Patterning a Complex Organ: Branching Morphogenesis and Nephron Segmentation in Kidney Development

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The two major components of the kidney, the collecting system and the nephron, have different developmental histories. The collecting system arises by the reiterated branching of a simple epithelial tube, while the nephron forms from a cloud of mesenchymal cells that coalesce into epithelial vesicles. Each develops into a morphologically complex and highly differentiated structure, and together they provide essential filtration and resorption functions. In this review, we will consider their embryological origin and the genes controlling their morphogenesis, patterning, and differentiation, with a focus on recent advances in several areas.

Introduction

The mammalian kidney is an elaborate organ, consisting of thousands of nephrons connected to a highly branched collecting duct system. Blood is filtered by the glomerulus, which consists of a capillary loop bound by mesangial cells, enveloped in podocytes, and enclosed by Bowman's capsule. The filtrate flows from the glomerular space through the nephron tubule, consisting in sequence of the proximal tubule, loop of Henle, distal tubule, and finally the connecting tubule, which joins a collecting duct (CD). The collecting system includes the cortical and medullary collecting ducts, calyces, papilla, and ureter.

Renal development has been extensively studied in animal models, including fish, amphibians, and mice, for several reasons. The ability to carry out the early phases of kidney development in vitro, and thus to manipulate and visualize the developing organ, has made animal models a powerful system for investigating the cellular basis of organogenesis. Many of the processes underlying renal development have common roles in other organs, such as epithelial-mesenchymal interactions, branching morphogenesis, stem and progenitor cell maintenance and differentiation, cell migration, oriented cell division, and cell-extracellular matrix interactions. On the other hand, kidney development includes some events that are unique to this organ, such as the mesenchymal-epithelial transition (MET) to form the nephron, and the differentiation of highly specialized structures, such as the glomerulus (Quaggin and Kreidberg, 2008). Furthermore, congenital anomalies of the kidney and urinary tract are among the most common birth defects (Airik and Kispert, 2007), and reduced nephron number is a significant risk factor for hypertension and renal failure later in life (Hoy et al., 2008). The information gained from studying animal models may aid in developing strategies to prevent or correct these defects.

During embryogenesis, the nephron epithelia and collecting system derive entirely from the intermediate mesoderm (IM), while other components such as the vasculature derive from other sources. Cells in the dorsal IM coalesce into the nephric duct (ND, also called the Wolffian duct) (Saxen, 1987), while the mesenchymal cell population in the ventral IM, called the

"nephrogenic cord," remains undifferentiated (Figures 1 A–1C). Interactions between the ND and the nephrogenic cord lead first to the formation of a transient group of primitive renal tubules, comprising the pronephric and mesonephric kidneys. Later, the IM becomes further specified along the rostro-caudal axis, forming a specialized region at the level of the hindlimb called the metanephric mesenchyme (MM). The MM serves a dual role: it contains progenitor cells for the nephrons of the metanephric (adult) kidneys, and beginning at E10.5, it also produces inductive signals that cause the ND to evaginate, forming a single ureteric bud (UB) near its caudal end. The UB extends into the MM and branches repeatedly to give rise to the collecting system. Simultaneously, it induces the MM to undergo mesenchymal epithelial transition (MET) and generate the nephron epithelia (nephrogenesis), thus forming the metanephric kidneys. Next, we will examine in detail how these events unfold.

Development of the Nephric Duct and Ureteric Bud: Origins of the Collecting System
Nephric Duct Development

The development of the ND involves several sequential processes that occur before UB outgrowth: specification from the IM, caudal extension, formation of a simple epithelial tube, and conversion of a caudal segment of the tube to a pseudostratified epithelium. Most of these events have been studied primarily in lower vertebrates (amphibians, birds, and fish); however, genetic approaches in the mouse are starting to provide insight into mammalian ND development.

The ND primordium extends caudally as the body axis elongates. Initially a solid cord of cells, it converts to an epithelial tube in a rostral-caudal sequence (Figures 1A–1F). In lower vertebrates, ND extension is mainly driven by the caudal migration and rearrangements of cells, although the recruitment of new IM cells into the growing end also plays a role in some species (Drawbridge et al., 2003; Schultheiss et al., 2003). Genetic fate mapping in the mouse suggests that ND cell migration and/or proliferation, rather than recruitment of new cells, drives the later stages of elongation (Mugford et al., 2008).
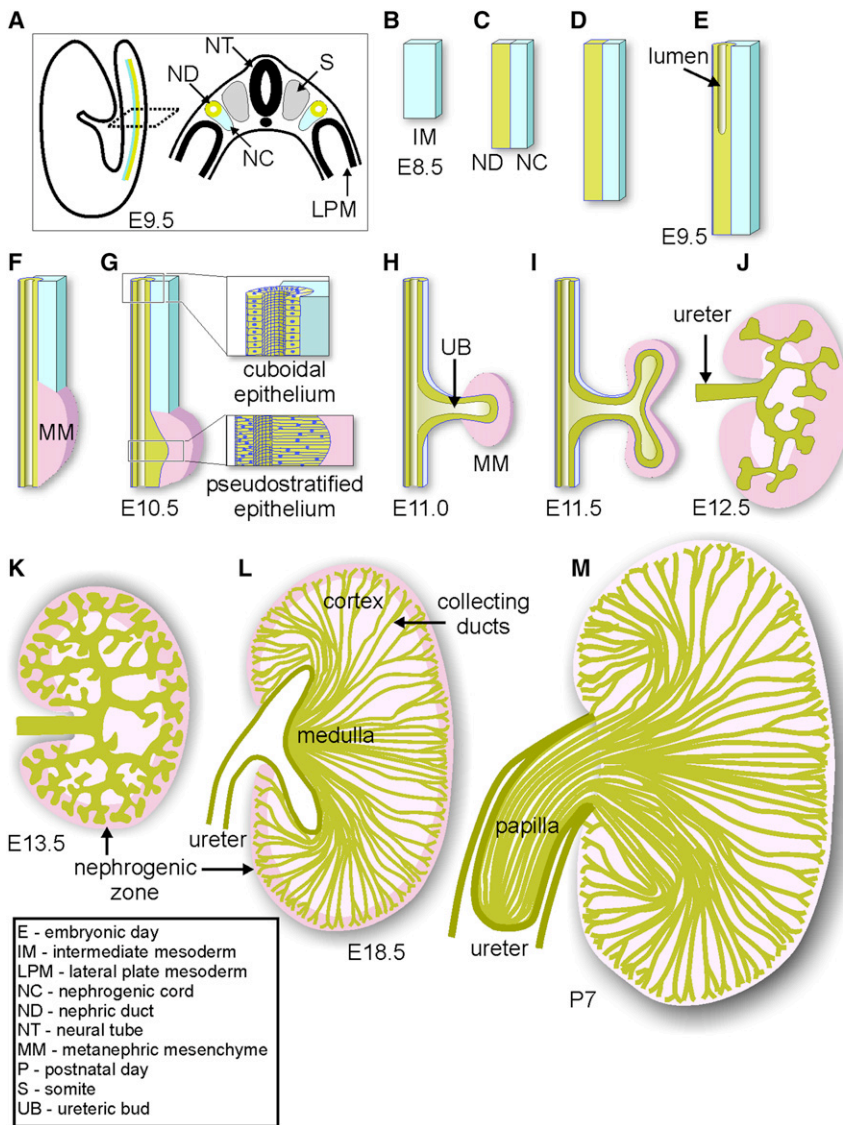
Figure 1. Development of Nephric Duct and Renal Collecting Duct System
(A) Left: diagram of E9.5 embryo, showing nephric duct (green, ND) and nephrogenic cord (blue, NC) in the caudal region of embryo. Right: schematic cross-section showing the ND, NC, neural tube (NT), somites (S), and lateral plate mesoderm (LPM).
(B) Intermediate mesoderm (IM) at ~E8.5.
(C) Formation of nephric duct (ND) primordium and nephrogenic cord (NC).
(D) ND elongation.
(E and F) Epithelialization of ND and formation of MM.
(G) Formation of pseudostratified epithelium in caudal ND, while rostral ND remains cuboidal epithelium.
(H) Outgrowth of UB.
(I) Initial branching of UB within MM.
(J) UB after 3–4 rounds of branching.
(K) Highly branched UB.
(L) Elongation of collecting ducts to form the medulla.
(M) Further CD elongation to form the papilla.

Gata3 transcription (Grote et al., 2008). *Ctnnb1* and *Gata3* are both required for the expression of RET, the tyrosine kinase receptor for the secreted protein GDNF (Bridgewater et al., 2008; Grote et al., 2008; Marose et al., 2008). However, the regulatory hierarchy among these genes does not fully explain the distinct phenotypes that occur in their absence. For example, while the ND fails to form in *Pax2/Pax8* mutants, the ND forms in *Lhx1* mutants but the caudal portion degenerates (Pedersen et al., 2005; Tsang et al., 2000). Lack of *Gata3* causes a more complex ND phenotype, with extensive ductal swelling and misdirected extension toward the surface ectoderm rather than the cloaca, its normal target. As a consequence, neither *Lhx1*$^{-/-}$ nor *Gata3*$^{-/-}$ mice form UBs or develop kidneys (Grote et al., 2006; Lim et al., 2000). Deletion of *Ctnnb1* also causes ND cells to prematurely differentiate and express markers characteristic of the renal CD (Marose et al., 2008). While *Ret* is expressed in the ND is important for later UB development (Costantini and Shakya, 2006), its absence has no visible effect on the ND (Chi et al., 2009), except for failure to fuse with the cloaca (C. Mendelsohn, personal communication). The existence of additional, yet to be identified target genes of Pax2/8, Lhx1, β-catenin, or Gata3 may explain the differences in mutant phenotypes.

The source and identity of signals that guide the caudal elongation of the ND remain to be identified; in the axolotl, signals from the surface ectoderm, including the extracellular matrix (ECM) protein laminin1, appear important (Morris et al., 2003). In the mouse, evidence for a role of laminin1 is ambiguous (Willem et al., 2002; Wu et al., 2009). Conversion of the ND from a mesenchymal cord to an epithelial tube requires signals from the ectoderm (at least in chick), apparently including

Surgical manipulation of chick and *Xenopus* embryos has shown that inductive signals secreted from the axial and paraxial mesoderm are involved in IM induction (Barak et al., 2005; James and Schultheiss, 2003, 2005; Mauch et al., 2000; Preger-Ben Noon et al., 2009; Seufert et al., 1999). A medio-lateral gradient of BMP and activin-like signals collaborate with an anterior-posterior gradient of retinoic acid signaling to assemble *Hox* gene expression domains; cells at specific values along these gradients will undergo ND specification. This process requires the coexpression of the transcription factors *Pax2*, *Pax8*, and *Lhx1* (Bouchard et al., 2002; Carroll and Vize, 1999).

Genetic studies in the mouse have identified several components of a gene regulatory network critical for normal ND development (Figure 2A). *Pax2* and *Pax8* are required for the continued expression of *Lhx1*, and all three may contribute to the expression of the transcription factor *Gata3* (Bouchard et al., 2002; Grote et al., 2006). *Gata3* expression also requires β-catenin (*Ctnnb1*), the effector of canonical Wnt signaling, which likely acts in parallel with *Pax2/8* to initiate and maintain
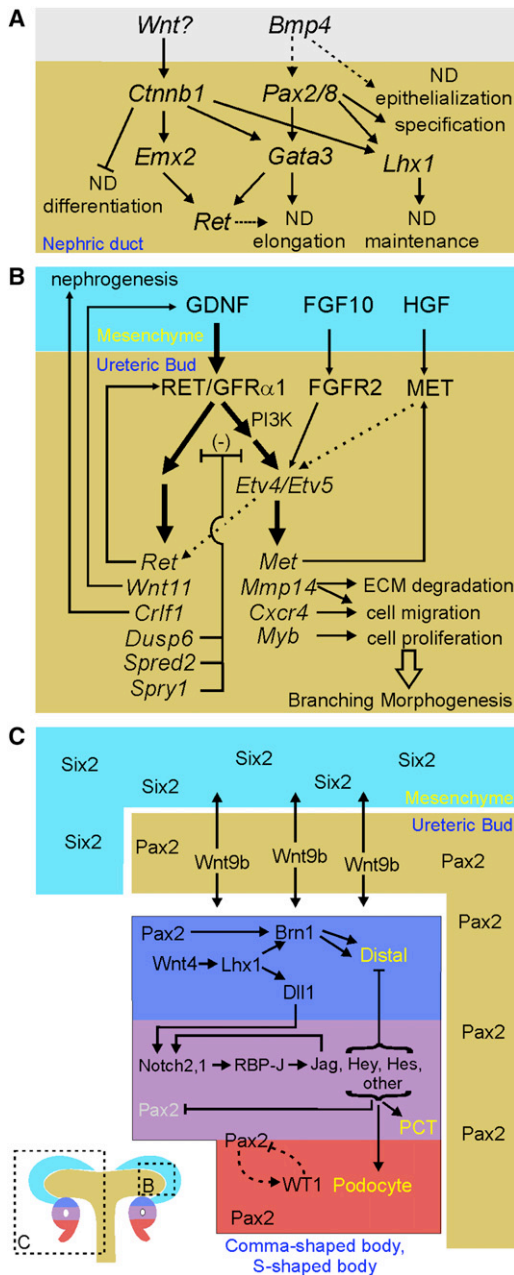
Figure 2. Genetic Networks Controlling Nephric Duct Development, Ureteric Bud Branching, and Nephron Segmentation
(A) Genetic network controlling nephric duct development (see text for details). Dashed arrows indicate effects that occur only at some stages of development.
(B) Genetic network downstream of RTKs that controls ureteric bud branching (see text for details). Bold arrows indicate major effects; dotted arrows, relationships that are uncertain. Modified from Lu et al. (2009).
(C) Genetic network involved in segmenting the nephron (see text for details) Pax2, Lhx1, and Brn1 are involved in establishing the distal segment; Notch2 (with some contribution from Notch1) is required to fix proximal identities and to establish proximal tubule precursors.
PCT, proximal convoluted tubule. Diagram at bottom left indicates the color code used in B and C.

BMP4 (Obara-Ishihara et al., 1999). Ectodermal signals are also required for the continued expression of *Lhx1* and *Pax2* in the ND, which in turn are important for maintenance of the epithelium

(James and Schultheiss, 2005; Torres et al., 1995). Accordingly, BMP inhibitors block ND differentiation in chick and *Xenopus*, establishing an evolutionary conserved role for this pathway in ND formation (Bracken et al., 2008; James and Schultheiss, 2005).

The ND remains a narrow, cuboidal epithelial tube until it fuses with the cloaca at ~E9.5. In the mouse, the caudal ND, from which the UB will later emerge, swells over the next 24 hr, displaying an elevated rate of cell proliferation and converting to a pseudostratified epithelium (Figure 1G). The entire ureteric bud will derive from this pseudostratified domain (Chi et al., 2009). Pseudostratified domains are found in several other epithelia that are about to produce outgrowths, including the mammary line (Veltmaat et al., 2004), otic placodes (Meier, 1978), and liver and thyroid buds (Bort et al., 2006; Fagman et al., 2006). While the importance of ND pseudostratification remains to be determined, the high cell density it generates in the prebudding region might permit rapid outgrowth and branching of the UB. Interestingly, the caudal WD apparently fails to become pseudostratified in $Osr1^{-/-}$ mutants, where the MM does not differentiate normally (see Figure 7 in Mugford et al., 2008). Since *Osr1* is expressed in the MM but not in the ND, this suggests that a signal from the MM may normally induce pseudostratification. This signal remains to be identified, for it is not GDNF as the ND becomes pseudostratified in $Ret^{-/-}$ mice (Chi et al., 2009).

Ureteric Bud Formation
Outgrowth of the UB (Figure 1H) is the initiating step in kidney development. Its correct positioning is critical: buds that form too rostrally or caudally fail to connect correctly to the bladder, and also invade the MM at a suboptimal position, compromising renal development (Airik and Kispert, 2007; Uetani and Bouchard, 2009). The signals from the MM that regulate UB formation have been extensively reviewed (Costantini, 2006; Costantini and Shakya, 2006; Dressler, 2006, 2009). GDNF is a major inducer, as UB outgrowth usually fails in $Gdnf^{-/-}$, $Ret^{-/-}$, or $Gfra1^{-/-}$ mutant mice (and humans with renal agenesis frequently have *RET* mutations) (Costantini and Shakya, 2006; Skinner et al., 2008). Many other mutant genes that cause renal agenesis encode transcription factors (e.g., *Sall1*, *Eya1*, *Pax2*, *Hox11* paralogs) or upstream signals or receptors (e.g., Nephronectin, α8β1 integrin, Gdf-11) required for normal GDNF expression (Bouchard, 2004; Boyle and de Caestecker, 2006). Conversely, mutations that result in the formation of multiple UBs (leading to duplex or triplex ureters and kidneys) impair genes that normally limit the domain of *Gdnf* expression (*Slit2*, *Robo2*, *Foxc2*) or negatively regulate the response to GDNF (*Spry1*). BMP4 also negatively regulates UB outgrowth and is locally suppressed by Gremlin1 to allow outgrowth in the right location (Costantini, 2006; Michos et al., 2007; Uetani and Bouchard, 2009). Recent findings in organ cultures have implicated neuropeptide Y (NPY) as a possible facilitator of GDNF-induced budding (Choi et al., 2009).

How does GDNF, together with other signals from the MM, induce formation of a single, discrete UB? Recent findings show that GDNF/Ret signaling promotes cell movements in the ND that lead to UB formation (Chi et al., 2009). In chimeric embryos containing a mixture of $Ret^{-/-}$ and wild-type (WT) cells, the mutant ND cells populated the ureter but were excluded from

the UB tips (Shakya et al., 2005). Because the tips contain the progenitors for the CD system (see below), the mutant cells were absent from most of the CD system as the kidney developed. Time-lapse imaging revealed that mutant and WT cells in the ND were sorted into different domains, a cluster of WT cells forming the UB tip, while $Ret^{-/-}$ cells were excluded (Chi et al., 2009) (Figure 3A). Analysis of chimeras containing cells of different genotypes revealed that ND cells are sorted according to their level of Ret signaling. For example, in chimeras between WT and S$pry1^{-/-}$ cells (which have elevated Ret signaling), the $Spry1^{-/-}$ cells preferentially formed the UB tips (Chi et al., 2009). In wild-type mice, ND cells are not equivalent as Ret activation (reflected by diphosphorylated Erk MAP kinase) was heterogeneous. Cells with elevated Ret signaling presumably undergo active rearrangements to form the first UB tip. This sorting hierarchy is reminiscent of the developing respiratory epithelium in *Drosophila*, where cells sort based on their levels of FGFR activation to form the tip of the air sac or tracheal branch (Cabernard and Affolter, 2005; Ghabrial and Krasnow, 2006). Why is sorting behavior common in branching epithelia? Perhaps it limits the number of cells that participate in bud formation, thus forming a single, discrete bud rather than a massive swelling.

Since other RTKs also activate the MAP kinase pathway, they might, in principal, be able to replace Ret in these events. Indeed, FGFs can induce budding of the ND in organ culture (Maeshima et al., 2007), and recent genetic data reveal that FGF signaling can substitute for Gdnf/Ret in vivo, under certain conditions. Surprisingly, removing *Spry1* in $Ret^{-/-}$ or $Gdnf^{-/-}$ mice restored UB formation and kidney development (Michos et al., 2010). But when *Fgf10* was also eliminated in $Gdnf^{-/-};Spry1^{-/-}$ mice, UB outgrowth failed. Thus, *Fgf10* can induce and correctly position UB outgrowth in $Gdnf^{-/-};Spry1^{-/-}$ mice (Michos et al., 2010). These experiments reveal that the mechanisms controlling UB formation and outgrowth can be accessed by other RTKs, including FGFRs, and thus, the cell rearrangements during normal ureteric budding are not uniquely driven by Ret signaling. Future studies are need to address the full range of signals that regulate UB formation to position it so precisely and the mechanisms that control cell movements in the ND.

UB Branching and Growth
Cell Lineages and Cellular Events in UB Branching and Elongation
After invading the MM, the UB undergoes about ten generations of repeated branching (Figures 1I–1K), a phase of organogenesis that can be carried out in culture and studied by time-lapse imaging (Srinivas et al., 1999). It is followed by a period of CD elongation with little branching, then by one to two rounds of branching before birth (Cebrian et al., 2004) (Figure 1L). There is a positive relationship between UB branching and nephron induction, so defects that reduce branching cause a reduced nephron number.

The UB grows via cell proliferation in both tips and trunks, the former predominating during the rapid branching phase, the latter during CD elongation (Karner et al., 2009; Michael and Davies, 2004). Tip cell proliferation increases the size of the terminal ampulla, which is then remodeled to form new branches (Watanabe and Costantini, 2004). As the tip cells divide, some
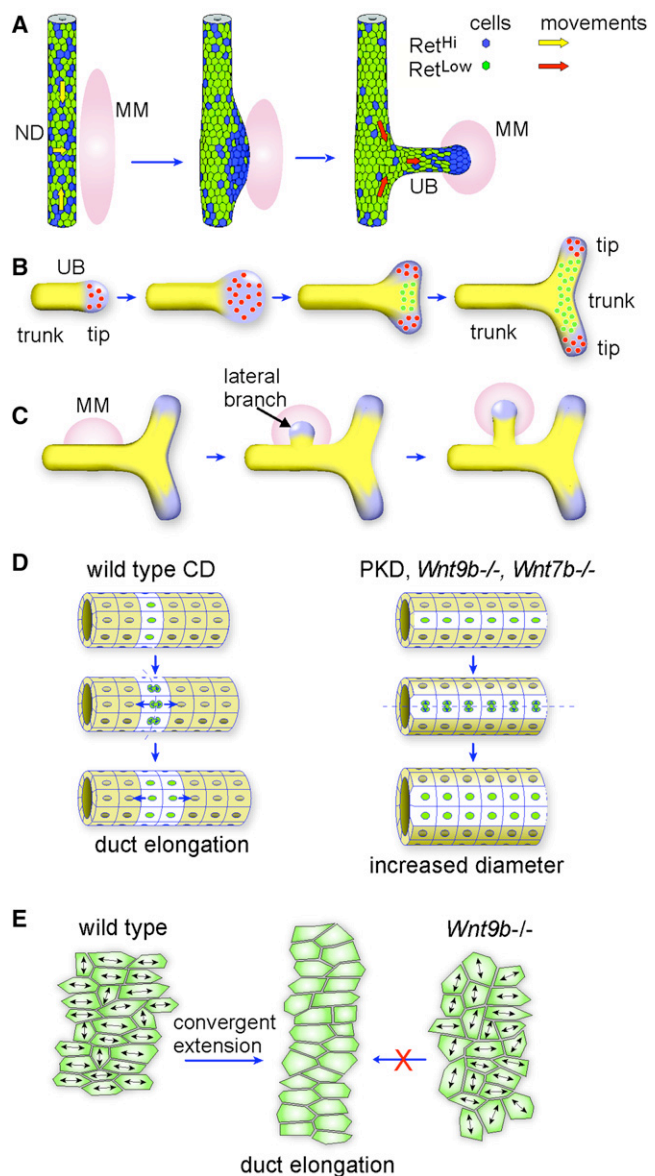

Figure 3. Cellular Mechanisms of Nephric Duct/Ureteric Bud/Collecting Duct Morphogenesis

(A) ND cell movements during the formation of the first UB tip domain. Cells in the ND with higher Ret signaling activity (blue) preferentially move (yellow arrows) to the dorsal ND, adjacent to the MM (pink oval), then form the first UB tip as it emerges. Cells with lower Ret activity (green) trail behind (red arrows) and populate the UB trunk.

(B) Bipotential nature of UB tip cells. UB tips are shown in blue and UB trunks in yellow. Cells initially in the UB tip (colored red) divide and give rise to new tip cells (red), as well as cells that are left in the growing trunk (green).

(C) During lateral branching, or branching induced by transplanted MM (pink), tip cells (blue) are regenerated from trunk cells (yellow).

(D) Left: in late fetal and postnatal CDs, mitoses are preferentially oriented along the long axis of the duct, leading to duct elongation. Right: in several mutants, mitotic orientation is randomized, leading to increased diameter (and cyst formation) rather than elongation.

(E) Most CD cells are elongated along the axis orthogonal to the CD long axis, suggesting that they undergo convergent extension movements leading to duct elongation. In *Wnt9b* mutants, however, cell orientation is altered, suggesting a defect in CE movements.

Diagrams adapted from Chi et al. (2009) (A), Shakya et al. (2005) (B), Karner et al. (2006) (D), and Karner et al. (2009) (E).

daughters remain in the tip (self-renewal), while others are "left behind" to form the elongating trunk (Figure 3B) (Shakya et al., 2005). Tip epithelial cells play a similar role as progenitors during branching morphogenesis of the lung (Rawlins et al., 2009) and pancreas (Zhou et al., 2007). The UB "tip to trunk" lineage does not represent an irreversible fate choice. In the "lateral" branching mode (Watanabe and Costantini, 2004), or when induced by transplanted MM (Sweeney et al., 2008), new buds form from an existing trunk and can then undergo further terminal branching; i.e., they appear to have normal tips, and thus, a new tip can be generated from the trunk lineage (Figure 3C).

Very little is known about the cellular events that cause the UB epithelium to branch. Mitotic cells are diffusely distributed around the ampulla (Michael and Davies, 2004), indicating that branching is not driven by localized proliferation; a similar conclusion was reached for lung bud branching (Nogawa et al., 1998). Some of the other processes that might underlie UB branching include cell movements within the epithelium (Chi et al., 2009), oriented cell division (OCD), or changes in cell shape (Meyer et al., 2004), but the importance of these mechanisms remains unclear. In contrast to branching, the elongation and narrowing of CDs at the later stages of kidney development (Figures 1L and 1M) are better understood at the cellular level. During postnatal growth, the elongation of medullary CDs is driven by mitoses that are aligned with the long axis of the duct (Figure 3D), a form of planar cell polarity (PCP) (Fischer et al., 2006). At this stage there is little cell migration or intercalation, so that longitudinally oriented cell division leads to CD elongation without a change in diameter. Interestingly, in several mutants that develop polycystic kidney disease (PKD), a condition in which CDs and nephron tubules enlarge to form cysts, OCD is randomized, leading to a progressive increase in ductal diameter (Fischer et al., 2006) (Figure 3D). One such mutant is in *Fat4*, a component of a PCP-signaling pathway conserved between flies and mammals, implicating this pathway in CD morphogenesis (Saburi et al., 2008).

One signal that controls OCD in the postnatal CDs is Wnt9b (Karner et al., 2009), which is expressed in the trunks of the branching UB and later in medullary CDs. In addition to its paracrine role in nephrogenesis, where it acts through the canonical Wnt pathway (see below), in CD cells Wnt9b seems to act in an autocrine mode to induce PCP through a noncanonical Wnt pathway involving Rho and JNK. CDs lacking *Wnt9b* dilate during fetal development, reflecting randomized OCD, and develop into large cysts postnatally (Karner et al., 2009). Fat4, noncanonical Wnt signaling, and the proteins encoded by nearly all cystic kidney disease genes are associated with the primary cilium, which may be critical for OCD (for a recent review, see Sharma et al. [2008]).

OCD is also important for development of the renal medulla and papilla, where it is controlled by *Wnt7b* (Yu et al., 2009). These structures form by CD elongation (Figures 1L and 1M), establishing a cortico-medullary axis along which the CDs and nephrons are patterned. In mice lacking *Wnt7b,* the CDs that would normally compose the medulla and papilla become abnormally wide, due to a loss of OCD. Unlike the apparent autocrine mode of Wnt9b in this process, Wnt7b is thought to signal to the interstitial cells via the canonical Wnt/β-catenin pathway, and these cells signal back (via an unknown mecha-

nism) to influence OCD and thus duct elongation (Yu et al., 2009).

Wnt9b also influences a second form of PCP that controls duct elongation and diameter (Karner et al., 2009). Most CD cells at E15.5 are elongated, with their long axes perpendicular to the long axis of the duct (Figure 3E). This asymmetrical shape was hypothesized to reflect a process of convergent extension (i.e., lateral intercalation), making the duct narrower and longer. In a *Wnt9b* hypomorph, cellular orientation was randomized and ductal diameter increased (Figure 3E). Thus, *Wnt9b* seems to act at different stages to regulate OCD and intercalative cell movements (Karner et al., 2009).

Finally, *Wnt7b* also acts as a survival signal in late fetal CD development. *Wnt7b* mutants display elevated apoptosis in papillary CDs (Yu et al., 2009), as do mice lacking the laminin receptor α3β1 integrin in CDs. Increased cell death affected the formation of the medulla (in β1 integrin mutants) (Wu et al., 2009) or papilla (in α3 integrin mutants) (Liu et al., 2009). The α3 mutant had reduced *Wnt7b* expression, and further studies revealed that α3β1 integrin acts in concert with the Met receptor tyrosine kinase to regulate *Wnt7b* expression and thus cell survival (Liu et al., 2009).

Factors that Control UB Branching
As the UB branches, the MM becomes distributed around the periphery of the kidney, surrounding the UB tips. Most MM-derived signals that regulate UB outgrowth (e.g., GDNF, FGFs, BMP4, and Gremlin1) also play a continued role in UB branching in the developing kidney. Additional factors that promote branching include Wnt11, pleiotrophin, VEGF-A, HGF, and EGF. While knockout (KO) of neither HGF or EGF (nor their receptors) had a clear effect on kidney development (Davies, 2002), UB-specific deletion of *Met* (the HGF receptor), when combined with a hypomorphic *Egfr* mutation, caused a significant branching defect. This revealed a redundant role for EGF and HGF in the later stages of branching (Ishibe et al., 2009). The ability of several RTKs to promote UB branching is consistent with the view articulated above that downstream signaling events that are common to all of these RTKs are critical in UB outgrowth. GDNF/Ret signaling is normally the predominant stimulus for branching, and when it is absent, the other stimulatory signals are unable to overcome the negative effects of *Spry1*. However, when *Spry1* is also absent, other signals are sufficient for extensive UB branching (Michos et al., 2010).

In contrast to factors emanating from the MM that promote UB branching, a number of signals produced by the MM or stroma inhibit this process, including BMP4 and other TGFβ family members, whose roles have been reviewed extensively (Cain et al., 2008; Costantini, 2006). Another class of signaling molecules implicated in the negative regulation of branching is the semaphorins. Knockout of *Sema3a* caused a transient increase in kidney size and UB branching, while the addition of SEMA3A to organ cultures had the opposite effect. SEMA3A binds to Neuropilin-1 (NRP1), a shared coreceptor for VEGF, which facilitates their signaling through VEGFR1. Both SEMA3A and NRP1 are expressed in the UB, suggesting a possible autocrine signaling mechanism, in which SEMA3A competes with VEGF-A (a promoter of branching) for binding to NRP1 (Tufro et al., 2008). Another semaphorin that plays a similar role, but through a different mechanism, is SEMA4D, the ligand of Plexin B1. Excess

SEMA4D reduced UB branching, apparently by activating the Rho-ROCK pathway, while *Plexnb1* KO caused increased branching (Korostylev et al., 2008). Interestingly, while either *Sema3a* or *Plexnb1* KO increased early branching at E12.5 – E13.5, the effects were transient and the extent of branching was normal by E15.5 (Korostylev et al., 2008; Tufro et al., 2008). This contrasts with mutations in genes that *decrease* early UB branching, which generally cause renal hypoplasia at later stages. It suggests that some independent, organ-size control mechanism limits kidney growth, overcoming the early growth-stimulatory effects of *Sema3a* or *Plexnb1* KO to coordinate organ size with the body.

Patterning UB Branching

Branching morphogenesis occurs in many developing organs, and while there are certain universal themes (e.g., mesenchymal-epithelial interactions), each organ uses somewhat different cellular mechanisms and generates a characteristic branching pattern (Davies, 2002). For example, at the cellular level, vascular branching occurs by the formation of unicellular extensions that secondarily develop a lumen, while in the kidney the lumen is ever present (Meyer et al., 2004). In the mammary gland, the branches grow via multilayered terminal end buds (Lu et al., 2006), while in the kidney (except at early stages) the UB tips are composed of a single-layered epithelium (Chi et al., 2009). Salivary gland branching involves cleavage of the epithelium by extracellular matrix fibers (Sakai et al., 2003), while the UB generates epithelial outpouches that extend to form new branches (Davies, 2002).

At the organ level, the branching "pattern" is defined by several parameters, such as the site and type of branching (terminal versus lateral, bifurcation versus trifurcation), branch angles, rates of elongation, and changes in tubular diameter. All of these processes are controlled to yield an overall pattern unique to each organ. In the kidney, UB branching and growth must also be coordinated with nephron induction and morphogenesis. In the lungs, studies of in vivo branching have revealed a highly stereotyped pattern, with specific, genetically encoded branching modules that are employed to generate the characteristic shape of the organ (Metzger et al., 2008). It is not yet clear to what extent UB branching is stereotyped in vivo.

The ability to branch and elongate is an intrinsic property of the UB epithelium, which requires growth factors and ECM components but can occur in the absence of mesenchyme (Qiao et al., 1999). The mesenchyme, however, influences the pattern of branching. One striking example came from tissue recombination experiments in which lung mesenchyme induced UB branching with a pattern characteristic of lung epithelium (i.e., increased lateral branching) (Lin et al., 2001). Thus, one model is that the local expression of growth promoters and inhibitors, by cells surrounding different domains of the UB, determines this pattern. The MM surrounding the tips produces growth- and branch-promoting factors, while nephron epithelia and stroma surrounding the trunks may promote elongation and suppress branching (Sweeney et al., 2008).

Based on this model, and on the chemoattractive properties of GDNF (Tang et al., 1998, 2002), it was suggested that UB branching is caused by the attraction of UB tips toward local sources of GDNF (Sariola and Saarma, 2003). A similar model involving FGF10 has been proposed for lung branching (Weaver et al., 2000). The ability of the kidneys to develop in the compete absence of *Ret* or *Gdnf* when *Spry1* is also absent, revealed that other signals besides GDNF must be capable of inducing UB branching in vivo (Michos et al., 2010). However, the kidneys that developed in *Gdnf*$^{-/-}$; *Spry1*$^{-/-}$ mice had a distinctly abnormal pattern of UB branching (Michos et al., 2010). This was not due simply to the absence of *Spry1* (it is not seen in *Spry1*$^{-/-}$ kidneys) but instead suggests a specific role of *Gdnf* in branch patterning. The mechanism of this effect remains unclear, and better methods to manipulate the spatial patterns of gene expression in developing kidneys are needed to address this issue.

Signaling Downstream of Ret

How does signaling by Ret and other RTKs induce UB growth and branching? Ret activates the Ras/Erk MAP kinase, as well as the PLCγ-Ca$^+$ and PI3K-Akt signaling pathways (reviewed by Takahashi [2001]), all of which are important for normal kidney development (Fisher et al., 2001; Jain et al., 2006; Tang et al., 2002; Wong et al., 2005). These lead to changes in gene expression that strongly influence UB morphogenesis. For example, in two feed-forward loops, Ret upregulates its own expression and Wnt11 in the UB tips (Pepicelli et al., 1997); Wnt11 then signals to the MM to upregulate GDNF (Majumdar et al., 2003). The upregulation of *Spry1* by Ret forms a critical negative feedback loop (Basson et al., 2005). Additional genes regulated by GDNF/Ret were identified in a microarray screen using isolated UB cultures (Lu et al., 2009), including the chemokine receptor *Cxcr4*, the cytokine *Crlf1*, the signaling inhibitors *Dusp6* and *Spred2*, and the transcription factors *Myb*, *Etv4* and *Etv5*. While the importance of many of these genes remains to be investigated, *Etv4* and *Etv5* were found to play a critical role downstream of Ret, and perhaps other RTKs, such as FGFRs and Met.

ETS transcription factors often act as mediators of RTK signals in metazoans. *Etv4* and *Etv5* are two closely related ETS factors, which are important in neuronal development, spermatogenesis, and limb development, downstream of GDNF, FGFs, or HGF (Chen et al., 2005; Helmbacher et al., 2003; Livet et al., 2002; Mao et al., 2009). In kidney, *Etv4* and *Etv5* are coexpressed in the UB tips, where they are upregulated by GNDF/Ret signaling via PI3K, as well as in the MM. Individual KO of *Etv4* or *Etv5* had little effect on kidney development, but double mutants were severely affected, revealing a redundant role for these two genes. *Etv4*$^{-/-}$; *Etv5*$^{+/-}$ mice had either renal agenesis or hypodysplasia (with severe branching defects), while double homozygotes never developed kidneys (Lu et al., 2009).

While the full set of *Etv4*/*Etv5* target genes remains to be defined, several downstream genes (either direct or indirect targets) were identified, including *Myb*, *Cxcr4*, *Met*, and *Mmp14*. Expression of these four genes was greatly reduced in *Etv4*/*Etv5* mutants as well as in *Ret*-hypomorphic mutant kidneys, suggesting that they are normally upregulated by GDNF/Ret signaling via *Etv4*/*Etv5* (Lu et al., 2009). Expression of MET is important for the ability of HGF to regulate UB branching (Liu et al., 2009). MMP14 is a matrix metalloproteinase, which may promote branching morphogenesis by remodeling the ECM, by releasing bound growth factors, or by a newly discovered autocrine signaling mechanism that promotes cell migration (Mori et al., 2009). MMP14 has been implicated in UB branching in culture (Kanwar et al., 1999; Meyer et al., 2004), and

Mmp14 −/− mice have UB branching defects (R. Zent and K. Riggins, personal communication).

Thus, an evolutionary conserved gene network involving RTKs and their downstream ETS transcription factors promotes and controls renal branching morphogenesis (Figure 2B).

Differentiation of the UB into Tip and Trunk Domains
As the kidney develops, there is an increasing degree of differentiation from the most proximal UB epithelium (tip) to the more distal (trunk). This may be a consequence of the way the UB grows (Figure 3B), where tip cells serve as progenitors of the entire epithelium (Shakya et al., 2005) (P. Riccio and F.C.,unpublished data). Differentiation of trunk from tip cells is evident in the spatial patterns of gene expression (Caruana et al., 2006; Lu et al., 2009; Schmidt-Ott et al., 2005). Many tip-specific genes are involved in growth and branching, whereas many trunk-specific genes have specific functions in the mature CD, e.g., as ion channels.

Little is known about the cues that trigger CD cell differentiation. It is likely that the surrounding MM, stroma, and nephron epithelia all have an influence—but even the isolated UB, growing in Matrigel without other cell types, becomes differentiated into tip and trunk domains (Sakurai et al., 2005). Therefore, lateral signaling within the UB epithelium may be involved; for example, the tips could secrete long-range signals that promote differentiation and short-range signals that prevent it. Alternatively, this process could be explained by a stochastic model, in which a random subset of tip cells are marked for differentiation, then excluded from the growing tip and left behind in the trunk.

One diffusible factor that promotes UB differentiation is heregulin (HRG)α, which signals through erbB RTKs, leading to the downregulation of tip markers and the upregulation of trunk markers, such as the water channel Aquaporin-2 and urea transporter Slc14a2 (Sakurai et al., 2005). On the other hand, the Wnt/β-catenin pathway is important to maintain UB cells in the undifferentiated state. Deletion of *Ctnnb1* in the ND/UB lineage (or culture with the Wnt antagonist Dkk) caused the premature expression, in the ND and early UB tips, of proteins normally expressed only in the more mature distal CDs (Bridgewater et al., 2008; Iglesias et al., 2007; Marose et al., 2008), including the zona occludens (ZO) protein ZO-1 and Aquaporin-3. Although *Ctnnb1* mutant cells fail to express *Ret* (Bridgewater et al., 2008; Marose et al., 2008), this does not appear to be the cause of premature differentiation, as *Ret* −/− NDs do not express ZO-1 (Grote et al., 2008). However, two genes that might be required to suppress differentiation are the transcription factors *Sox9* and *Emx2*, whose expression requires β-catenin (Bridgewater et al., 2008; Marose et al., 2008).

Establishment of Specialized Cell Types within the Collecting System
The mature CDs connect the cortically located distal nephron segments to the ureter and consist of at least two functionally distinct cell types that are required for normal acid-base homeostasis and water and electrolyte balance. These include the α and β intercalated cells (ICs), which regulate pH homeostasis by secretion of H$^+$ or HCO$_3^-$ into the urine, and the more numerous principal cells (PCs), which concentrate the urine by absorbing water and regulating Na$^+$ homeostasis. These functionally distinct cell types are intermingled along the entire CD system with a gradual increase in the PC:IC ratio toward the medulla. During differentiation, CD cells first express markers of both PCs (aquaporin2; *Aqp2*) and ICs (carbonic anhydrase II; *Car2*) (Blomqvist et al., 2004). Further differentiation requires the forkhead transcription factor Foxi1, as *Foxi1* −/− mice develop distal renal tubular acidosis (dRTA) due to the absence of IC (Blomqvist et al., 2004).

Recent studies have identified parallels between the differentiation process of IC/PC in the mouse CD (Jeong et al., 2009), the ciliated versus Clara cells in the lung (Morimoto et al., 2010; Tsao et al., 2009), and the multicillated versus absorptive cells in the zebrafish pronephros (Liu et al., 2007; Ma and Jiang, 2007). In all three systems, Notch signaling restricts the numbers of the minority cell population. In the lung and the pronephros, loss of Notch signaling results in expansion of ciliated (lung) or multiciliated cells (pronephros) at the expense of secretory Clara cells or absorptive pronephros cells. In the mouse CDs, inactivation of the E3 ubiquitin ligase Mind-bomb1, a protein required in the ligand-expressing cells for the efficient activation of Notch, resulted in increased numbers of ICs and reduced numbers of PCs. The PC deficiency in *Mib1*-deficient CDs is functionally similar to the loss of *Aqp2* function (McDill et al., 2006), resulting in an inadequate resorption of water and sodium, reminiscent of nephrogenic diabetes insipidus (NDI), and leading to progressive hydronephrosis (Jeong et al., 2009). Whereas Notch signaling-deficient lungs suffer complete loss of Clara cells, loss of *Mib1* in the CDs affects the PC:IC ratio, but both cell types form in all regions of the CDs. This may reflect compensation for the loss of *Mib1* by upregulation of *Mib2* (Jeong et al., 2009), or alternatively Notch signaling is redundant with another mechanism used to suppress *Foxi1* expression in bipotential progenitors. The character of CD cells expressing Notch receptors and the timing of Notch activation during PC specification remains unresolved.

The future will likely reveal that Wnt, TGFβ, and Hedgehog pathways also impact patterning of cell fates within the CDs. In this regard, conditional inactivation of *Wnt7b* within the CD results in a phenotype superficially similar to Mib1 inactivation: an inability to concentrate urine and sometimes hydronephrosis (Yu et al., 2009). The primary reason ascribed for this phenotype was that without *Wnt7b,* OCD was randomized, failing to elongate the medullary CDs (as discussed above), and secondarily producing an insufficient number of principle cells. However; a quantitative analysis of the PC to IC ratio was not performed. Paradoxically, in the mature CD, the most common adverse effect of lithium, a GSK3β inhibitor widely used to treat bipolar disorder, is NDI (Grunfeld and Rossier, 2009). In rodent models, lithium-induced NDI is accompanied by a dramatic reduction in the PC:IC ratio, which is thought to be a physiologic adaptation to the initial improper IC function (Christensen et al., 2004). A recent proteomic analysis of inner medullary CD cells in lithium-treated rats confirmed that lithium resulted in increased cytosolic β-catenin, mimicking an activated state of the Wnt pathway and consistent with a role for Wnt in this fate selection. Several studies reveal that the mature CD remodels its population in response to metabolic changes. It will be of interest to know whether the same signaling pathways that initially drive

PC versus IC differentiation, such as Notch, are involved in these physiologic responses.

Origins of the Metanephric Mesenchyme from the IM

As mentioned in the Introduction, the ventral IM remains organized in a loose mesenchymal structure (the "nephrogenic cord"), which will give rise to all nephrons (both meso- and metanephric). The nephrogenic cord expresses *Rarb*, *Osr1*, and, in the posterior, *Hox11* paralogs, whereas the ND expresses *Hoxb7*. The earliest known marker of the IM progenitor population is *Osr1,* and genetic fate mapping studies with *Osr1-CreERT2* illustrate clearly the progressive fate restriction occurring as progenitors are specified along the rostro-caudal axis. Whereas nearly all the cell types in the developing ureter and kidney, including UB, nephron, stromal, endothelial, mesangial, and smooth muscle cells, derive from progenitor cells that expressed *Osr1* before E9.5, hormone pulses given between E9.5 and E11 no longer label the UB or endothelial cells but continue to label the MM, pericytes, mesangium, kidney capsule, and interstitial stroma populations (Mugford et al., 2008), as well as a fraction of smooth muscle cells. Around E11, after the UB invades the MM, *Osr1* expression becomes restricted to the MM population, which will only contribute descendants to the nephron epithelia (Mugford et al., 2008).

Importantly, although *Osr1*-expressing cells give rise to both interstitial and epithelial lineages, individual *Osr1*-positive cells produced either epithelial (Pax2/Six2$^+$) or interstitial (Foxd1$^+$) progenitors but never both, indicating that these two lineages separate early in IM development (Mugford et al., 2008). Genetic fate mapping in the mouse seems to indicate a common origin for both stromal and nephron progenitors from an *Osr1*$^+$ precursor born before E9.5. However, *Osr1* is also expressed in lateral plate mesoderm. It thus remains unclear if a single early *Osr1*$^+$ progenitor begets both mesodermal populations or if two distinct and unrelated *Osr1*$^+$ progenitors exist. The latter model is supported by direct cell tagging experiments in chick embryos, which are particularly amenable to this type of fate mapping (Guillaume et al., 2009). Lineage mapping studies show that some renal stromal cells originate in the paraxial (presomitic) mesoderm and not in the IM (Guillaume et al., 2009). This raises the possibility that while nephron progenitors have a common origin in an *Osr1*$^+$ cell in several species, the progenitors of the stromal cells could arise from precursors residing in the IM and also in the paraxial mesoderm.

At the transcriptional level, *Osr1* is required within MM progenitors to induce/maintain the expression of *Eya1* and *Pax2* (James et al., 2006; Mugford et al., 2008). Hox11 paralogs form a complex with Eya1 and Pax2 to induce/maintain the expression of the homeobox-containing gene *Six2* and *Gdnf* (Gong et al., 2007; Wellik et al., 2002). In addition to these factors, the transcription factor *Foxd1 (Bf-2)* is expressed in IM cells that do not express *Six2* and *Pax2*. In the absence of *Foxd1,* nephrons form but the stroma is severely depleted (Hatini et al., 1996), suggesting that *Foxd1* marks a progenitor population distinct from the nephron progenitor. LacZ insertion into the *Foxd1* locus confirmed that *Foxd1* expression was restricted to the stroma and the renal capsule (Levinson et al., 2005). Loss of stroma impaired UB branching and nephron elongation (Hatini et al., 1996), possibly due to loss of the *Wnt7b*-dependent signal secreted by the stroma (Yu et al., 2009). In addition, *Foxd1*-deleted renal capsule secretes BMP4, which interferes with branching and with nephrogenesis (Levinson et al., 2005).

The MM cells form a cap around the UB tip. Based on gene expression studies, the cap mesenchyme can be subdivided into the "capping mesenchyme" (Six2$^+$, *Cited1*$^+$, *Wnt4*$^-$) and the "induced mesenchyme" (*Six2*$^+$, *Cited1*$^-$, *Wnt4*$^+$) (Mugford et al., 2009) (Figure 4). The capping mesenchyme seems to contain the stem cell population for the entire nephron, which first expands and than becomes depleted as kidney development nears completion.

Maintenance of the Nephron Progenitor Population

Fate mapping studies using hormone-inducible Cre targeted to either the *Six2* or the *Cited1* locus confirmed that *Six2*$^+$, *Cited1*$^+$ cells give rise only to renal epithelial (nephron) cells, but never to any nonepithelial lineages within the kidney. This is consistent with *Six2/Cited* expression marking the committed progenitor pool of the renal epithelium (Boyle et al., 2008; Kobayashi et al., 2008). Therefore, *Six2* and *Foxd1* define two committed progenitor populations within the MM, which together contribute to all renal lineages other than the UB, endothelial, and smooth muscle cells (Dressler, 2009). These studies also demonstrated that the nephron progenitor population expands while retaining its muiltipotency, surviving long enough to allow the formation of ~13,000 nephrons in the mouse and up to ~1,500,000 in humans. To generate this large number of nephrons, *Cited1*$^+$, *Six2*$^+$ cells undergo at least four rounds of replicative divisions from E11 to birth in the mouse to expand ~16 fold (Kobayashi et al., 2008). Chimera analysis indicated that although *Six2*$^{-/-}$ cells could contribute to all cell fates within the nephron, they could not self-renew (Kobayashi et al., 2008). Unlike *Six2*, *Cited1* and other markers of epithelial progenitors (Boyle et al., 2008) are not required for progenitor maintenance (Boyle et al., 2007), consistent with a specific role for *Six2* in maintaining the "stemness" of the progenitor population.

How stemness is maintained in the MM, and why it is lost after birth, is an important question with great implications to the clinic. Maintenance of the progenitors requires autonomous expression of WT1 (Hartwig et al., 2010) and Sall1 (Nishinakamura and Osafune, 2006), the receptors FGFR1 and 2 (Poladia et al., 2006), and sources of FGF8 (Grieshammer et al., 2005; Perantoni et al., 2005) and BMP7 (Dudley et al., 1999; Kazama et al., 2008; Luo et al., 1995). In the absence of WT1, BMP7, or FGF signaling, the MM cells undergo apoptosis and at most, only a few nephrons form. *Sall1*, a putative BMP target, can allow a single cell to produce multiple nephron lineages in a clonogenic assay when overexpressed, indicating a function in maintaining stem cell identity. Accordingly, in the absence of *Sall1*, MM is formed but UB induction fails, perhaps due to a role together with Hox genes in GDNF maintenance (Kawakami et al., 2009; Nishinakamura, 2003). Wnt signaling may also play some role in stem cell maintenance, since *Wnt4* null kidneys exhaust the MM rapidly, forming only a few renal vesicles (Kispert et al., 1998).

Although cells residing in the induced mesenchyme (*Six2*$^+$, *Cited1*-, *Wnt4*$^+$) have begun to respond to Wnt9b by elevating *Wnt4*, it remains unclear whether or not this reflects a loss of stemness. In contrast, the capping mesenchyme (CM) cells
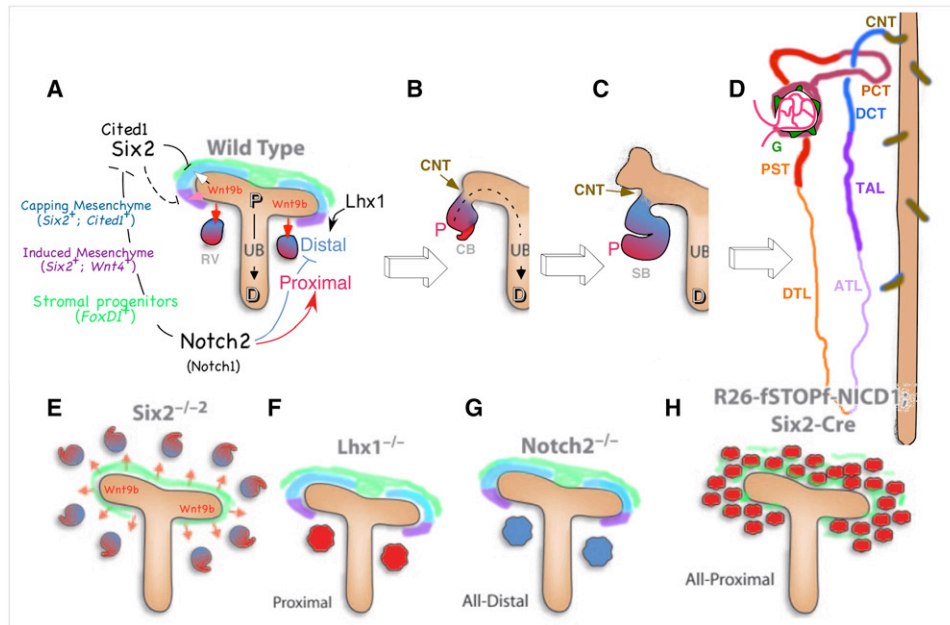
Figure 4. Molecules Involved in Maintaining the Nephron Progenitor Pool, Inducing and Polarizing the Nephron

(A) The MM contains at least two populations of progenitors, the capping mesenchyme (CM; in which the cells express *Six2* and *Cited1* and are resistant to Wnt9b) and induced mesenchyme (in which the cells lose *Cited1* and begin to respond to Wnt 9b by expressing *Lef1* and *Wnt4*). After MET, the nascent epithelial renal vesicles (RV) form a proximo-distal axis, where the surface facing the cortex is distal (blue) and the medullary surface is proximal (red). Note that this axis appears inverted relative to the UB axis: the RV distal end is near to the UB proximal tip (P), and the UB distal end is the ureter.

(B) The distal cells of the comma-shaped body (CB) invade the proximal tip of the UB and fuse to form one continuous P/D axis (dashed gray arrow).

(C) The proximal segment of the S-shaped body (SB) is thought to give rise to the future glomerulus and proximal tubule.

(D) The mature nephron is further subdivided to glomerulus (G), proximal convoluted tubule (PCT), the straight segment of the proximal tubule (PST or S3), and the loop of Henle with a descending thin limb (DTL) and ascending thin limb (ATL). The thick ascending limb (TAL) connects to the distal convoluted tubule (DCT), which connects to the collecting duct via a short connecting tubule (CNT). The identity of molecules and the processes involved in subdividing the nephron is not yet known.

Maintenance of progenitors and RV polarization:

(E) Loss of *Six2* leads to premature differentiation of renal epithelia, indicating that *Six2* acts to antagonize the Wnt9b signal within the MM (A).

(F) The *Lhx1* gene is required to secure the distal identity of the CB; in its absence, the remaining cells will acquire the most proximal fate of the Bowman's capsule.

(G) *Notch2* is required for maintaining the proximal identities (in its absence, the remaining cells acquire a distal identity and fuse with the UB).

(H) Constitutive activation of Notch1 (which makes a small contribution to maintaining proximal fates) converts the entire MM into loosely organized cluster of proximal tubule-like epithelia lacking polarity (compare (E) with (H)), suggesting that Notch activity can suppress both the distal most and proximal most (podocyte, Bowman's capsule) nephron fates and either augment the Wnt signal or act independent of it to induce the epithelialization of the entire MM. Diagrams adapted from Kopan et al. (2007).

($Six2^+$, $Cited1^+$, $Wnt4$-) do not express *Wnt4* and proved resistant to ectopic activation of β-catenin in organ culture (Mugford et al., 2009). Thus, the CM may contain the "true" stem cells, and *Cited1* may collaborate with *Six2*, helping it to resist the *Wnt9b* signal within the cap mesenchyme. This activity of *Cited1* may be mediated by its ability to enhance BMP signals at the expense of Wnt responses (Mugford et al., 2009). However, since *Cited1* can be removed without loss of progenitors (Boyle et al., 2008), the mechanism involved in maintaining stemness in the CM remains an open question.

In principle, a self-sustaining stem cell-like population could remain in the adult kidney. However, *Six2* expression disappears shortly after birth in the mouse with the subsequent conversion of the remaining MM cell into nephrons (Hartman et al., 2007). Why these cells are not maintained after formation of a species-appropriate number of nephrons, and what cells replace them in the adult, remains a mystery. The kidney, however, has many more nephrons than necessary for survival (as evident from live organ donation), and therefore retention of stem cells may have not been strongly selected. Instead, adult renal epithe-

lial cells retain the ability to dedifferentiate and repair the kidney following injury (Humphreys et al., 2008).

Forced *Osr1* expression can prevent MET in the chick (James et al., 2006), most likely by maintaining *Six2* expression (see below) or by assisting Six2$^+$ cells to resist Wnt signals. To allow the transition from strict self-renewal in the CM to an induction-permissive state, *Cited1* (or a yet to be identified factor) needs to be turned off. To undergo MET and fully respond to induction, both *Osr1* and *Six2* expression need to be turned off. How *Cited1*, *Six2*, and *Osr1* expression is extinguished at the appropriate interface is not known.

The Earliest Steps of Specification: Renal Vesicle Polarization, Establishment of Distal Fates, and Fusion to the Duct

As noted above, after the Six2-expressing MM cells extinguish *Cited1*, they begin to stabilize β-catenin in response to Wnt9b, which can instruct MM cells to undergo MET. Nascent tubular structures normally appear beneath the UB tips, while the MM above the tips remains undifferentiated (Saxen, 1987). Mice

deficient in *Wnt9b* fail to undergo MET (Carroll et al., 2005), whereas mice deficient in *Six2* undergo exuberant MET: they form renal vesicle (RV)-like epithelial aggregates above and below the UB branches (Figure 4E) (Self et al., 2006). Expression of a stabilized β-catenin can also induce ectopic RV markers within *Six2* expressing cells that would normally remain undifferentiated (Park et al., 2007). Since mice that are simultaneously deficient for both *Six2* and *Wnt9b* cannot undergo MET (Kobayashi et al., 2008), Six2 activity in cells within the capping mesenchyme most likely blocks MET by antagonizing the β-catenin stabilizing effects of Wnt9b and thus maintaining the progenitor cell population (Carroll et al., 2005; Kispert et al., 1998; Kobayashi et al., 2008; Kuure et al., 2007; Majumdar et al., 2003; Park et al., 2007; Self et al., 2006).

Regional specification within the nephron, however, is not predetermined in these founder cells, as fate mapping of *Six2*- or *Cited1*-expressing cells conducted at low tamoxifen concentrations indicates that individual Six2+ or Cited1+ cells contribute descendants to all structures along the entire axis of the nephron (Boyle et al., 2008; Kobayashi et al., 2008). How are these regional identities acquired?

Proximal-distal polarity in the RV appears to be dependent on signals, perhaps a Wnt gradient, emanating from the UB (Kopan et al., 2007). A polarized response to Wnt is first seen in the induced mesenchyme in the polarized distribution of two *Wnt* responsive genes, *Wnt4* and *Lef1* (Mugford et al., 2009). Once formed, the polarization axis of the RV along the proximal/distal (or P/D) axis remains, in addition to a cellular apical-basal polarity that leads to formation of a lumen (Figures 4A–4C). At the molecular level, recent observations identified nearly 100 genes with polarized P/D expression within the pretubular aggregate (Mugford et al., 2009) and the RV (Brunskill et al., 2008; Georgas et al., 2009). Within the newly formed RV, the distal domain is defined by the restricted expression of many genes, including *Lhx1* and its transcriptional target, the Notch ligand *Dll1* (Kobayashi et al., 2005), the POU domain-containing transcription factor *Brn1* (Nakai et al., 2003), *Dkk1, Jag1,* and *Bmp2* (Georgas et al., 2009). The proximal pole is defined by elevated *Tmem100* and *Wt1* expression (Georgas et al., 2008, 2009). Although *Brn1*−/− kidneys develop a normal number of mature glomeruli and proximal tubules, a dramatic reduction is seen in the length and number of the mature loop of Henle segments, macula densa (the region secreting renin) and distal convoluted tubules (Figure 4D). As a consequence, nephron development is arrested at a primitive stage. *Brn1* is thus acting downstream of *Lhx1* to specify the distal domain within the RV. Although proximal development proceeded independently of distal development, differentiation of distal derivatives—the loop of Henle, the macula densa, and the distal convoluted tubule—required *Brn1* (Kobayashi et al., 2005; Nakai et al., 2003).

Using detailed marker analysis and 3D reconstructions, Georgas et al. (2009) uncovered evidence that cells located at the distal end of the RV penetrate and fuse with the UB to form the "connecting segment." These new findings demonstrate a more advanced differentiation state of distal RV cells relative to their siblings at the proximal end. Accordingly, many more markers are expressed in the distal RV relative to the proximal RV (Georgas et al., 2009). Polarity becomes apparent morphologically as the RV becomes the comma-shaped body; these stereotypical structures were so named because the first cells to elongate, change shape, and form a "slit" are located at the proximal end (Saxen, 1987). The mirror image symmetry of the nascent comma-shaped body is another indication of organizing signals emanating from the UB. Since Wnt9b (and its target, *Wnt4* [see below]) can induce *Lhx1* expression, it is safe to assume that, as in the fly imaginal disc, an early Wnt9b gradient provides the polarizing signal; cells near the source (receiving relatively higher levels of signal) will express *Lhx1*, whereas cells further away (receiving lower levels of signal) will be the first to elongate and acquire proximal fates. Importantly, fate mapping confirms that, like *Six2*−/− cells, *Wnt4*−/− cells can only contribute to the RV and its subsequent structures but can no longer contribute to the progenitor pool (Kobayashi et al., 2008; Shan et al., 2009). Interestingly, Wnt4 and Wnt9b are not interchangeable: both factors are capable of inducing RV formation and tubulogenesis in isolated MM, but Wnt9b-expressing cells cannot induce differentiation in *Wnt4*−/− MM, whereas Wnt4 can induce RV formation and tubulogenesis in competent *Wnt9b*−/− mesenchyme (Carroll et al., 2005). What are the special properties of Wnt4, and why must activated β-catenin be downregulated to fully rescue epithelialization (Park et al., 2007)? These questions are subject to ongoing investigation.

Establishment of Proximal Cell Fates along the Nephron by Notch Signaling

Conditional inactivation of *Lhx1* in the MM results in the formation of RVs that express *Wnt4*, *Pax8* and *Fgf8* but lack the distal-specifying factor *Brn1* and the Notch ligand *Dll1*. The failure of *Lhx1*−/− RVs to regionalize along the P/D axis arrests nephron development at this stage and no S-shaped bodies (SB) are formed (Figure 4F) (Kobayashi et al., 2005). In chimera experiments, *Lhx1*−/− embryonic stem cells can initially contribute to the entire RV, but in the mature nephron, *Lhx1*−/− cells can only form the Bowman's capsule and podocytes, the most proximal cells in the nephron (Kobayashi et al., 2005). This indicates that *Lhx1* plays a role not only in regulating distal fate but also in establishing the midproximal region (the proximal tubule). Because *Lhx1* activates *Dll1*, it may set in motion a Notch-dependent process that determines proximal tubule (PT) and podocyte identities (Cheng et al., 2007). In accord with this view, Cheng et al. (2007) observed that *Dll1* hypomorphic animals lose proximal segments and display a severe reduction in nephron numbers. *Dll1* is thus the first Notch ligand to act in the RV; *Lhx1* acts downstream of *Wnt* signaling during mesenchymal epithelial transition and upstream of *Brn1* (inducing distal fates [Nakai et al., 2003]) and Notch (inducing proximal fates, below) in nephron segmentation.

A pharmacological block of γ-secretase applied during metanephric development (Cheng et al., 2003) defined a window during which Notch activity is required for the fixation of a proximal cell identity. These cells are subdivided further into podocyte precursors (that will lose Notch activity, perhaps due to an increase in the Notch antagonist COUP-TFII [Suh et al., 2006]) and proximal tubule precursors that depend on Notch activation. Interestingly, γ-secretase inhibition applied to the SB cannot prevent the emergence of molecularly recognizable proximal cell types (Cheng et al., 2007; Kopan et al., 2007), suggesting that these identities were fixed by the time the SB is visible.

Notch activation involves γ-secretase-mediated proteolysis and generation of a neo-epitopes, some recognizable by antibodies. These antibodies identified cleaved Notch1 within the RV, indicating that Notch1 is activated early in nephrogenesis. However, genetic analysis indicated that Notch2 provides most of the needed signal (Cheng et al., 2007; Kopan et al., 2007), whereas Notch1 contributions are only revealed when the levels of Notch2 are severally compromised (Surendran et al., 2010a).

Given that *Lhx1*, *Dll1* and *Notch1* are properly expressed in *Notch2* deficient RVs, why does the RV fail to segment? Forced expression of activated Notch1 within the *Six2* expression domain resulted in complete conversion of the MM to immature podocytes and LTL-binding, *Cdh6* expressing, PT-like structures at the expense of all other markers (Figure 4H). This result suggests that Notch1 *can* promote PT fates. Why, in the *Notch2* mutant, Notch1 activity is too low to compensate for the loss of Notch2 is unclear, but one clinical consequence of this shortcoming is Alagille syndrome in which either one allele of the ligand *jagged1* (*JAG1*) is mutated (ALGS1), or one allele of *NOTCH2* is mutated (ALGS2). It was recently demonstrated that lowering the activity of Notch1 inhibitors such as MINT may help treat this syndrome (Surendran et al., 2010a).

In the absence of *Notch2*, the initial separation of the RV into Wt1^{+} and Pax2^{+} domains occurs, but is halted and reversed due to the ineffectiveness of Notch1 (Figure 4G) (Cheng et al., 2007). Critical evidence to support an exclusively cell-autonomous role for Notch in specifying the PT and podocyte is lacking however. Only lineage studies will determine the developmental potential of SB cells under normal circumstances.

After the proximal nephron forms, podocytes function normally in the absence of Notch (Surendran et al., 2010b). Whereas Notch signaling blockade may assist in preventing glomerulosclerosis (Niranjan et al., 2008), Notch1 and 2 cell autonomously regulate proximal tubular diameter by ensuring that the division plane is perpendicular to the basement membrane (Surendran et al., 2010b). In their absence, proximal tubule cysts form, which can progress to form papillary renal cell carcinoma-like structures (Surendran et al., 2010b). Wnt9b (Karner et al., 2009) and Wnt7 (Yu et al., 2009) also control proximal tubule diameter and loop of Henle elongation, respectively.

Concluding Remarks
As this and other recent reviews (Dressler, 2009) describe, considerable progress occurred in recent years in identifying the genes that regulate the morphogenesis and differentiation of the nephron and collecting system of the kidney. The invaluable information from gene knockouts, which first moved the field of kidney development from the morphological to the molecular level, has been greatly augmented by the application of new genetic tools, such as conditional/tissue-specific knockouts, genetic lineage tracing, chimeric analysis, and fluorescent in vivo markers. Further advances in genetic manipulation are needed to allow modifications of specific, disease-relevant, or developmentally distinct groups of cells; for example, a specific region of a branching UB or specific mesenchymal populations. Data on spatial and temporal gene expression patterns are accumulating on a large scale and at a rapid pace (Brunskill et al., 2008; McMahon et al., 2008) (http://www.gudmap.org; http://www.euregene.org; http://www.genepaint.org), and several

gene regulatory networks controlling different aspects of kidney development are beginning to be defined. While organ culture and imaging techniques have contributed greatly to recent advances, improved culture methods are needed to allow later stages of kidney development to be examined and manipulated, to preserve the 3D structure of earlier organs, and ultimately to allow renal filtration and physiology to be reconstituted in vitro. At higher resolution, progress in imaging methods, including in vivo reporters of cell signaling and other intracellular processes, seem to be forthcoming at a rapid rate and should have many applications to this field. Methods for culturing isolated components such as the ND, UB, or nephron are giving hope that strategies to reconstitute the kidney from its separate components could be developed. The introduction of exogenous cells (e.g., stem cells), an approach that could be extremely informative, promises also to have important applications in regenerative medicine. Finally, a computation-driven systems approach, a field that is gradually emerging as large data sets are collected, will be needed to interpret and synthesize all this information (Brunskill et al., 2008; Choi et al., 2009; Tsigelny et al., 2008).

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