data not shown). Cell size however did not appear to be affected (data not shown). It is important to note that the cellular numbers calculated for later stage (E15.5-P1) mutants are most likely an underestimate of actual values. In order to assure that only epithelial cross sections were evaluated, we did not analyze tubules that varied significantly from being perfect circles (see Chapter 4: Materials and Methods). At later stages, due to drastically increased diameter, most mutant tubules were grossly misshapen and were excluded from the analysis. Therefore, the mutant tubules assessed were the most “wild type” examples leading to an underestimate of the true number of cells per mutant tubule wall.

Another expected outcome from defects in convergent extension movements is that mutant tubules should be shorter along their proximal/ distal axis. Once again, this appeared to be the case. The length of individual collecting ducts was significantly decreased in mutants compared to wild type (1.2 +/- 0.05 mM for wild type, 0.83 +/- 0.05 uM for mutants. P= 0.001 by two tailed students T test.). Although their convoluted nature prevented an accurate measure of their length, the distance from the cortical to the medullary tip of individual proximal tubules was also consistently and significantly reduced in mutants (data not shown).

Wnt9b signals through a non-canonical pathway to regulate tubule elongation.

Previous studies suggested that during tubule induction, Wnt9b signaled through the canonical/β-catenin dependent signal transduction branch (Chapter 2) [16, 122]. We next sought to determine whether the role for Wnt9b in establishing and maintaining tubule diameter was also 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 3-12A). 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 [157, 199-201]. 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 time point where there are clear cellular defects (Figure 3-12B 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 3-12C and data not shown).

In addition to the lack of significant change in the expression of β-catenin or β-catenin target genes in Wnt9b mutants, we also failed to

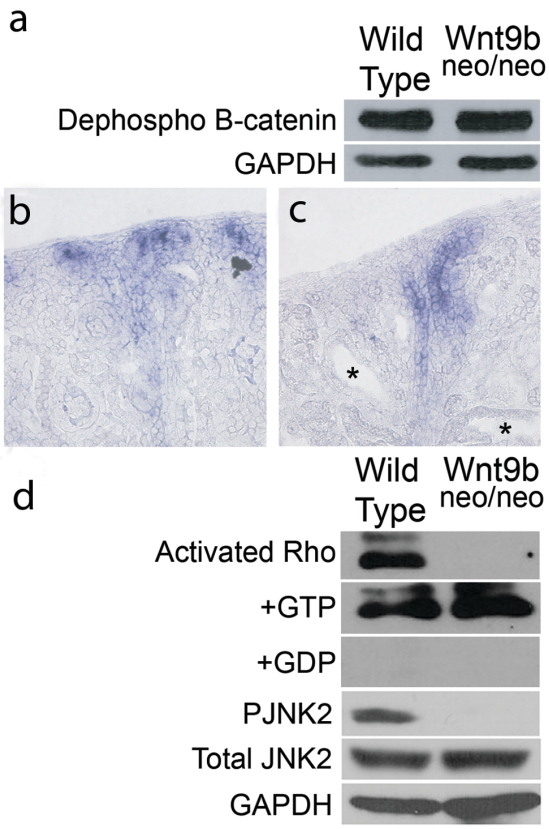

Figure 3-12: Wnt9b signals through the non-canonical pathway to regulate tubule diameter.

(a) Protein blots of total protein extracted from wild-type and Wnt9b$^{neo/neo}$ kidneys probed with an antibody specific to the dephosphorylated (active) form of β-catenin show no significant differences in canonical Wnt activity compared to wild-type. (b,c) Section in situ hybridization with a probe for the b-catenin target Axin2 also shows no significant decrease in canonical activity in P1 Wnt9bneo/neo kidneys (c) compared to wild-type (b). Note that there is no ectopic Axin2 expression in cystic proximal tubules (asterisks in c). (d) Protein blots indicate that activated Rho is significantly decreased in Wnt9bneo/neo kidneys at P1 relative to total (+GTP control) Rho levels. Addition of GDP (+GDP) to inactivate Rho was used as a negative control. Phosphorylated Jnk2 is also significantly decreased in Wnt9bneo/neo kidneys at P1 relative to total levels of Jnk2. Blots shown are representative examples of data gathered from at least three different blots from three independent protein extractions.

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} [202]) 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 in mice with activated β-catenin alone (*KspCre*;*catnb*^{exon3flox}) are mildly dilated at birth, and the mice die by P2 (Figure 3-13C 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 (Figure 3-13I).

These data suggest that Wnt9b signals through one of the non-canonical pathways to regulate tubule diameter. The most obvious candidate pathway is the PCP branch as this pathway has previously been implicated in cell orientation and convergent extension movements.

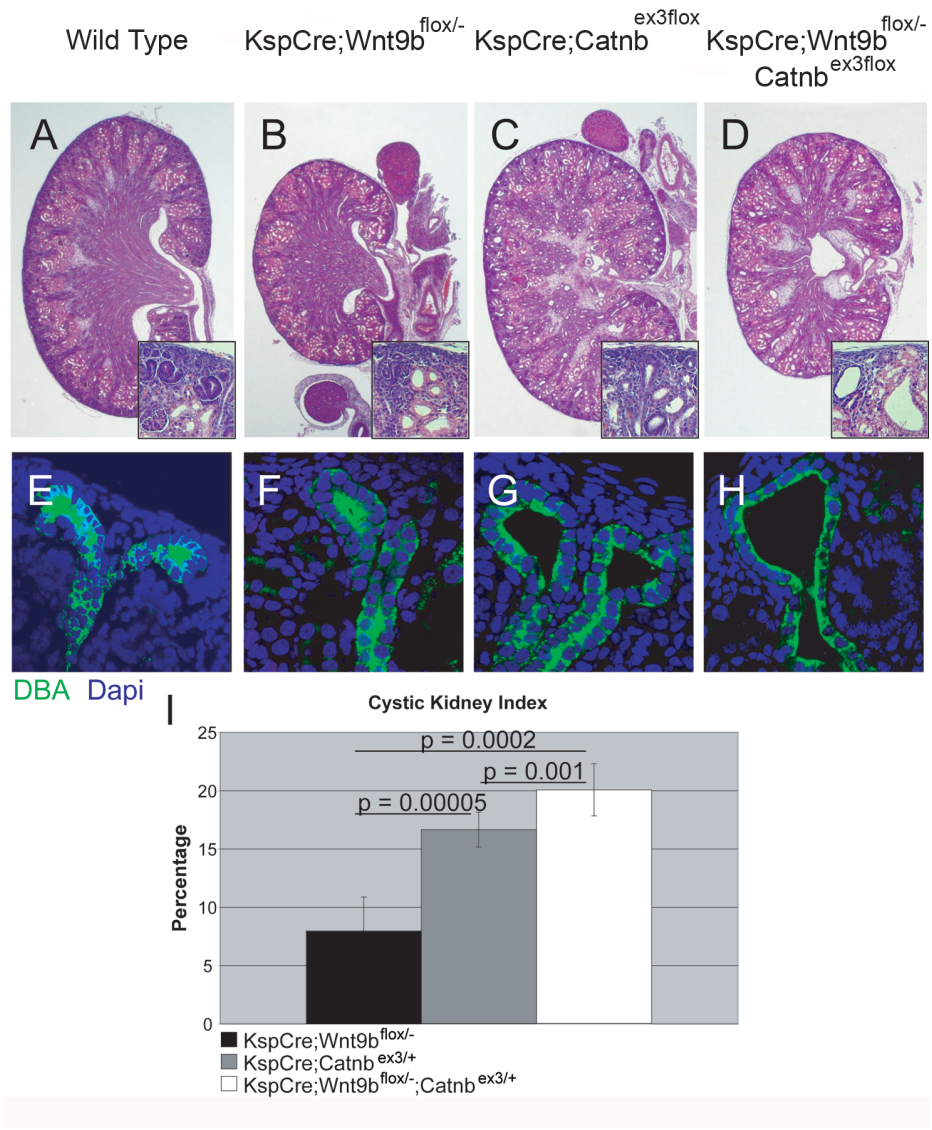

Figure 3-13: Activation of β-catenin in a Wnt9b mutant background enhances cystogenesis.

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

Although there is no established molecular readout of PCP in vertebrates, it has been shown that signaling through this pathway can activate the Rho-GTPases and Jun kinase (Jnk) [203-205]. Activated (GTP bound) levels of Rho (but not Cdc42 or Rac) were significantly decreased relative to total Rho levels in mutants (Figure 3-12D and data not shown). Further, we found a significant decrease in the level of phosphorylated Jnk2 (relative to total Jnk2) in P1 Wnt9b mutant kidneys (Figure 3-12D). These data support the hypothesis that Wnt9b signals through the non-canonical/ PCP pathway to regulate convergent extension and oriented cell division during kidney tubule morphogenesis.

Discussion

In this study, we demonstrate that, in addition to its initial role in renal vesicle formation, Wnt9b plays a later role in renal tubule morphogenesis. Mice carrying a hypomorphic mutation of Wnt9b or mice that have had a floxed allele of Wnt9b deleted with either KspCre or the tamoxifen inducible *CaggCreErTm;Wnt9b*$^{-/flox}$ develop cystic kidneys. Cystogenesis does not appear to be caused by increased cell numbers as we have not detected significant differences in the rates of cell proliferation or apoptosis in mutant epithelia either prior to or concurrent with cyst formation. Instead, we hypothesize that cyst formation is the result of defects in planar cell polarity. We show that cells within the epithelial tubule are elongated perpendicular to the proximal/distal axis of the tubule and that this process is dependent on Wnt9b. We hypothesize that proper cell orientation is required for convergent extension movements and oriented cell divisions. Although cells within the normal collecting ducts and proximal tubules of embryonic kidneys divide in a random orientation, the number of cells composing the wall of the tubule decreases during the embryonic period. We hypothesize that convergent extension movements drive the decrease in the number of cells within the circumference (or wall) of a tubule as the tubule elongates. Thereby (at least in part) establishing the tubule diameter and contributing to tubule length. Once the tubule diameter is established, cell division becomes oriented parallel to the proximal/distal axis to ensure that the kidney

tubules continue to elongate while they maintain their diameter. Our data suggest that Wnt9b plays essential roles in both of these processes, perhaps by mediating cell orientation.

In stark contrast to its β-catenin dependent/canonical role during tubule induction (see chapter 2), We show here that the role of Wnt9b in establishing and maintaining tubule diameter is β-catenin independent. Instead, Wnt9b appears to signal through the non-canonical Rho/Jnk pathway during tubule morphogenesis. Notably, recent studies showed that attenuation of Rho kinase led to shorter, wider tubules in cultured kidneys [206], a phenotype that may reflect attenuation of Wnt9b signaling. Our data support a hypothesis whereby Wnt pathway usage is not determined by the individual Wnt ligand but instead by the cellular environment in which the signal is received. Depending on the cell type, Wnt9b can signal through both pathways within the same organ system.

Several factors involved in cystic kidney diseases are localized to, and/or are necessary for the function of, the apical mono-cilia [126, 133, 136, 207-216]. In addition, recent studies suggest that the primary cilium may play a role in inhibiting canonical Wnt signaling (perhaps promoting non-canonical Wnt signaling) in early mouse and zebrafish embryos [139, 140]. A simple model for Wnt pathway usage in the kidney is that the cilia and/or ciliary factors block canonical signaling by Wnt9b and promote non-canonical signaling. Indeed, we see no defects in the expression or localization of several ciliary factors such as Pc-1 and Pc-2 (factors

involved in the progression of human autosomal dominant PKD) [213, 217] and inversin (*Inv*) mRNA (the ortholog of the gene mutated in Nephronophthisis type II) [215] in Wnt9b mutants nor is Wnt9b mRNA expression affected in *Inv*$^{-/-}$ [8] or *Pkd1*$^{-/-}$ [217] mice (data not shown).

Although this study has revealed a great deal about the mechanisms that regulate tubule diameter, several questions remain unanswered. One such question is why Wnt9b cysts are more severe in the cortex of the kidney? The simplest answer is that another molecule compensates for Wnt9b in the medullary region. Several Wnts, including Wnt5a, Wnt7b, Wnt4 and Wnt11, are expressed in the medullary region of both wild type and Wnt9b mutant kidneys ([31] and Karner and Carroll, unpublished observations) and any one of these factors may compensate for loss of Wnt9b. Alternatively, there may be a parallel, Wnt independent, signaling pathway that regulates PCP in the medulla. A recent study showed that mice lacking the PCP determinant Fat4 developed kidney cysts primarily within the medullary region [130]. Compensation by either another Wnt or Fat4 would explain the paucity of medullary cysts in Wnt9b mutants. However, it is important to note that we did not observe increased numbers of cells within the circumference of the Wnt9b mutant collecting ducts as well as defects in cell orientation during embryonic stages, but but failed to observe cysts in this nephron segment until post-natal stages. Other processes, such as defects in fluid secretion or cellular

growth (hypertrophy) most likely contribute to cyst formation and are more severely affected in the proximal tubules of Wnt9b mutants.

Another question raised by these findings is how Wnt9b, produced by the collecting ducts, affects planar cell polarity in the relatively distant proximal tubules? One possibility is that Wnt9b signals directly to (i.e. its receptor is expressed in) the cells of the proximal tubule. If this were the case, the ligand could travel several different routes (e.g. through the intervening stroma, through the lumen of the tubules or through the plane of the epithelium (transcytosis)). A second possibility is that the effects on diameter are all indirect (e.g. Wnt9b could be affecting the expression of a second molecule in the stroma or mesenchyme and this molecule directly regulates PCP). A final possibility is that Wnt9b functions to polarize the cells of the collecting duct and that this polarizing information is passed on to the renal vesicles and their derivatives (in an epigenetic fashion) after these structures fuse with the collecting duct. Directly addressing these hypotheses has been complicated by the fact that we do not know at precisely what step during tubule morphogenesis Wnt9b acts nor do we know the identity of its receptor. Our results with the *KspCre*;*Wnt9b*$^{-/flox}$ mice have led us to hypothesize that Wnt9b acts during the later stages of morphogenesis. If this is the case, then travel through the lumen (potentially in a direction that is opposite the flow of urine) or through the plane of the epithelium seems unlikely. However, at this point we cannot rule out the possibility that Wnt9b functions during the early stages of

tubule formation (perhaps even affecting the polarity of the mesenchyme itself) and that all of our mutants represent a reduction of Wnt9b signaling to this cell population. Identification of the frizzled receptor and molecular targets of Wnt9b will allow us to determine which of these mechanisms is utilized.

A final question is whether Wnt9b contributes to human forms of PKD. Wnt9b continues to be expressed in the adult kidney but ablation of Wnt9b in P14 mice has no effects on kidney homeostasis suggesting that the pathway may not be active in a healthy adult. However, it is possible that it becomes activated and is required for kidney repair after injury. Recent studies suggest that cystic kidney diseases may in fact be the result of defects in the tubular repair process [218, 219]. Correct orientation of cell division is required for proper tubular repair after kidney injury [135]. It is possible that Wnt9b is required for the orientation of cell divisions (and perhaps convergent extension) that occur after injury. An additional role for Wnt9b in PKD may be in the regulation of β-catenin levels. An inability to properly regulate pathway usage after injury (through loss of the cilia or the polycystins) might contribute to PKD. As mentioned, β-catenin levels are increased in multiple models of cystic kidney disease and it has recently been suggested that apical epithelial mono-cilia normally attenuate Wnt activity [139, 140]. However, loss of the apical mono-cilia alone is not sufficient to drive increased levels of β-catenin; a Wnt ligand must be present [139]. We think it is plausible that in

various kidney ciliopathies, canonical Wnt9b signaling is no longer attenuated resulting in increased levels of β-catenin while non-canonical signaling is impaired. At a molecular level, this would resemble our studies simultaneously ablating Wnt9b while β-catenin is stabilized. If this is the case, Wnt9b may represent an interesting therapeutic target for treating PKD.

In sum, our findings show that Wnt9b, produced by the kidney collecting ducts, non-autonomously regulates morphogenesis of the developing kidney tubules. We suggest that Wnt9b is required for PCP and the PCP dependent cellular processes convergent extension and oriented cell division. These processes are in turn required to establish and maintain the tubular diameter and length during the embryonic period but are dispensable in healthy, differentiated tubules. A better grasp of the regulation and downstream targets of Wnt9b will significantly impact our understanding of epithelial tubule morphogenesis and the treatment of polycystic kidney disease.

CHAPTER FOUR

Materials and Methods

Generation of Wnt9b mutant mice and genotyping.

The $Wnt9b^{-}$ and $Wnt9b^{neo}$ alleles were previously described [16]. The $Wnt9b^{neo}$ allele has a PGK-Neo cassette inserted in the 2^{nd} intron and loxp sites flanking the 2^{nd} exon. The neomycin cassette in the $Wnt9b^{neo}$ mice was flanked by flp recombinase target sites (frt). To generate the $Wnt9b^{flox}$ mice, $Wnt9b^{neo/+}$ animals were crossed to mice carrying a ubiquitously expressed flippase gene. Removal of the neomycin cassette was confirmed by Southern blot. Males and females that had had the neomycin cassette excised were crossed to each to generate ($Wnt9b^{flox/flox}$) animals. These mice were maintained as a homozygous line. To generate the conditional null kidneys, KspCre;$Wnt9^{+/-}$ or CaggCreERTM;$Wnt9b^{+/-}$ males were crossed to $Wnt9b^{flox/flox}$ females. Noon of the day of vaginal plugging was considered E0.5.

Genotyping of mice was performed by digesting a 0.5 cm piece of tail in tail lysis buffer (Viagen) at 55 degrees overnight. The floxed and null alleles were amplified in a single reaction using the conditions previously described [16]. The null allele generates a 500 bp band, the flox allele a 240 base pair band and the wild-type allele a 200 bp band. The *KspCre* and *CaggCreERTM* alleles where amplified using the primers CCATGAGTGAACGAACCTGG and TGATGAGGTTCGCAAGAACC to give a 400 base pair band using the conditions previously described. The

β-catenin exon3flox mice were provided by Mark Taketo [202] and the exon3flox allele was amplified using the primers: AACTGGCTTTTGGTGTCGGG and TCGGTGGCTTGCTGATTATTTC. Using a 55^{o} extension, the wild type allele gives a 291 base pair band while the exon 3 floxed allele gives a 400 base pair band.

Immunohistochemistry.

Specimens were fixed in 4% paraformaldehyde in PBS (EMS) for 16 hours at 4 degrees C, washed 3 times with PBS and cryoprotected in 30% sucrose for 16 hours at 4 degrees C. Specimens were then embedded in OCT and cryosectioned at the thicknesses indicated. Specimens were washed 3 times in 0.1% TritonX/PBS for 5 minutes each wash and blocked for a minimum of 1 hour at room temperature in 5%FBS/ 0.1%TritonX/PBS. Sections were incubated in primary antibody for either 2 hours at room temperature or at 4 degrees overnight followed by 3 washes in 0.1%TritonX/PBS. Sections were then incubated in secondary antibody for 2 hours at room temperature, washed 3 times in PBS, mounted with Vectashield and examined by scanning laser confocal microscopy (Zeiss LSM-510). Sections were incubated with the following lectins or antibodies: *Dolichos bifloris* lectin (DBA, Biotinylated, 1:500 Vector Laboratories), *Lotus Tetragonolobus* lectin (LTL, Biotinylated, 1:500 Vector Laboratories), anti-Laminin (Rabbit polyclonal, 1:300 Sigma), anti-Tamm-Horsfall protein (Rabbit, 1:300 Biomedical Technologies), anti-E-cadherin (Rat, 1:500 Zymed), anti-Ki67 (Rabbit, 1:800 Novocastra), anti-

cleaved caspase-3 (Rabbit, 1:300 Promega), anti-GFP (Rabbit, 1:1000 Abcam), anti aPKC (Rabbit, 1:500 Santa Cruz), and the nuclear dye Sytox Green (1:5000, Invitrogen).

Western blotting.

Wild type and Wnt9b$^{neo/neo}$ (P1) kidneys were homogenized in a medium containing 20mM Hepes (Ph 7.4), 10mM NaCl, 1.5mM MgCl2, 20% glycerol, 0.1%Triton X-100, 1Mm DTT, 1.5mM sodium orthovanadate and protease inhibitor mix (Complete Mini, one protease inhibitor cocktail tablet per 20 ml of medium. Roche Molecular Biochemicals, Catalog: 04693124001) in a dounce homogenizer by giving 40 strokes. The lysate was centrifuged at 3400 rpm for 3 min in 4^0C to separate the cytosolic and nuclear fractions. Supernatant was used as the cytosolic fraction. Protein concentration was estimated by the method of Bradford.

Protein (50ug) was resolved on 10% polyacrylamide gel and subjected to immunoblot analysis using the respective antibodies. GAPDH was used as a loading control. Antibodies against pJnk1/2 (1:500 dilution, Biosource, Catalog: 44-682G), total Jnk2 (1:1000 dilution, Cell signaling Technology, Catalog: 4672), dephosphorylated β-catenin (1:1000 dilution, Upstate Cell signaling solutions, catalog: 05-601) and GAPDH (1:3000 dilution, Santa Cruz Biotechnology, Catalog: sc25778) were used to detect the respective protein levels in wild-type and Wnt9b$^{neo/neo}$ cytosolic fractions. The immunoblots were blocked for one hour at room temperature in 5% Non-fat dry milk (1x TBS, 0·05% Tween) followed by an

overnight incubation at 4^0C in their respective diluted primary antibody solutions. Membranes were then washed three times using TBS/Tween 0·05% (5 min/15 ml) and further incubated with the secondary antibody, HRP goat anti rabbit (Invitrogen, Catalog: G21234) in 5% Non-fat dry milk (1xTBS/Tween 0·05%) for 1 h at room temperature (1:5000 to detect pJnk1/2, 1:2000 to detect total Jnk2 and 1:10,000 to detect GAPDH levels). Dephosphorylated β-catenin was detected using HRP Goat anti mouse (1:5000, Pierce, catalog: 1858413) using the same conditions as described above. All the blots were developed using the Pierce Super signal West Femto maximum sensitivity substrate kit. Quantification of the proteins levels was done using Image J software. Each experiment was done a minimum of three times with at least two independently prepared protein samples.

Rho Pulldown.

Activated Rho was pulled down from Wild type and $Wnt9b^{neo/neo}$ P1 kidneys using EZ-Detect™ Rho Activation Kit with slight modification to the manufacturer's protocol (Pierce, Catalog: 89854). The kidneys were homogenized in the lysis buffer provided in the kit with the addition of protease inhibitor mix (Complete Mini, one protease inhibitor cocktail tablet per 10 ml of medium, Roche Molecular Biochemicals, Catalog: 04693124001) in a dounce homogenizer by giving 10-15 strokes. The lysate was centrifuged at 14,000 rpm at 4^0C for 10 minutes. Supernatant was separated and used for the assay. 1mg of protein was used for each

pull down assay. In vitro control treatments were done by the addition of GTP (positive control) or GDP (negative control) to activate or inactivate Rho respectively.

Protein was resolved on 12% polyacrylamide gel and subjected to immunoblot analysis using anti-Rho antibody. The immunoblots were blocked in TBS containing 3% BSA at room temperature for 2 hrs followed by an overnight incubation in primary antibody solution (1:500 dilution in 3% BSA in TBS/Tween-0.05%) at 4^0C. Membranes were washed three times using 15 ml of TBS/Tween-0.05% (5 min each wash) and further incubated with the secondary antibody, HRP goat anti Mouse (Invitrogen, Catalog: G21040) in 5% NFDM (1xTBS/Tween 0.05%) for 1 h at room temperature (1:10000 dilution). Immunoblots were developed using Pierce Super signal West Femto maximum sensitivity substrate kit.

In situ Hybridization.

In situ hybridization was performed on 30 or 16 uM cryosectioned kidneys as previously described [16]. Sections labeled with DBA lectin where washed 3 times in PBS after the color reaction, fixed in 4% paraformaldehyde in PBS (EMS) for 1 hour at room temperature, washed 3 times with PBS and processed for immunohistochemistry as described. Kidneys stained for X-gal were fixed (1% PFA, 0.2% gluteraldehyde in PBS) for 1 hour, washed 3 times with 0.02%NP-40/PBS, stained (5mM K3Fe(CN)6, 5mM K4Fe(CN)6, 2mM MgCl2, 0.01% NaDeoxycholate,

0.02% NP-40, 1mg/ml X-gal) for up two 2 hours at 37 degrees. Staining was stopped by washing in PBS followed by fixation in 4% PFA + 0.2% gluteraldehyde for 30 minutes. Samples were then processed for whole mount *in situ* hybridization as previously [16]. Sections to be stained for X-gal were fixed as for whole mount staining and processed for cryosectioning as described above. 14 uM sections were washed 3 times with PBS and stained for β-galactosidase activity for a maximum of 1 hour at 37 degrees C. Staining was stopped by washing 3 times for 10 minutes in PBS followed by a 10 minute fixation in 4% PFA before proceeding to section *in situ* hybridization.

LiCl induction

E11–E11.5 wild-type or Wnt9b$^{-/-}$ kidneys were incubated at room temperature for 80 seconds in 2.25% pancreatin, 0.75% trypsin in Tyrodes solution pH 7.4 followed by inactivation with fetal bovine serum for 10 minutes on ice. Metanephric mesenchyme was mechanically separated from the ureteric bud in PBS and cultured at the air media interface on Nucleopore filters (Whatman #110410) as indicated.

IW treatment

E11.5 urogenital systems were microdissected in sterile PBS and then cultured on Nucleopore filters at the air media interface for up to 48 hours in the presence of either DMSO, 5uM IWP2 or 100uM IWR1. The media

supplemented with DMSO, IWP2 or IWR1 was changed at 12 hour intervals for the duration of the experiment.

Surface Heterochronic *In Vitro* Recombinations (SHIVR)

Metanephric mesenchyme from either E11.5 wild type or E13.5 Wnt9b$^{-/-}$ animals were isolated as described above. Once isolated the metanephric mesenchyme was recombined with the E11.5 wild type ureteric bud and cultured on a nucleopore filter at the air media interface for 48 hours. Control mesenchymes were recombined with E13.5 Wnt9b$^{-/-}$ ureteric bud.

Tubule diameter counts

To quantitate the number of cells making up the cross-sectional wall of individual tubules, 10uM kidney sections were stained with segment specific markers (DBA or LTL), antibodies to the extracellular matrix protein laminin and the nuclear marker Dapi. For the collecting ducts, we excluded the cortical most epithelia to avoid branching tubules. To assure that only cross sections were being analyzed, the diameter of the tubule was measured at two intersecting lines that were perpendicular to each other. If the two measured diameters varied by more than 10% (making the shape of tubule more of an oval than a circle), the section was assumed to be frontal and therefore excluded from analysis. If a tubule was considered to be transverse, the number of nuclei in the tubular cross section was averaged. This was performed for both the collecting ducts

and the proximal tubules at multiple embryonic and postnatal time points (E13.5, 15.5, 17.5 and P1). Statistical differences between wild-type and mutants were assessed by Student's T-test.

Tubule length measurements

100μM sections of P1 kidneys were cut using a vibratome. Individual sections were incubated with LTL or DBA and anti-E-cadherin and distinct fluorescent secondary antibodies and photographed with a Zeiss fluorescent stereoscope and an MRc5 camera. Zeiss axiovison software was used to trace the entire length of individual collecting ducts. Only ducts that could be traced unbroken from the renal pelvis to the distal tubules were measured. For proximal tubules, a line was drawn from the proximal end to the distal end of individual intact LTL positive tubules. Axiovision software was used to measure the length of the line.

Measuring the orientation of cell division

To evaluate the orientation of cell division we utilized a protocol similar to that described by Fischer *et al* with slight modification [129]. 50 uM thick E13.5, 15.5, P1 and P5 kidney sections were labeled with an anti-laminin antibody, a tubule specific marker (DBA or LTL) and the nuclear dye Sytox green. For the collecting ducts, we excluded the cortical most epithelia to avoid branching tubules. Labeled tubules containing anaphase nuclei where manually identified and a Z-stack was taken using the Zeiss LSM-510. These images where reconstructed using the Imaris software and Cartesian coordinates where assigned for the mitotic spindles and

basal lamina of the tubule (Figure 3-9 C,D). The angle between the resulting vectors was determined according to [129]. The randomness of cell division was determined by the Kolmogorov-Smirnov Goodness of Fit Test.

Measurement of cell elongation and orientation

To determine if cells were elongated, sections of E15.5 kidneys were stained with DBA, E-cadherin and aPKC. The cortical most epithelia were excluded to avoid branching tubules. Z-stacks were captured and sections were identified that were frontal through the collecting duct and that fell one frame below (basal to) the aPKC staining. Using Image ProPlus software, two roughly parallel lines were drawn on opposite sides of every cell in the image where E-cadherin staining outlined the entire cell (Cells on the edges that had discontinuous E-cadherin staining were not measured). The software then calculated the average distance between those two lines and assigned a length to width ratio for each individual cell, with the length being the longer of the two sides. Cells that possessed a length to width ratio of greater than 1.2 were considered elongated. To measure the orientation of elongated cells, a vector was assigned for the elongated axis of the cell and the elongated axis of the tubule. The angle between these two vectors was determined using Image ProPlus software. The percentage of total cells that fell within each 10^{o} bin was calculated. Statistical analysis for the wild-type and mutant populations was performed according to the Mann-Whitney U test.

CHAPTER FIVE

Summary and Conclusions

Abstract

This work has provided novel insights into many diverse developmental processes. Here I have shown that in addition to it's previously described role in renal vesicle induction, Wnt9b regulates both the proliferation/expansion of the nephron progenitor cells and the morphogenesis of the renal vesicle after induction. The molecular mechanisms by which Wnt9b regulates these diverse processes is controlled by the cell receiving the signal. Wnt9b signals canonically to regulate both differentiation and progenitor maintenance. Wnt9b appears to continue to signal to the RV after induction. However, at this stage Wnt9b appears to activate Rho and Jnk to regulate morphogenesis of the RV.

Canonical Wnt9b signaling regulates both differentiation and progenitor cell expansion.

In this study, I have identified novel Wnt9b target genes and demonstrate that *Wnt9b* functions in multiple processes during kidney development. Initially, Wnt9b activates β-catenin to induce the cells of the metanephric mesenchyme to aggregate and undergo a mesenchymal-to-epithelial transition to form the RV. Interestingly, Wnt9b also activates β-catenin in the renal progenitors. The transcription factor Six2 is necessary

to modify the outcome of the Wnt9b signal from one that induces the progenitor cells to differentiate to a signal that regulates proliferation/self renewal.

While it was known that Wnt9b induced the mesenchymal progenitor cells to differentiate and form RV **[16]**, the molecular pathway Wnt9b activated was unknown. It has long been established that canonical Wnt/β-catenin signaling is sufficient to induce the mesenchyme to differentiate **[121-124]**. However, determining the pathway Wnt9b activated was complicated by the fact that Wnt4 is up-regulated in response to Wnt9b and can functionally replace Wnt9b **[16]**. Wnt4 signals through β-catenin to regulate differentiation **[122]**. To answer this question I undertook an unbiased approach comparing mRNA profiles from wild type and *Wnt9b*$^{-/-}$ MM at E11.5. This analysis yielded novel Wnt9b targets, including C1qdc2, that are expressed in the pre-tubular aggregates. The Wnt9b target genes, Pax8 and C1qdc2, are both induced by LiCl in isolated MM indicating that Wnt9b signals through β-catenin to regulate differentiation. This analysis also identified novel Wnt4 target genes, like the gene *Daple* in the PTA which are also inducible by LiCl. These data confirm the conclusions of Park et al that *Wnt4* signals canonically to regulate both gene expression and differentiation **[122]**.

My data indicate that both Wnt9b and Wnt4 signal canonically to regulate gene expression and differentiation. However these data are confusing due to Wnt9b being unable to compensate for the absence of

Wnt4 despite the fact that both signal canonically **[16]**. Collectively these date imply that Wnt9b and Wnt4 are signaling through separate receptors. It is difficult to test this hypothesis as individual Fzd mutants have been generated and none present a kidney phenotype. However all 10 Fzd receptors are expressed during kidney development and significant redundancy between these receptors in this system is likely **[27-30]**. A separate possibility is that Wnt4 activates a separate non-canonical pathway necessary for RV induction that Wnt9b is unable to activate. Non-canonical Wnt4 signaling activates RhoA and Jnk in a colony forming assay although this appears to regulate progenitor cell expansion and not tubulogenesis **[146]**. This hypothetical non-canonical pathway involvement may be receptor or co-receptor dependent or may reflect intracellular differences in the dorsal Six2 positive progenitor cells with respect to the PTA.

Alternatively Wnt9b may activate a non-canonical pathway that promotes a progenitor fate while Wnt4 cannot activate this pathway leading to differentiation. A recent report indicated that in the context of the *Xenopus* embryo Wnt5a and Wnt11 form homodimers that interact to activate both canonical and non-canonical pathways **[220]**. An interesting finding in this paper was that disrupting the balance between Wnt5a and Wnt11 proved to have an inhibitory effect on the signaling pathway. An intriguing possibility during kidney development is that a Wnt ligand homodimeric combination regulates the choice between differentiation and

renewal. It is plausible that Wnt9b homodimers alone (or possibly complexed with Wnt6 or Wnt11) induce a progenitor fate. As a cell traverses the UB towards the ventral domain the composition of these homodimeric complexes would be predicted to increasingly include Wnt7b homodimers. The combination of Wnt9b and Wnt7b homodimers would lead to differentiation. This scenario seems unlikely as Wnt7b mutants do not have tubulogenesis defects; however there is likely much redundancy in this system **[31]**.

Another possibility is that spatial differences in the expression of intracellular cofactors regulate β-catenin activity. Many diverse regulators of Wnt signaling are expressed in the developing kidney and may act to fine tune the response to either Wnt9b or Wnt4. Many of these genes, like *Daple*, *Idax* and *Lef1*, are expressed in the PTA and may modify either the binding specificity (Lef1) or the activation of β-catenin (Daple and Idax) downstream of Wnt4 leading to the observed disparity.

Interestingly, many putative Wnt9b target genes were expressed in the progenitor compartment. These data indicated Wnt9b actively signals to the progenitor compartment. These genes, including *Pla2g7*, *Tafa5*, *Cldn9*, *Rik593027L02* and *Rik2310045a20* were all induced by LiCl and inhibited when Wnt/β-catenin signaling was ablated by culturing in either IWR1 or IWP2. From these data we conclude that Wnt9b signals through β-catenin to regulate gene transcription in the progenitor domain. This novel activity for Wnt9b in the progenitor cells is not inconsequential as

there is a significant decrease in proliferation in the absence of Wnt9b. My data indicate that Wnt9b is not necessary for the specification of the progenitor domain as the progenitors form normally and are competent both expand and differentiation in Wnt9b mutants. We observed that the progenitor cells are completely lost within 48 hours after the removal of Wnt9b using the KspCre deleter line. While Wnt9b regulates progenitor cell proliferation, a loss of proliferation alone cannot explain the complete ablation of this domain. The simplest hypothesis is that Wnt9b is necessary for survival of the MM, however Wnt9b does not appear to regulate apoptosis as there is no significant change in Tunel staining in the MM at E11.5 in *Wnt9b*$^{-/-}$ animals. Future studies should evaluate apoptosis in the progenitor population of KspCreWnt9b$^{c/-}$ animals.

While Wnt9b is not necessary for the specification of the progenitor cells, these cells are completely eliminated from the kidney by E15.5. As there is no change in apoptosis initially in Wnt9b mutants these data indicate a second signal is necessary for progenitor survival. Perhaps this survival signal emanates from the renal vesicles as these are absent in Wnt9b mutants. Fgf signaling inhibits apoptosis in many organ systems including the kidney **[221-224]**. Fgf2 is expressed in the UB and promotes survival of the MM **[222-224]**. However, since apoptosis is not affected at e11.5 in Wnt9b mutants it is logical to assume that Fgf2 is unaffected as well. I hypothesize that a separate signal from the RV, possibly *Fgf8* or *Fgf9*, signals to the progenitor cells and acts as an survival cue while

Wnt9b acts as a proliferative cue leading to the expansion of the progenitor cells. Future studies should focus on RV signaling to the progenitor cells, specifically if Fgf signaling from the RV acts as to block cell death in the progenitor population.

How does Six2 regulate Wnt9b activity?

The data indicating that both PTA and progenitor Wnt9b targets are regulated by β-catenin present a conundrum. How does Wnt9b induce such a disparate response to β-catenin signaling and what factors mediate the decision between differentiation and self-renewal? No doubt the molecule Six2 is an important player in this cell fate decision. Six2 is necessary and sufficient for the maintenance of the progenitor compartment **[141]**. I demonstrate here that Six2 is necessary to alter the response to the Wnt9b signal at least at a transcriptional level. The question then becomes how does Six2 alter the transcriptional output of β-catenin in the progenitors? It is plausible that Six2 regulates the expression of progenitor-specific factors that interact with or alter the specificity of β-catenin. This could include transcription factors or pathway modulators that might fine tune β-catenin activity. One such factor is the canonical Wnt agonist Rspo1. The expression pattern of Rspo1 would be predicted to result in increased levels of β-catenin signaling in the progenitors. In fact it would seem that the progenitor domain “sees” the highest level of β-catenin signaling. Coupling this data with the expression of many canonical Wnt antagonists in the PTA and it becomes conceivable

that a gradient of β-catenin activity might regulate the decision between differentiation and proliferation. Cells in the progenitor compartment expressing Rspo1 would receive the highest level of β-catenin signaling. As these cells begin to traverse the UB the levels of Wnt ligand (and thus β-catenin) they are receiving would begin to be attenuated due to the expression of secreted antagonists in the PTA like Sfrp2 and Dkk1 from the stalk of the UB **[225]**. Cells in this position, receive lower levels of β-catenin signaling and have reduced or no Six2 present, differentiate and undergo MET under the guidance of Wnt4. However, this hypothesis seems unlikely as Rspo1 is expressed at slightly lower levels but is present in the progenitor cells of *Six2*$^{-/-}$ animals.

It seems much more likely that Six2 cooperates with β-catenin to regulate the progenitor state. Many progenitor cell markers that are reduced in *Six2* mutants (Eya1, Meox and Pax2 **[141, 143, 163]** are also reduced in *Wnt9b* mutants by microarray. Evaluation of these genes in *Six2*$^{-/-}$;*Wnt9b*$^{-/-}$ double mutants should determine if Six2 and Wnt9b cooperate to regulate the progenitor fate. An alternate possibility is that Six2 may directly interact with β-catenin. Any interaction between Six2 and β-catenin would be predicted to alter either the binding specificity or transcriptional activity of β-catenin leading to a progenitor fate. A simple model would be that the combination of β-catenin and Six2 is the deciding factor between progenitor expansion and differentiation. Cells that express Six2 and do not receive a canonical Wnt signal (i.e. Wnt9b

mutant) do not differentiate or proliferate and are quiescent. Cells that express Six2 and receive a Wnt9b signal are induced to proliferate and maintain the progenitor pool. Proliferation would lead to cells on the ventral aspect of the population with decreased Six2. These cells that receive the Wnt9b signal would then be induced to differentiate, turn on Wnt4 and epithelialize. This model would depend on the relative levels of each molecule to determine the molecular readout. Again molecules like Rspo1 would modulate β-catenin signaling resulting in progenitor cells having high levels of β-catenin and Six2 leading to expansion. Cells closer to the PTA would have Six2 and lower levels of β-catenin which could trigger the differentiation process.

One confusing aspect is that for differentiation to occur the progenitor cells must down-regulate Six2 expression. As such, the mechanism that regulates the down-regulation of Six2 in the progenitors prior to differentiation is worthy of future studies. It is likely that a signal from the PTA down-regulates Six2 in the progenitor cells. This signal would prime these cells for differentiation. However, the mechanism by which the initial dorsal ventral pattern is established is unknown. It is possible that UB invasion patterns the MM. Cells adjacent to the invasion point receive Wnt9b initially and begin to differentiate while the rest of the MM adopts a progenitor fate. The differentiating cells would then signal to adjacent progenitors to regulate Six2 expression while dorsal progenitors proliferate and expand during development. If this hypothesis is correct

then removing the ureteric bud at e10.5 and reinserting it 180 degrees from the entry point should reverse the observed pattern with progenitors being found dorsally and differentiating cells found dorsally. However, if a separate signal from the stalk of the UB, such as Wnt7b, is necessary to maintain this pattern then this experiment will not affect the pattern. Nevertheless, determining the signal or signals that set up the original pattern in the MM will be a necessary endeavor to answer this question.

It is likely that Wnt9b sets up the initial pattern in the metanephric mesenchyme. In this model, Wnt9b is secreted from the Wolffian duct and signals out to the adjacent mesenchyme. I would hypothesize that originally the mesenchyme is not patterned with an equal potential to differentiate or adopt a progenitor fate. A gradient of Wnt9b activity from the WD might pattern the mesenchyme. Cells adjacent to the Wolffian duct would initially receive the highest levels of Wnt9b signaling and up-regulate C1qdc2, Pax8 and Wnt4. Cells further away from the WD receive lower levels of Wnt9b, do not turn on these genes and adopt a progenitor fate. In the absence of Wnt9b, forced expression of activated β-catenin (Rarb2cre;Wnt9b$^{-/-}$;Bcat^{exon3flox}) induces the expression of the progenitor target Tafa5, however the patterning of the mesenchyme is defective. Tafa5 is expressed in a punctate pattern on both the ventral and dorsal aspects of the UB. Similar results are observed with Rarb2cre;Bcat^{exon3flox} animals. In this model, theoretically all cells of the MM receive high levels of β-catenin which imparts no patterning information in the absence of

Wnt9b (Rarb2cre;Wnt9b$^{-/-}$;Bcat^{exon3flox}) or overrides the patterning information imparted by a gradient of Wnt9b activity (Rarb2cre;Bcat^{exon3flox}). A non-canonical Wnt aspect might influence pattern formation as the forced progenitor expression of Wnt1 (Rarb2Cre;Wnt9b$^{-/-}$;Rosa^{Wnt1eGFP}) results in much more uniform rescue of Tafa5 (not shown). However, there appears to be a mixing of progenitor and PTA fates indicative of defective patterning. Further evaluation of the role Wnt9b plays in patterning the MM is needed.

Wnt9b signals non-canonically to regulate tubule morphogenesis

In addition to these two early roles for Wnt9b, I show that Wnt9b regulates the PCP of cells within the developing tubule. Mice carrying a hypomorphic mutation of Wnt9b or mice that have had a floxed allele of Wnt9b deleted with either KspCre or the tamoxifen inducible *CaggCreErTm;Wnt9b$^{-/flox}$* develop cystic kidneys. Cystogenesis appears to be caused by defects in PCP. I show that cells within the epithelial tubule are elongated perpendicular to the proximal/distal axis of the tubule and this orientation is dependent on Wnt9b. In other systems, cellular orientation is regulated by PCP and is a hallmark of tissues that are undergoing for convergent extension movements [52, 66, 198]. Contrary to what has been observed in adult kidney tubules [129, 130, 135, 226], cell division is randomly oriented within the cells of normal collecting ducts and proximal tubules in embryonic kidneys. Although cell division in the manner would be predicted to result in a net increase in the number of

cells that comprise the wall of the tubule, the number of cells composing the wall of the tubule actually significantly decreases during the embryonic period [39, 129, 135]. My data indicate that convergent extension movements drive the decrease in the number of cells in the tubule wall as the tubule elongates. The observed randomly oriented cell division would be inconsequential during this stage as directed cell movements would compensate for any aberrant cell division. After the diameter of the tubule has been established (around birth), cell division becomes oriented parallel to the proximal/distal axis. At this point, cell division oriented in this manner protects against increases in tubule diameter. My data suggest that Wnt9b plays essential roles in both of these processes, perhaps by mediating the orientation of cells in the developing tubule.

In contrast to its canonical Wnt/β-catenin dependent role during tubule induction and progenitor cell expansion, I show that the role of Wnt9b in establishing and maintaining tubule diameter is β-catenin independent. Instead, Wnt9b activates the non-canonical Rho/Jnk pathway to regulate these processes. This is important as it appears Wnt9b is activating both pathways simultaneously, the canonical in the MM and non-canonical in the RV. This fact is of note as it lends credence to the hypothesis that pathway usage is not determined by the individual Wnt ligand but instead by the cellular environment in which the signal is received.

The factors that mediate these disparate responses to the same Wnt9b ligand are unknown. Wnt9b induces the expression of many so-called “pathway switches” such as Daple, Idax and Daam1 in the RV. Each of these molecules interacts with Dsh and are thought to inhibit the canonical Wnt pathway, while activating the non-canonical pathways. It seems plausible then that Wnt9b regulates PCP of the RV by inducing the expression of these molecular switches. Expression of genes like Daple, Idax and Daam1 would be predicted to shuttle Wnt9b (or Wnt4) activity away from the canonical pathway to PCP signaling. However any role for these genes regulating PCP establishment in the developing kidney remain to be elucidated. However, it is important to note the Wnt9b receptor is unknown and may also bias the choice between β-catenin and Rho/Jnk activation.

Interestingly, recent studies showed that attenuation of Rho kinase led to shorter, wider tubules in cultured kidneys [206]. This phenotype may be due to attenuation of signal transduction downstream of Wnt9b and concomitant defects in convergent extension processes. Although we have detected differences in the levels of activated Rho and Jnk in Wnt9b mutants, we have not seen any other defects in the formation or polarity of the cytoskeleton (Data not shown). Further analysis of the cellular events involved in tubule formation will be greatly enhanced by the ability to examine cell movements and shape changes in live tissue.

Although this study has revealed new insights into the mechanisms that regulate tubule diameter, several questions remain unanswered. One such question is why Wnt9b cysts are more severe in the cortex of the kidney? There are several possible answers. The simplest answer is that another molecule compensates for Wnt9b in the medullary region. Several Wnts, including *Wnt5a*, *Wnt7b*, *Wnt4* and *Wnt11*, are expressed in the medullary region of both wild type and *Wnt9b* mutant kidneys ([31] and not shown) and any one of these factors may compensate for loss of Wnt9b. Alternatively, there may be a parallel, Wnt-independent, signaling pathway that regulates PCP in the medulla. The PCP determinant Fat4 is expressed in cortical and medullary stroma of the kidney (Figure 5-1A and [227]). A recent study showed that mice lacking the PCP determinant Fat4 developed kidney cysts primarily within the medullary region [130]. Compensation by either another Wnt or Fat4 would explain the paucity of medullary cysts in Wnt9b mutants.

Planar cell polarity establishment

The manner in which PCP is set up or directed remains elusive. Many PCP genes are expressed during kidney development, however members of both the core and upstream groups are expressed in unique non-overlapping patterns. The upstream genes Ds, Fjx and Fat4 are expressed in the stroma and progenitor cells with Fjx also being expressed in epithelial structures of the Comma-Shaped Body and S-shaped body [227]. In contrast to this, the core genes Celsr1, Celsr2,

Vangl1 and Vangl2 are expressed predominantly in epithelial structures, while the Fzds are expressed in epithelial, stoma and progenitor compartments [30, 228, 229]. These disparate expression patterns of core and upstream genes imply that the simple two tier hypothesis for the establishment of PCP may be different with respect to the kidney. It is plausible these individual groups are sufficient to polarize the cells they are expressed in. In this model, the upstream group genes might polarize the stroma while the core group would polarize the epithelial tissues. This is similar to the PCP establishment model proposed in [33]. In this model, the two PCP gene groups act in parallel to establish PCP. Alternatively, the polarizing information may come from interactions between the different tissue types. For example, upstream group genes in the stroma, such as Fat and Ds, would act as polarizing cues to establish PCP in the epithelial structures. Epithelial-stroma interactions would then bias the activity and localization of Fzd, Celsr, and Vangl in the developing epithelia with respect to the stroma.

During kidney development, Fat4 expression is restricted to the cortical and medullary stroma, perhaps implying this cellular compartment is important for the establishment of PCP (Figure 5-1 and [227]). As mentioned, Fat4 mutants have defects in PCP and form cysts similar to the Wnt9b mutants [130]. The similarity in the two mutant phenotypes suggest Wnt9b and Fat4 act in parallel to polarize the developing tubules. Normally Fat4 is localized to the cortical side of developing tubules (Figure

5-1). However, Fat4 is significantly up-regulated in Wnt9b$^{neo/neo}$ mutants at both E15.5 and P1 (Figure 5-1 and not shown). In Wnt9b$^{neo/neo}$ animals, the Fat4 expression domain completely encompasses some tubules. If Fat4 from the stroma acts to polarize the developing tubule then either too much, or too little Fat4 would have the same result: defective PCP and cyst formation. To test this hypothesis it will be necessary to generate mice that express Fat4 in an inducible and cell type-specific manner. Expressing Fat4 from the medullary side of the Comma-Shaped Body or from the UB would be expected to perturb the establishment of PCP and lead to cyst formation if this hypothesis is correct.

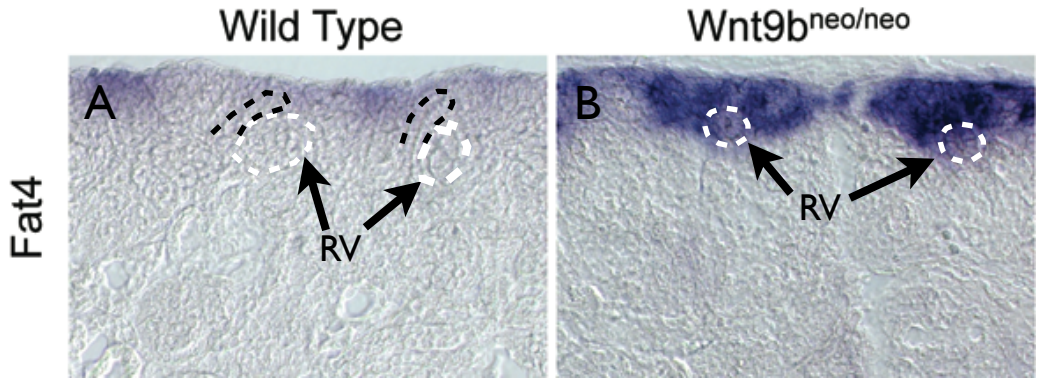

Figure 5-1: Fat4 is up-regulated in Wnt9b$^{neo/neo}$ P1 kidneys.
Section *in situ* hybridization comparing the expression of the gene *Fat4* in wild type and Wnt9b$^{neo/neo}$ newborn kidneys (A, B). (A) In wild type kidneys, Fat4 is normally restricted to the cortical stromal. Normally Fat4 is expressed on the cortical aspect of the ureteric bud (outlined in black) and developing tubules (outlined in white). (B) In Wnt9b$^{neo/neo}$ kidneys, Fat4 expression is expanded and now surrounds the the developing tubules (RV).

While we are currently unable to test if too much Fat4 results in cyst formation, the removal of Fat4 in a Wnt9b$^{neo/neo}$ background results in a

slight enhancement of cystogenesis (Figure 5-2). These results are difficult to decipher as the Wnt9b$^{neo/neo}$ phenotype is extremely variable and this rescue could reflect this variability. Repeating these experiments utilizing the KspCre;Wnt9b$^{c/-}$ mice should clarify this issue. However, Wnt9b$^{+/-}$ Fat4$^{+/-}$ compound heterozygotes have a cystic phenotype that is very similar to Fat4$^{-/-}$ animals indicating that Wnt9b and Fat4 interact genetically (Figure 5-2). However, a more detailed cellular characterization of this phenotype should be performed to determine if Wnt9b and Fat4 cooperatively regulate cellular orientation and either OCD or CE.

Another question raised by these findings is how Wnt9b, produced by the collecting ducts, affects planar cell polarity in the relatively distant proximal tubules? There are several possibilities. One possibility is that Wnt9b signals directly to (i.e. its receptor is expressed in) the cells of the proximal tubule. If this is the case, there are several different routes that the ligand could travel (e.g. through the intervening stroma, through the lumen of the tubules or through the plane of the epithelium (transcytosis)). A second possibility is that the effects on diameter are all indirect (e.g. Wnt9b could be affecting the expression of a second molecule in the stroma, like Fat4, or mesenchyme and this molecule directly regulates PCP).

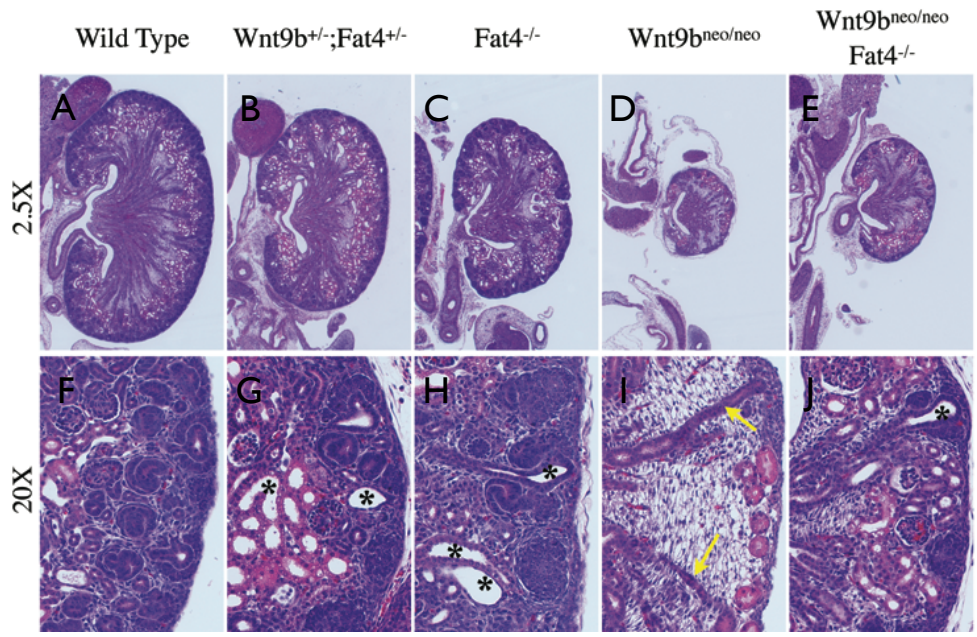

Figure 5-2: Wnt9b and Fat4 genetically interact to regulate tubule diameter.

H&E sections of P1 wild type (A,F), *Wnt9b*$^{+/-}$*;Fat4*$^{+/-}$ (B,G), *Fat4*$^{-/-}$ (C,H), Wnt9b$^{neo/neo}$ (D,I) and *Wnt9b*$^{neo/neo}$*;Fat4*$^{-/-}$ (E,J). (A-E) show low magnification (2.5X) images of of the whole kidney while (F-J) show high magnification (20X) images focusing on the cortical aspect of the kidneys. *Fat4*$^{-/-}$ kidneys are smaller at birth with some obvious cystic epithelia (asterisks in H). Wild type kidneys do not contain cysts (F). *Wnt9b*$^{+/-}$*;Fat4*$^{+/-}$ (B,G) kidneys are smaller kidneys than wild type (compare A and B) but larger than Fat4$^{-/-}$ mutant kidneys (compare B and C). *Wnt9b*$^{+/-}$*;Fat4*$^{+/-}$ kidneys also form cysts comparable to *Fat4*$^{-/-}$ kidneys (compare F,G and H). Wnt9b$^{neo/neo}$;*Fat4*$^{-/-}$ kidneys are larger that Wnt9b$^{neo/neo}$ kidneys and show an increase in cyst formation (compare arrows and asterisks in I and J). Collectively these data indicate that Wnt9b and Fat4 interact genetically to regulate tubule diameter.

As mentioned above Wnt9b negatively regulates the expression of Fat4 in the stroma (Figure 5-1), but the pathway Wnt9b signals through or if Wnt9b signals directly or indirectly to the stroma to regulate Fat4 expression is unknown. Fat4 contains multiple consensus β-catenin binding sites, perhaps indicating that Wnt9b signals directly to the stroma to negatively regulate Fat4 expression. A separate possibility is that Wnt9b regulates Fat4 expression indirectly by repressing the stroma cell lineage. In this scenario, the observed increase in *Fat4* mRNA would be due to an increase in the number of stromal cells that express Fat4, not due to an increase of Fat4 expression *per se*. There are many scenarios in which this could occur. Wnt9b could signal directly to the stromal cells. Multiple Fzd receptors are expressed in this compartment making this situation a plausible possibility. Alternatively, the progenitor compartment may interact with and inhibit expansion of the stromal lineage. In this scenario, reduction in Wnt9b signaling would result in the loss of Wnt9b mediated progenitor cell expansion. If the progenitor cells have an antagonistic effect on stomal expansion then the loss of the progenitor lineage observed in Wnt9b mutants would be predicted to result in secondary stromal cell expansion.

Similar to what is observed in both chick [62] and *C. elegans* [97, 101], Wnt9b may act instructively to polarize either the cells of the MM or the collecting duct. Cells in the MM that receive the Wnt9b signal would be polarized relative to the source of Wnt9b (UB) and develop normally.

However, attenuation of the Wnt9b signal (in this study using either the $Wnt9b^{neo/neo}$ or KspCre;$Wnt9b^{flox/-}$ alleles) may interfere with the polarizing signal while leaving the inductive signal intact. A final possibility is that Wnt9b functions to polarize the cells of the collecting duct and that this information is passed on to the renal vesicles and their derivatives (in an epigenetic fashion) after these structures fuse with the collecting duct. This final possibility seems unlikely due to the fact that cysts form in animals where Wnt9b is removed at birth (*CaggCreERTM;*$Wnt9b^{flox/-}$). If Wnt9b simply functioned to polarize the collecting duct and this information was passed on to the RV upon fusion, removal of Wnt9b at birth should have no effect on this as the collecting duct should be properly polarized at this stage. These data argue for a role for Wnt9b continuously signaling to polarize the RV.

Directly addressing these hypotheses has been complicated by the fact that we do not know at precisely what step during tubule morphogenesis Wnt9b acts nor do we know the identity of its receptor. All 10 Fzd receptors are expressed in the developing kidney. However, these Fzd receptors are expressed in unique cellular compartments. For example Fzd4, 5, 7, 8 and 10 are all expressed in the renal vesicle, Comma-Shaped Body and S-shaped body making these the highest priority targets for being a putative Wnt9b receptor in the RV. While Fzd 1, 2 and 10 are expressed in the stroma making these the highest priority targets to be a Wnt9b receptor in the stroma. Pertaining to the step at

which Wnt9b acts, the *KspCre*;*Wnt9b*$^{-/flox}$ mice results have lead us to hypothesize that Wnt9b acts during the later stages of morphogenesis. If this is the case, then travel through the lumen (potentially in a direction that is opposite the flow of urine) or through the plane of the epithelium seems unlikely. However, at this point we cannot rule out the possibility that Wnt9b functions during the early stages of tubule formation (perhaps even affecting the polarity of the mesenchyme itself) and that all of our mutants represent a reduction of Wnt9b signaling to this cell population. Identification of the frizzled receptor and molecular targets of Wnt9b will allow us to determine which of these mechanisms is utilized.

A final question that remains is whether Wnt9b contributes to human forms of PKD. Wnt9b continues to be expressed in the adult kidney but ablation of Wnt9b in P14 mice has no effects on kidney homeostasis suggesting that the pathway may not be active in a healthy adult. These data are reminiscent of results with the removal of Kif3a and Pkd1 indicating that once the tubule diameter is established (after P12) it is difficult to perturb without injury [135, 230]. It is possible that Wnt9b becomes activated and is required for kidney repair after injury. Recent studies suggest that cystic kidney diseases may in fact be the result of defects in the tubular repair process [218, 219]. It has been demonstrated that correct orientation of cell division is required for proper tubular repair after kidney injury [135, 226]. It is possible that, similar to the developing tubule, Wnt9b is required for oriented cell divisions (and perhaps

convergent extension) that occur after injury. If this is the case, Wnt9b may represent an interesting therapeutic target for treating PKD. However, my preliminary data indicate no role for Wnt9b during the repair process after injury (not shown). A better grasp of the regulation and downstream targets of Wnt9b will significantly impact our understanding of epithelial tubule morphogenesis and the treatment of polycystic kidney disease in the future.

VITAE

Courtney Michael “Moose” Karner was born in Lincoln, Nebraska on September 23 1980, the son of Miles and Dawnelle Karner. From an early age Moose was fascinated by the natural world and took up bowhunting at the age of 10. His love of wild animals and good food drove him to harvest over 8 distinct game species culminating with the harvest of an 154 inch (B&C) white tail deer buck in November 2002. These outdoor activities nearly derailed his scholastic endeavors as he frequently took to cutting class to satiate his bloodlust. Moose excelled in both athletics and academics at Altus High School, Altus, Oklahoma, where he graduated in the top 3.5616439% of his class in May of 1999. Courtney was a 3 year letterman at AHS and was awarded the Oklahoma Football Coaches Association Scholar Athlete of the Year award, was named to the numerous All-Star teams and represented the state of Oklahoma in the 60th annual Maskat Shrine Oil Bowl Classic where Oklahoma defeated Texas 41-13. Courtney matriculated at The East Central University in the fall of 1999. While at ECU Moose excelled both athletically, mastering the aquatic art of fishing without the benefit of pole or bait (aka noodlin’) and academically. He enjoyed a successful career as the long snapper and right tackle for the ECU Tiger football team. For his efforts he was named Academic All-American in 2003, named to the Lone Star Conference (LSC) All-Academic Team 3 times, the LSC All-Conference Team in 2003 and was named the LSC Male Scholar Athlete

of the year in May 2004. Moose also excelled academically at ECU where he was a NASA Fellow and was awarded the Frank G. Brooks award for excellence in student research in 2003 and was named an East Central University Top 10 graduating senior in 2004. Moose received the degree of Bachelor of Science with a major in Biology and minors in Chemistry and Mathematics from ECU in May 2004. In June 2004, Courtney entered the Graduate School of Biomedical Sciences at the University of Texas Southwestern Medical Center at Dallas. After seven rotations Courtney met Dr. Thomas J Carroll and the rest is as they say, history. He was awarded the degree of Doctor of Philosophy in November of 2009.

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