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Ret-Dependent Cell Rearrangements in the Wolffian Duct Epithelium Initiate Ureteric Bud Morphogenesis

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SUMMARY

While the genetic control of renal branching morphogenesis has been extensively described, the cellular basis of this process remains obscure. GDNF/RET signaling is required for ureter and kidney development, and cells lacking *Ret* are excluded from the tips of the branching ureteric bud in chimeric kidneys. Here, we find that this exclusion results from earlier *Ret*-dependent cell rearrangements in the caudal Wolffian duct, which generate a specialized epithelial domain that later emerges as the tip of the primary ureteric bud. By juxtaposing cells with elevated or reduced RET activity, we find that Wolffian duct cells compete, based on RET signaling levels, to contribute to this domain. At the same time, the caudal Wolffian duct transiently converts from a simple to a pseudostratified epithelium, a process that does not require *Ret*. Thus, both *Ret*-dependent cell movements and *Ret*-independent changes in the Wolffian duct epithelium contribute to ureteric bud formation.

INTRODUCTION

Development of the kidney is initiated when the caudal region of the Wolffian duct (WD), an epithelial tube derived from intermediate mesoderm, swells and then buds out into the adjacent metanephric mesenchyme (MM). This evagination, the ureteric bud (UB), then undergoes a complex process of branching morphogenesis to give rise to the renal collecting duct system, while it induces MM cells to form the nephron epithelia. UB growth and branching are critical for normal urogenital development and their failure leads to birth defects such as renal agenesis, hypoplasia, or congenital obstructive uropathy (Costantini, 2006; Dressler, 2006; Schedl, 2007; Shah et al., 2004). While many signals and receptors that control these events have been identified, little is known about the specific responses of WD or UB cells to these signals and how they lead to epithelial morphogenesis. Similar questions remain in other organs that develop through branching morphogenesis (Davies, 2002; Sternlicht et al., 2006). Studies in simpler organisms have yielded considerable insight into the cellular events that shape epithelial structures (Affolter et al., 2003; Pilot and Lecuit, 2005), and advances in genetic manipulation and imaging now make it feasible to address these questions in the context of mammalian organogenesis.

A key growth factor that promotes UB outgrowth and branching morphogenesis is GDNF, which is expressed by the MM and signals to WD and UB epithelial cells expressing the RET receptor tyrosine kinase and coreceptor GFRα1. In developing kidneys, *Ret* and *Gfrα1* are expressed at the UB tips, a specialized domain where most of the cell proliferation and epithelial growth and branching occurs (Lin et al., 2001; Michael and Davies, 2004; Watanabe and Costantini, 2004), but not in the more differentiated UB "trunks." In mice lacking *Ret*, *Gdnf*, or *Gfrα1*, the UB fails to form, leading to renal agenesis, or else it grows and branches very poorly, leading to severe renal hypodysplasia (reviewed in Costantini and Shakya, 2006). Humans with renal agenesis also have a high frequency of *RET* mutations (Skinner et al., 2008). Additional mouse genetic studies revealed that *Ret* and *Gdnf* are central components of a regulatory network that controls UB morphogenesis and kidney development (Schedl, 2007).

Specifically how GDNF/RET signaling alters the behavior of Wolffian duct and UB cells to promote branching remains unclear. To investigate this issue, we previously developed an experimental system in which to analyze the fate of $Ret^{-/-}$ cells in the developing kidney (Shakya et al., 2005). $Ret^{-/-}$ ES cells carrying the WD/UB transgenic marker *Hoxb7/GFP* were injected into wild-type (WT) blastocysts and their contribution to the resulting chimeric kidneys was examined. When the UB first grew out from the WD, the mutant cells contributed extensively to the WD and the UB trunk but were largely absent from the tip. At later stages of branching, $Ret^{-/-}$ cells

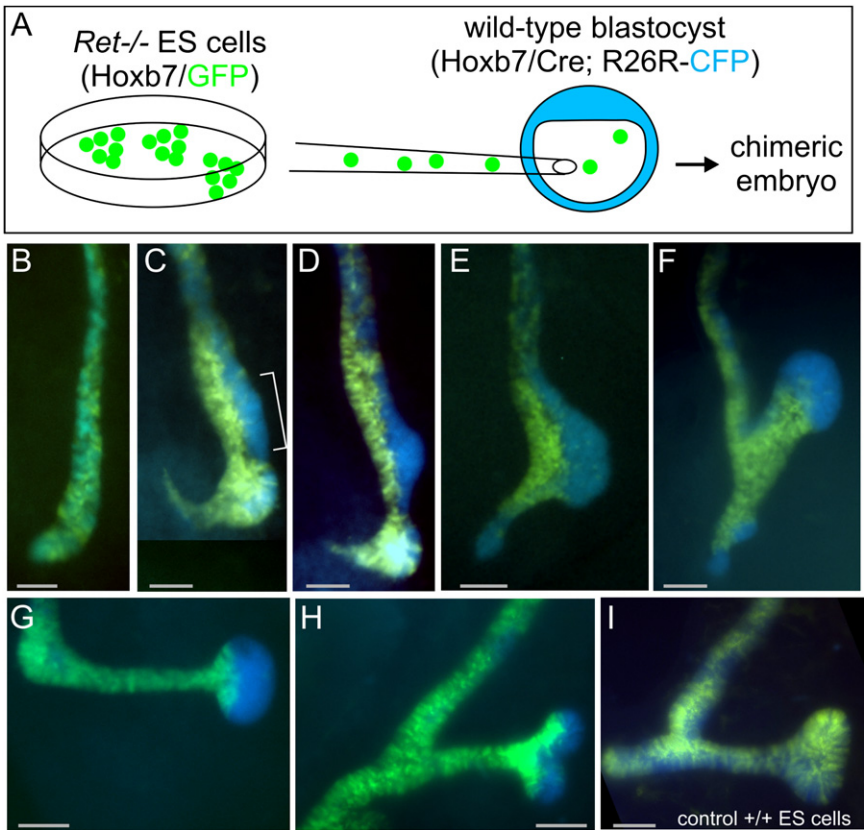


Figure 1. Behavior of WT and *Ret*$^{-/-}$ Cells during the Formation, Outgrowth, and Initial Branching of the UB

(A) *Ret*$^{-/-}$ ES cells carrying Hoxb7/GFP were injected into WT blastocysts, which were generated by crossing *Hoxb7/Cre* (Yu et al., 2002) and *R26R-CFP* mice (Srinivas et al., 2001). Therefore, WD/ UB cells derived from the *Ret*$^{-/-}$ ES cells expressed GFP, while those derived from the WT host expressed CFP. Dissected urogenital regions were examined in whole mount.

(B) A chimeric WD at ~E9.5, before the beginning of UB formation. GFP^{+} and CFP^{+} cells appear randomly distributed.

(C) At ~E10.0, the dorsal side of the caudal WD, where the UB will later emerge, becomes highly enriched for WT (CFP^{+}) cells (bracket).

(D–E) As the UB emerges (~E10.5), it is composed almost entirely of WT cells.

(F) As the UB elongates (~E11.0), WT cells generate the tip, while mutant cells follow behind and contribute to the trunk.

(G–H) During the first UB branching event (~E11.5), mutant cells contribute to the proximal sides of the branches, while only WT cells form the tips.

(I) Control chimeric UB generated using WT (*Ret*$^{+/+}$) ES cells, which contribute uniformly.

Scale bars: 100 μM.

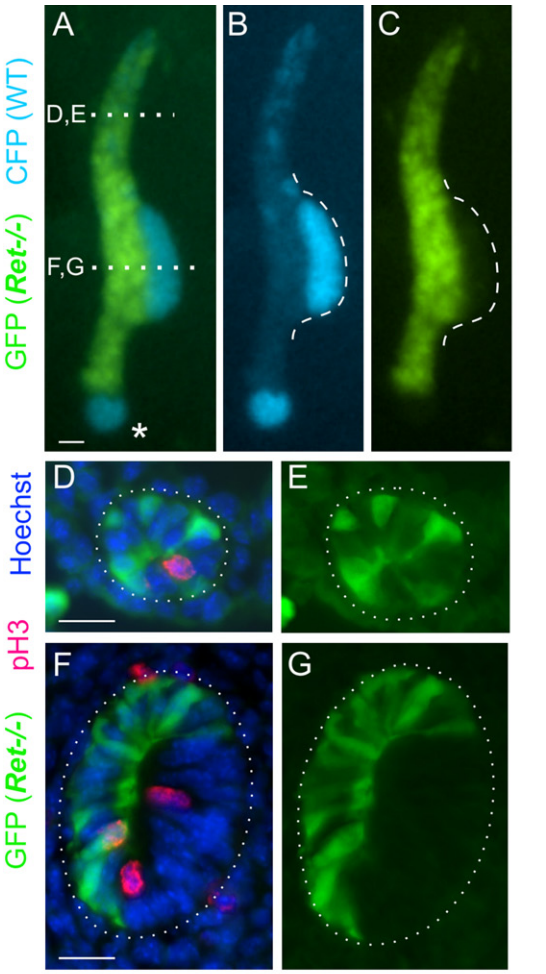
contributed to some of the UB trunks but rarely to the more distal branches. These results showed that *Ret*$^{-/-}$ cells have a cell-autonomous inability to contribute to the "tip domain" of the primary UB. As this domain contains the progenitors for most of the UB epithelium (Shakya et al., 2005; unpublished data), this early defect led to their exclusion from most of the collecting duct system.

These findings raised the questions of what cellular events lead to the formation of the UB and why *Ret* is required for a cell to contribute to the tip, but not the trunk? Here, we address these questions by following the behaviors of fluorescently marked mutant versus WT cells in time-lapse movies of organ cultures. We find that defects in the behavior of *Ret*$^{-/-}$ cells in chimeric embryos are visible even before the UB starts to form, when WT cells undergo extensive movements within the Wolffian duct epithelium to generate a domain that will become the first UB tip. Next, we compare the ability of cells with increased or reduced RET signaling activity to contribute to the tip domain. The results show that WD cells can compete with each other, based on their level of RET signaling, to generate the UB tip domain. In normal (nonchimeric) Wolffian ducts, RET signaling is heterogeneous from cell to cell, and cells can move independently of their neighbors, suggesting that *Ret*-dependent cell competition is a normal feature of the budding process. We also observed that the swelling of the caudal WD before UB outgrowth reflects a transient conversion from a simple to a pseudostratified epithelium, but this process is independent of *Ret*. Thus, both *Ret*-dependent and *Ret*-independent cell behaviors contribute to UB formation.

RESULTS

In the First Step in UB Formation, WT Wolffian Duct Cells Are Rearranged to Form the Primary Tip Domain, while *Ret*$^{-/-}$ Cells Are Excluded

To investigate how *Ret*$^{-/-}$ cells were excluded from the first UB tip (Shakya et al., 2005), we generated *Ret*$^{-/-}$ ↔ WT chimeric embryos using host embryos that expressed CFP in the WD and UB (Figure 1A), so that the WT (CFP^{+}) and mutant (GFP^{+}) cells could be distinguished in live specimens. At E9.5, before the caudal WD began to swell, the first step in UB formation, *Ret*$^{-/-}$ cells were broadly distributed, apparently at random, throughout the WD (Figure 1B). However, as the caudal WD swelled, it developed a localized region on the dorsal side composed almost entirely of wild-type cells (which we term the "primary UB tip domain") (Figures 1C, 1D, 2A–2C, 2F, and 2G). As the UB began to evaginate, nearly all the mutant cells remained behind in the WD, while the wild-type cells formed the leading portion of the bud (the "primary UB tip") (Figures 1D and 1E). Only after the UB had begun to elongate did *Ret*$^{-/-}$ cells follow the WT cells into the UB trunk (Figure 1F). As the UB began to branch, mutant cells entered the proximal parts of the first two branches, while the two tips and the distal side of the branches were composed of WT cells (Figures 1G and 1H). In contrast, in control chimeras, WT ES cells contribute randomly throughout the UB (Figure 1I) (Shakya et al., 2005). Thus, the absence of *Ret*$^{-/-}$ cells from the primary UB tip is a consequence of the rapid enrichment of WT cells in a Wolffian duct domain that will give rise to the UB tip, even before budding is initiated.



H

	% pH3+ WT cells	% pH3+ Ret-/- cells
rostral Wolffian duct	2.2 +/- 1.1 *	2.8 +/- 2.0
caudal Wolffian duct	3.8 +/- 0.7 *	2.9 +/- 1.0
entire Wolffian duct	3.0 +/- 0.6	2.7 +/- 0.7

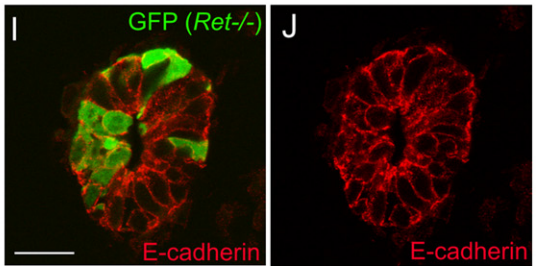


Figure 2. Spatial Distribution and Properties of Mutant and WT Cells in the Prebudding *Ret* $^{-/-}$ ↔ WT Chimeric Wolffian Duct

(A–C) Whole-mount images of a chimeric E10.5 WD, in which the primary UB tip domain (right side, dorsal) is composed mainly of WT (CFP$^+$) cells, while the ventral WD (left side) is depleted of WT cells. Asterisk marks the CND, also highly enriched in WT cells. Dotted lines in (A) show approximate planes of the sections in (D), (E), (F), and (G) (which show a different chimeric embryo).

(D–G) These panels show one section through the rostral WD (D and E) and one through the caudal WD (F and G) of an E10.5 chimera. The host embryo did not carry CFP, and the blue nuclear stain is Hoechst. The rostral WD has a random distribution of mutant (GFP$^+$) and WT cells, while in the caudal WD, mutant cells are absent in the primary UB tip domain (dorsal region, on right) but enriched in the ventral region. Red stain in (D) and (F) is anti-pH3.

(H) Percentage of mutant or WT cells that were pH3$^+$ in the rostral, caudal, or entire WD of chimeric embryos (mean ± SD). Asterisks indicate a significant difference ($p < 0.01$) between percentate of WT cells that were pH3$^+$ cells in rostral versus caudal WD. However, the percentage of mutant versus WT pH3$^+$ cells was not significantly different.

(I–J) E-cadherin staining (red) of a chimeric WD shows similar expression in WT and mutant (GFP$^+$) cells. GFP was detected by fluorescence (A–C) or anti-GFP (other panels).

Scale bars: 20 μM.

These observations raised the question: what is the cellular mechanism by which WT but not *Ret*$^{-/-}$ cells form the UB tip domain? Among the possibilities were differences in cell proliferation, survival, migration, and adhesion. We previously speculated that GDNF/RET signaling might cause the WT cells to proliferate rapidly in the forming tip domain and overgrow the mutant cells, which cannot respond to GDNF (Shakya et al., 2005). Indeed, it was recently shown that *Fgfr2*$^{-/-}$ cells are displaced by proliferation of *Fgfr2*$^{+/-}$ cells in chimeric mammary gland terminal end buds (Lu et al., 2008). However, measurements of phosphohistone H3$^+$ (pH3$^+$) cells showed no significant difference between the mitotic index of WT versus *Ret*$^{-/-}$ cells in chimeric Wolffian ducts (Figures 2D, 2F, and 2H) or in nonchimeric WT versus *Ret*$^{-/-}$ embryos (data not shown). Therefore, the paucity of mutant cells in the tip domain is not due to a proliferative defect. We also saw no evidence of cell death that could explain their depletion in this region (data not shown).

The occurrence of extensive cell rearrangements was first revealed by comparing the distribution of WT and *Ret*$^{-/-}$ cells in the rostral versus caudal Wolffian duct at E10.5. In the rostral duct, mutant cells appeared randomly distributed (e.g., Figures 2D and 2E), but in the caudal region of the same duct (Figures 2F and 2G), not only were mutant cells absent from the forming tip domain on the dorsal side, but they were highly enriched on the adjacent ventral side. This indicated that WT and mutant cells had sorted to different domains. In some situations, cells sort out based on differing expression of adhesion molecules, such as E-cadherin (Steinberg, 2007). We found that *Ret*$^{-/-}$ cells expressed E-cadherin similarly to WT cells (Figures 2I and 2J); however, a role for other cell adhesion molecules has not been excluded.

The Caudal Wolffian Duct Forms a Pseudostratified Epithelium, in a Ret-Independent Process, before UB Outgrowth

While examining WD sections, we observed that the caudal WD epithelium at E10.5 is multilayered (e.g., Figures 2F, 2I, and 2J). While the caudal WD obviously increases in diameter before budding, its cellular organization has not been previously described. Studies using *Hoxb7/myr-Venus* transgenic mice, which express a membrane-bound Venus fluorescent protein in the WD/UB lineage (Chi et al., 2009), showed that while the rostral WD at E10.5 is single layered, the caudal region is multilayered (Figures 3A–3C′). Both the dorsal and ventral sides of the duct are multilayered, but the dorsal side (where the UB will form) is often thicker. While the nuclei are located at different levels along the apical-basal axis (Figure 3C′), many cells contact

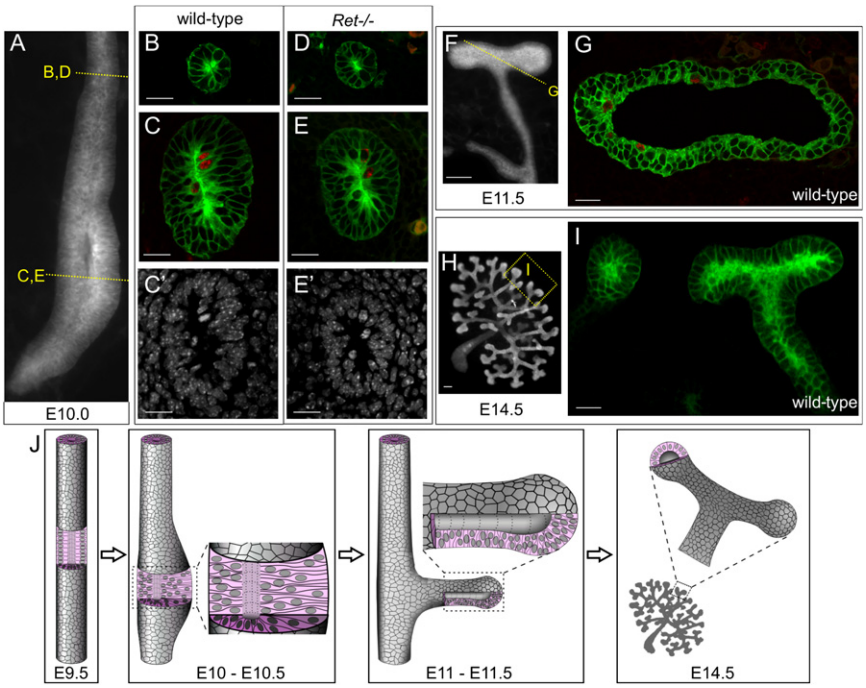


Figure 3. The Caudal Wolffian Duct Forms a Transiently Pseudostratified Epithelium in Both WT and *Ret*$^{-/-}$ Embryos

The WD and UB epithelium was visualized using the *Hoxb7/myr-Venus* transgene.

(A–E′) WT and *Ret*$^{-/-}$ WDs at E10.0. (A) Whole-mount image of WT Wolffian duct. Yellow lines indicate approximate planes of the sections at right, which show rostral WD from a WT (B) or *Ret*$^{-/-}$ (D) embryo, and pseudostratified caudal WD from a WT (C and C′) or *Ret*$^{-/-}$ (E and E′) embryo. B–E were stained with anti-GFP to detect myr-Venus (green) and anti-pH3 (red). (C′ and E′) Hoechst nuclear stain of the sections in (C) and (E).

(F and G) WT UB at E11.5 in whole mount. Yellow line indicates approximate plane of section in (G).

(H and I) WT UB at ~E14.5 in whole mount. Yellow box shows approximate area of the optical section through a terminal UB branch in (I).

(J) Schematic diagram of the formation of pseudostratified epithelium preceding ureteric budding and reversion to a simple epithelium by E14.5.

Scale bars: 100 μM in A, F, and H; 20 μM in other panels.

both the apical and basal surfaces (Figure 3C). This is most easily seen in chimeric ducts, where only a fraction of cells express GFP (Figures 2F and 2G) or myr-Venus (Figure 5B). Thus, the caudal WD is a pseudostratified rather than a truly stratified epithelium.

Pseudostratified epithelia are characterized by interkinetic nuclear migration (shuttling of nuclei between the apical and basal surfaces during the cell cycle), which causes M phase nuclei to be found only near the apical surface (Bort et al., 2006). Staining with anti-pH3 (which labels late G2 and M phase nuclei) showed that nearly all pH3^{+} nuclei (138/140) in the caudal E10.5 WD were apical (e.g., Figures 3C and 3E). At E11.5, the UB epithelium remains multilayered (Figures 3F and 3G), but by E14.5 it is again a simple epithelium (Figures 3H and 3I), consistent with previous analyses (Chi et al., 2009; Meyer et al., 2004). Thus, the pseudostratified state is a transient phase that begins before initial budding and ends during early renal branching (Figure 3J). Since the transition to pseudostratified epithelium occurs at the same stage as *Ret*-dependent cell rearrangements, we asked whether *Ret* played a role in pseudostratified epithelium formation. In *Ret*$^{-/-}$↔WT chimeric Wolffian ducts, both WT and mutant cells were found in the pseudostratified region (e.g., Figures 2F and 2I). Furthermore, in *Ret*$^{-/-}$ (nonchimeric) embryos, the caudal WD was pseudostratified (N = 6) (Figures 3E and 3E′). Thus, this change in epithelial organization occurs independently of RET signaling.

The Distribution of WT and Mutant Cells Is Altered by *Ret*-Dependent Cell Movements within the Wolffian Duct Epithelium

To investigate the processes by which WT and *Ret*$^{-/-}$ cells occupy different domains of the WD, we performed time-lapse imaging of urogenital organ cultures. Figures 4A and 4B and Movie S1 (available online) show a culture begun at ~E10.0 (32 somites) in which formation of the primary UB tip domain could be followed. Initially, the WT and mutant cells appeared randomly interspersed throughout the WD, but by 24 hr a cluster of WT (CFP^{+}) cells could be observed at the prospective site of ureteric budding (arrows). In the merged GFP/CFP images (Figure 4A) the behavior of CFP^{+} versus GFP^{+} cells could not be distinguished until the tip domain had formed. However, the CFP channel revealed that the WT cells were at first widely dispersed but gradually moved together into a cluster (Figure 4B, brackets), while the mutant cells remained dispersed (Figure 4A). Urogenital cultures initiated at this early stage rarely progress to the stage of UB outgrowth, so to examine subsequent events we started cultures at ~E10.5 (36–38 somites).

Figures 4C–4F (Movies S2, S2A, and S2B) illustrate primary UB tip domain formation as well as UB outgrowth in a *Ret*$^{-/-}$↔WT chimera. The primary UB tip domains had already formed (Figure 4C, arrows), but during culture several ectopic buds emerged from the WDs and their origins could be followed from an early stage (*Ret*$^{-/-}$↔WT chimeras often form ectopic UBs in addition to the normal UB; see Discussion). In the merged GFP/CFP images (Figures 4C and 4D), the sequence of budding events was similar to that illustrated in Figure 1. However, the CFP channel (Figures 1E and 1F; Movies S2, S2A, and S2B) showed that the WT cells were at first broadly distributed along the Wolffian ducts (brackets, 0 hr), then converged into tight clusters (8–17 hr). They next changed direction and emerged as the tip of a UB (Figures 1E and 1F; 17–28 hr). The mutant (GFP^{+}) cells did not appear to undergo any such concerted movements (Movie S2). Based on extensive analysis of sections and confocal images (Figure 2; data not shown), the moving cells do not delaminate, but remain part of the WD epithelium throughout the process. These observations indicate that an important function of *Ret* in the WD is to mediate the *rearrangement* of epithelial cells to form the primary UB tip domain.

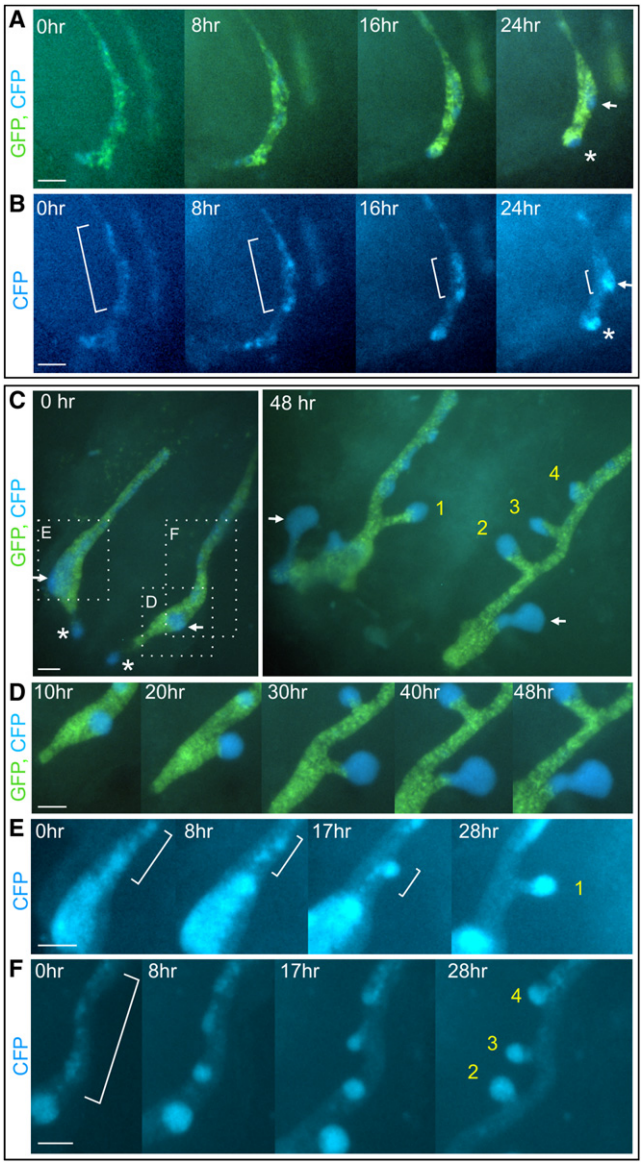


Figure 4. WT Cells in the Chimeric *Ret* $^{-/-}$ ↔ WT Wolffian Duct Converge to Form the Primary UB Tip Domain

(A and B) Culture of a *Ret*$^{-/-}$↔WT chimera (~E10.0) in which formation of the primary UB tip domain was visualized (Movie S1). (A) shows CFP and GFP images merged, revealing interspersion of WT (CFP$^+$) and mutant (GFP$^+$) cells in the Wolffian duct at 0 hr, but enrichment of WT cells at the primary UB tip domain (arrow) and common nephric duct (CND, *) by 24 hr. (B) shows the CFP channel, revealing rearrangement of WT cells to form the UB tip domain (brackets) and CND.

(C–F) Culture of a *Ret*$^{-/-}$↔WT chimera from E10.5 (Movies S2, S2A, and 2B). The boxes in (C) indicate the areas enlarged in (D)– (F). At 0 hr [(C), left], the normal primary UB tip domains had already formed (arrows). By 48 hr [(C), right], the two normal UBs had grown out (arrows) and four ectopic buds (1–4) had also emerged. (D) Enlargements from Movie S2 showing outgrowth, elongation, and ampulla formation of a normal UB. Between 30 and 48 hr, CFP$^+$ cells from the UB tip give rise to part of the trunk, consistent with lineage analyses (Shakya et al., 2005). (E and F) Enlargements from Movies S2A and S2B showing cell movements preceding evagination of buds 1–4. The CFP$^+$ WT cells, which are initially dispersed (brackets), converge into clusters before evaginating as the UB tips.

Scale bars: 100 μM.

The caudal-most segment of the WD, the common nephric duct (CND), also became enriched for WT cells by the comigration of initially dispersed WT cells (asterisks, Figures 2A and 4A–4C). During ureter maturation, CND expansion followed by apoptosis is crucial for separating the ureter from its original insertion site in the WD and repositioning it to its final insertion site in the bladder neck (Batourina et al., 2005). This process depends on *Ret* expression in the CND, but it has been unclear at which stage in CND remodeling *Ret* acts. The observation that the expanded CND in *Ret*$^{-/-}$↔WT chimeras is composed mainly of WT cells suggests that *Ret*-dependent cell movements are important to generate other components of the urogenital system in addition to the UB tip.

Despite the specialized junctions between epithelial cells that maintain cell contacts and epithelial integrity, there are extensive cell movements in many growing epithelial structures, requiring that the cell junctions be frequently remodeled (Gumbiner, 2005; Lecuit, 2005). Extensive, but apparently random, epithelial cell rearrangements have been previously observed during branching morphogenesis in the developing kidney (Shakya et al., 2005), mammary gland (Ewald et al., 2008), and salivary gland (Larsen et al., 2006). In contrast, the present observations reveal a different type of rearrangement that appears tightly orchestrated, is controlled by *Ret*, and patterns the Wolffian duct into distinct functional domains.

Wolffian Duct Cells Compete Based on the Level of RET Signaling to Form the UB Tip Domain

In the developing *Drosophila* respiratory epithelium, cell movements to the tip of the air sac or tracheal branch are governed by competition among cells based on FGF signaling levels (Cabernard and Affolter, 2005; Ghabrial and Krasnow, 2006). To ask if WD cells can compete in a similar manner, based on RET signaling, we used a genetic approach employing a *Sprouty1* (*Spry1*) null allele that increases RET signaling and a hypomorphic *Ret* allele that decreases it. *Spry1* is a feedback inhibitor of receptor tyrosine kinase (RTK) signaling, which is required to negatively regulate GDNF/RET signaling (Basson et al., 2005). *Spry1*$^{-/-}$ mice develop multiple UBs, leading to multiple ureters and multiplex kidneys, due to increased sensitivity to GDNF (Basson et al., 2005; Basson et al., 2006). Thus, *Spry1*$^{-/-}$ Wolffian duct cells have elevated RET signaling and, according to a competition model, should prevail over WT cells to form the UB tip domain.

Spry1$^{-/-}$ ES cells carrying *Hoxb7/myr-Venus* were used to produce *Spry1*$^{-/-}$↔WT chimeras. In a chimera cultured from ~E9.5 (Figure 5A), the mutant (green) cells were initially dispersed along the Wolffian duct among WT (CFP$^+$) cells but were later rearranged into two clusters, one corresponding to the presumptive UB tip domain and one to the CND. This is *opposite* to the rearrangements in *Ret*$^{-/-}$↔WT chimeras, where WT cells form the UB tip domain and CND (Figures 4A and 4B). In chimeric Wolffian ducts at ~E10, the *Spry1*$^{-/-}$ cells were distributed in a pattern *opposite* to that seen with *Ret*$^{-/-}$ cells: they were *enriched* in the dorsal side, where the tip domain is forming (Figure 5B).

Time-lapse imaging (Figure 5C-D; Movies S3 and S4) illustrates the behavior of *Spry1*$^{-/-}$ versus WT cells during UB outgrowth and branching. Figure 5C shows a chimeric WD that

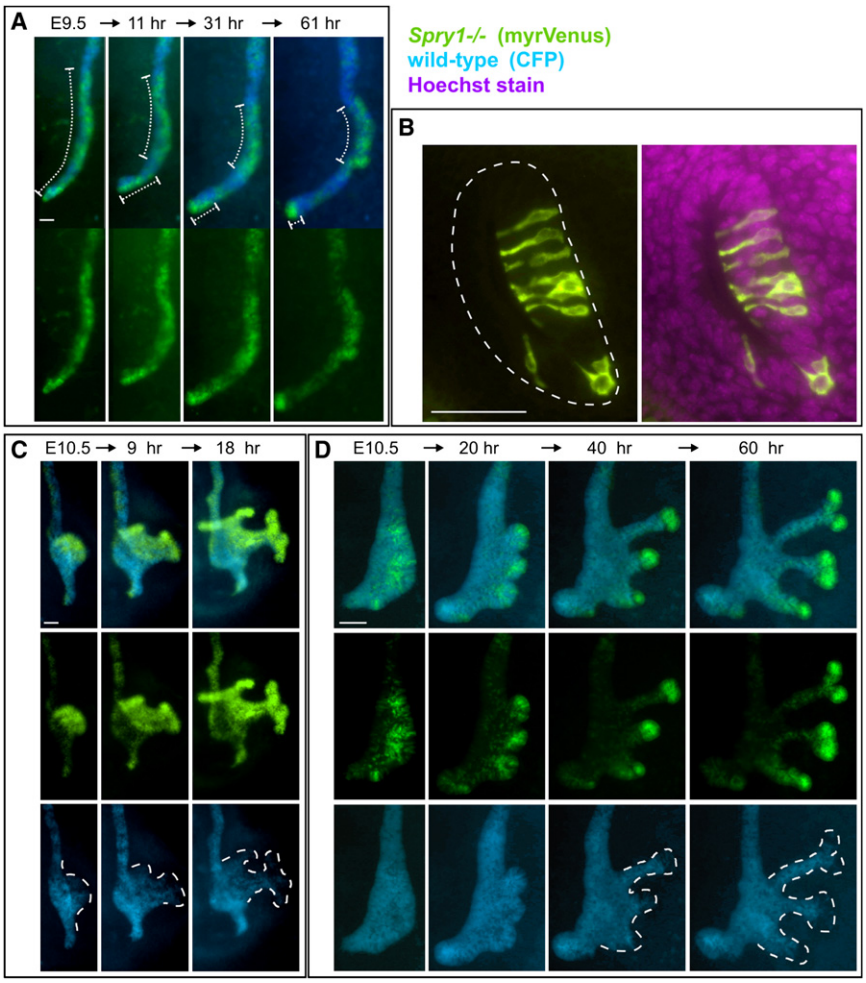


Figure 5. In *Spry1*$^{-/-}$↔WT Chimeras, *Spry1*$^{-/-}$ Cells Preferentially Populate the UB Tip Domain while WT Cells Are Excluded

(A) Movements of *Spry1*$^{-/-}$ cells in the Wolffian duct during culture of a *Spry1*$^{-/-}$↔WT chimera isolated at ~E9.5 (produced by injecting *Spry1*$^{-/-}$, *Hoxb7/myr-Venus* ES cells into *Hoxb7/Cre;R26R*$^{CFP+}$ blastocysts; see Figure 1A). The chimeric WD is shown at four time points. Brackets indicate the changing distribution of *Spry1*$^{-/-}$ cells as they converge into two clusters, one at the site of future ureteric budding and one at the CND.

(B) In a section through the caudal Wolffian duct of a *Spry1*$^{-/-}$↔WT chimera at ~E10.0, *Spry1*$^{-/-}$ cells preferentially populate the primary UB tip domain on the dorsal (right) side.

(C and D) show the Wolffian ducts and UBs of two *Spry1*$^{-/-}$↔WT chimeras cultured from ~E10.5. In both cases, *Spry1*$^{-/-}$ cells predominate over WT cells to form the UB tips (dashed lines). See Movies S3 and S4.

Scale bars: 100 μM in A, C, D, 50 μM in B.

had formed a single UB tip domain at the early budding stage that was highly enriched in *Spry1*$^{-/-}$ cells. As the UB branched, *Spry1*$^{-/-}$ cells were preferentially found in the tips and distal branches, where WT cells were underrepresented. Figure 5D shows another chimeric WD that generated three UBs, as do many *Spry1*$^{-/-}$ WDs (Basson et al., 2005). The *Spry1*$^{-/-}$ cells, which were already enriched on the dorsal side at E10.5, segregated into three clusters that evaginated as the tips of three UBs, while WT cells were depleted in the tips. Thus, the behavior of WT cells varies drastically depending on whether they are competing with *Ret*$^{-/-}$ cells or *Spry1*$^{-/-}$ cells.

To further test the model of RET signaling-based competition, we used a hypomorphic *Ret* mutant, *Ret*$^{tm2(RET)Vpa}$ (de Graaff et al., 2001), as the host strain. *Ret*$^{tm2(RET)Vpa/tm2(RET)Vpa}$ homozygotes have milder defects than *Ret*$^{-/-}$ homozygotes, including small kidneys with reduced branching, but not renal agenesis. Like *Ret*$^{+/-}$ mice, *Ret*$^{tm2(RET)Vpa}$ heterozygotes are normal. The *Ret*$^{-/-}$ ES cells, or control *Ret*$^{+/-}$ ES cells, were injected into *Ret*-hypomorphic or control host blastocysts, and their contribution to the UB was analyzed. When injected into normal host embryos (Figures 6A, 6B, 6E, and 6F), *Ret*$^{-/-}$ cells were found mainly in the ureter and early UB branch generations, with very few in the distal branches or tips, as previously observed (Shakya et al., 2005). In contrast, when the same *Ret*$^{-/-}$ ES cells were injected into *Ret*-hypomorphic hosts, a higher proportion of mutant cells was located in the distal branches and tips (Figures 6C, 6D, 6G, and 6H). The control *Ret*$^{+/-}$ ES cells contributed evenly to all regions of the UB in a WT host (Figure 6I), as previously reported (Shakya et al., 2005), consistent with the observation that loss of one *Ret* allele has no effect on kidney development (Schuchardt et al., 1994). In *Ret*-hypomorphic hosts, the control ES cells also contributed to all regions of the UB, but appeared somewhat enriched in the tips compared to trunks (Figure 6J).

Thus, the fate of *Ret*$^{-/-}$ cells in a chimeric UB is altered by the *Ret* genotype of the host embryo, further supporting a competitive model of UB tip domain formation. This indicates that the *Ret*$^{-/-}$ cells are not *inherently* unable to contribute to the UB tip (perhaps because of functional overlap between Ret and other RTKs), and they contribute more successfully in a host with reduced RET signaling.

Wolffian Duct Cells in Normal Embryos Display Heterogeneous RET Signaling and Independent Cell Movements

What do the cell rearrangements in embryos chimeric for *Ret* (summarized in Figures 7A–7C) reveal about normal development, where every cell is WT? One possibility is that a subset of WD cells normally achieves higher levels of RET signaling than their neighbors and preferentially migrates to contribute to the UB tip domain. Two predictions from this model are that (1) WD cells display locally heterogeneous levels of RET expression and/or signaling, and (2) some WD cells move independently of their neighbors during budding.

While *Ret* gene expression was homogenous among WD cells (Figure 7D), RET signaling appeared heterogeneous. Erk MAP

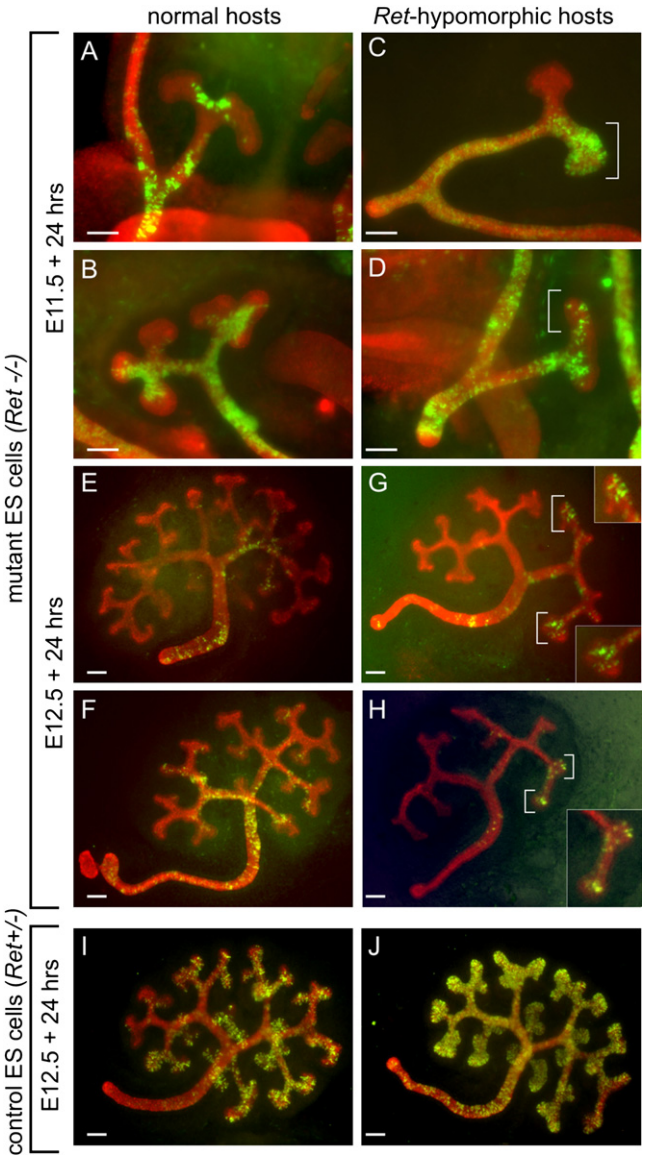


Figure 6. In *Ret*-Hypomorphic Host Embryos, Unlike Normal Hosts, *Ret* $^{-/-}$ Cells Frequently Contribute to the UB Tips

Ret $^{-/-}$ ES cells (A–H) or control *Ret* $^{+/-}$ ES cells (I–J), both carrying *Hoxb7/GFP*, were injected into normal (left column) or *Ret*-hypomorphic blastocysts (right column). Kidneys were excised at E11.5 (A–D) or E12.5 (E–J) and cultured for 24 hr. The kidneys were stained with anti-cytokeratin (red) to visualize the UB epithelium (as host embryos did not carry the *R26R*^{CFP} gene), and the ES cell contribution was detected with anti-GFP (green). Brackets in (C), (D), (G), and (H) indicate *Ret* $^{-/-}$ cells in the UB tips. Insets in (G) and (H) show magnified images of the UB tips. Note that the presence of many *Ret* $^{+/-}$ cells in (J) corrected the branching defect seen in *Ret*-hypomorphic kidneys (G and H). Scale bars: 100 μM.

kinase is phosphorylated upon RET signaling (Takahashi, 2001), and the diphosphrylated form (dp-Erk) is an established indicator of RTK signaling (Corson et al., 2003; Lunn et al., 2007). In E10.5 WT embryos, dp-Erk staining was localized in the dorsal part of the caudal WD (Figure 7E, arrows), corresponding to the site of primary tip domain formation (Figures 1 and 2) and adjacent to the GDNF-expressing MM (dashed circles). In contrast, the *Ret* $^{-/-}$ WD was dp-Erk-negative (Figure 7F), indicating that dp-Erk is a reporter of RET activity in the WD. Sections from E10–10.5 wild-type WDs revealed extensive cell-to-cell heterogeneity in dp-Erk staining (Figures 7G–7J), indicating that neighboring WD cells can differ considerably in RET signaling levels.

To examine cell movements in nonchimeric Wolffian ducts, we induced expression of YFP by crossing a *R26R*^{YFP} Cre-reporter (Srinivas et al., 2001) to an inducible *Cre* allele expressed in the WD (*Ret*^{CreERT2}) and inducing Cre activity with Tamoxifen. Recombination leading to YFP expression occurs in a subset of cells, which should be random with respect to differences in RET signaling level. In several cultured explants with low percentages of YFP^{+} cells, it was possible to follow cell movements during budding. Figures 7K and 7L show two examples in which several neighboring cells displayed independent movements, some contributing to a forming bud (yellow brackets) and others remaining behind in the duct (red brackets). Thus, not only in chimeras, but also in Wolffian ducts genetically uniform for *Ret*, some cells undergo extensive movement with respect to their neighbors. Together, these observations suggest that the competitive sorting of Wolffian duct cells in *Ret* ↔ WT or *Spry1* ↔ WT chimeras reflects a normal process of *Ret*-dependent cell rearrangements.

DISCUSSION

Branching morphogenesis is a critical process in the development of many organs, including the kidneys (Davies, 2002). However, its cellular basis is poorly understood, particularly in mammals for which there are few experimental systems to manipulate and visualize the process of organogenesis. In this paper, we used time-lapse imaging of chimeric organ cultures to investigate the processes by which the first UB tip is generated, a *Ret*-dependent event crucial for ureter and kidney development. We find that *Ret*-expressing Wolffian duct cells undergo extensive movements to generate a specialized epithelial domain that gives rise to the first UB tip, while cells lacking *Ret* are excluded. Chimeric studies that juxtapose cells with different levels of RET activity, as well as analyses of cell movements and RET signaling in the WT Wolffian duct, suggest that these rearrangements result from competition among cells based on the level of signaling, a mechanism with interesting parallels to those described in branching fly epithelia (Affolter and Caussinus, 2008). We also found that ureteric budding is preceded by the formation of a pseudostratified epithelium in the caudal WD, a process that is independent of *Ret*. These studies provide new insight into cellular events during the initiating steps of renal development and how they are influenced by GDNF/RET signaling.

The Wolffian Duct Forms a Pseudostratified Epithelium Independently of *Ret* Activity

The WD derives from a cord of cells that converts to a simple epithelial tube, which elongates caudally between ~E8.5 and E9.5. At these stages, *Ret* is not yet required for cells to contribute to any region of the WD. At ~E10.0, the caudal WD starts to swell as the epithelium becomes pseudostratified (Figure 3). This region exhibits higher cell proliferation than the

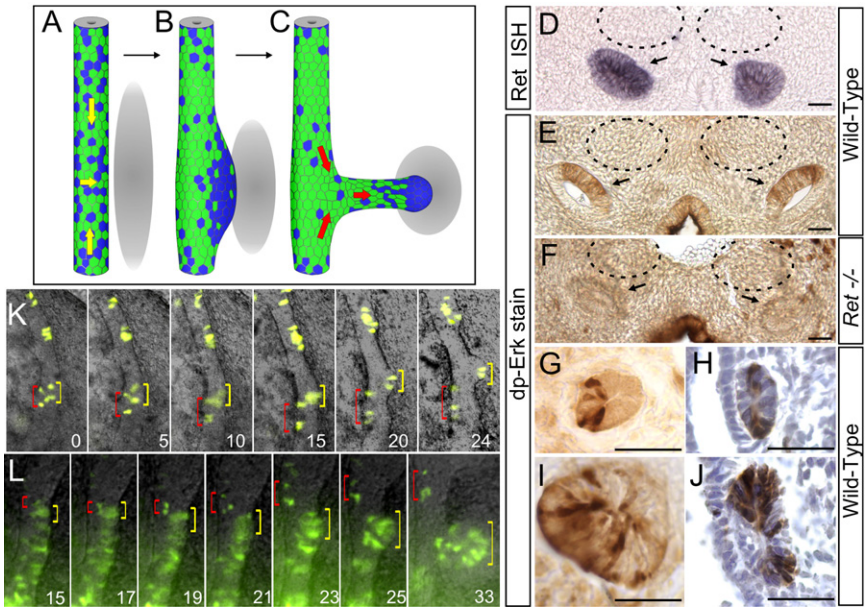


Figure 7. Independent Cell Movements and Heterogeneous RET Signaling in the WT Wolffian Duct

(A–C) Diagram illustrating rearrangement of WT (blue) and mutant (green) cells in *Ret* $^{-/-}$↔WT chimeras. Gray ovals represent metanephric mesenchyme. The initially dispersed WT cells (A) converge along the long axis of the Wolffian duct and also move dorsally (yellow arrows) to form the primary UB tip domain (B). When the UB grows out, WT cells lead, forming the tip, while *Ret* $^{-/-}$ cells follow [(C), red arrows].

(D–J) Transverse sections of ~E10.0-10.5 embryos. (D) *Ret* mRNA is expressed uniformly in cells of the Wolffian duct (arrows). Dotted circles indicate GDNF-expressing MM. (E) In WT (37 somite) embryo, anti-dp-Erk specifically stains the WD domain that will form the UB tip (arrows; compare to Figures 2F and 2G). (F) In a *Ret* $^{-/-}$ embryo, the WD is dp-Erk-negative. (G–J) Wolffian duct sections (34–36 somite WT embryos) showing heterogeneous dp-Erk staining. (I) and (J) are caudal and (G) and (H) more rostral sections. (H) and (J) were counterstained with Hematoxylin.

(K and L) Independent movements of neighboring cells during budding in nonchimeric WDs. A random subset of duct cells was YFP-labeled by Tamoxifen treatment at E7.5 (K) or E9.5 (L) of *Ret* $^{CreERT2/+}$; *R26R* $^{YFP/+}$ embryos (in which all cells have the same *Ret* $^{+/-}$ genotype). Urogenital regions were cultured from ~E10.5 for indicated number of hours. In each sequence, several YFP$^+$ cells enter the tip of a forming UB (yellow brackets) while other neighboring YFP$^+$ cells remain behind in the WD (red brackets).
Scale bars: 25 μM.

rostral WD (Figure 2H) (Michael and Davies, 2004), which may generate the extra cells needed to form a pseudostratified region. Interestingly, pseudostratified domains are formed in several other epithelial structures that are about to produce an outgrowth, including the ectodermal mammary line (Veltmaat et al., 2004) and otic placodes (Meier, 1978) and the endodermal liver and thyroid buds (Bort et al., 2006; Fagman et al., 2006). In liver bud development, the *Hex* homeobox gene is required for pseudostratification and for the subsequent delamination of hepatoblasts into the stroma (Bort et al., 2006). In the WD/UB lineage, the pseudostratified epithelium is also a transient state, but cells do not delaminate. Instead, part of the pseudostratified region evaginates, giving rise to the UB. While the importance of WD pseudostratification remains to be elucidated, we speculate that pseudostratified epithelium formation may serve to generate a high cell density in the prebudding region, which permits rapid outgrowth and branching of the UB.

The mammary epithelium also contains multilayered regions at the terminal end buds (TEBs), which are important for branching (Sternlicht et al., 2006), but the TEBs are not pseudostratified. Another difference is that the UB reverts to a simple epithelium by E14.5, while still growing and branching; thus, the pseudostratified epithelium is not required for later stages of UB branching morphogenesis.

Ret-dependent cell movements to form the primary UB tip domain occur while the caudal WD is becoming pseudostratified. However, *Ret* $^{-/-}$ cells contribute to the pseudostratified regions of chimeric WDs (although rarely to the dorsally-located primary UB tip domain), and in *Ret* $^{-/-}$ homozygotes the caudal WD becomes pseudostratified, despite the failure of ureteric budding in most *Ret* $^{-/-}$ embryos (Schuchardt et al., 1996). Therefore, GDNF/RET signaling is dispensable for pseudostratified epithelium formation. Given the proximity of the metanephric mesenchyme to the caudal WD, other signals from the MM are likely to induce this event. In support of this hypothesis, in embryos lacking *Osr1*, a transcription factor expressed in the MM and required for its normal differentiation from the intermediate mesoderm, the caudal WD remains a narrow tube (James et al., 2006, Figure 3; Mugford et al., 2008, Figure 7), failing to become pseudostratified.

The Primary UB Tip Domain Is Generated by Cell Movements in the Wolffian Duct Stimulated by Ret

While the caudal WD is becoming pseudostratified but before budding, extensive cell rearrangements are observed in cultured *Ret* $^{-/-}$↔WT chimeras. Many of the WT cells converge toward the future site of budding, forming a dorsal domain of the epithelium that is composed almost entirely of WT cells and later emerges to form the first UB tip. Cell movements are visible mainly along the rostral-caudal axis of the WD, but they also seem to occur along the dorsal-ventral axis, as the dorsal side becomes enriched and the ventral side depleted of WT cells. These studies provide strong evidence that *Ret*-dependent cell movements are important for epithelial morphogenesis during the initiation of ureter and kidney development.

What directs these cell movements? RET signaling might alter the adhesive properties of cells, so that WT cells are passively sorted from mutant cells, based on adhesive differences (Lecuit, 2005; Steinberg, 2007). However, it seems unlikely that cell adhesion *alone* could cause the observed cell movements to a specific location where the UB will form, and the time-lapse movies suggest a directed migration. GDNF can serve as a chemoattractant for *Ret*-expressing cells in culture (Tang et al., 1998) and for enteric neuroblasts (Natarajan et al., 2002).

However, the broad expression domain of GDNF (Figure 7A) and the predominantly longitudinal cell movements (perpendicular to the source of GDNF) argues against a model in which cells are simply migrating toward the GDNF source. Another possibility is that RET signaling induces the WD cells to express other molecules that promote attraction (e.g., secreted chemoattractants and/or receptors), leading to their convergence. Later, when the UB tip starts to emerge and grow laterally into the MM, it is likely that GDNF serves as a chemoattractant (Sainio et al., 1997). However, since the UB emerges and grows toward the MM in some embryos that lack Ret or *Gdnf* entirely (Costantini, 2006), other mesenchymal signals must also participate.

As noted above, it was surprising that $Ret^{-/-} \leftrightarrow$ WT chimeric Wolffian ducts often formed one or more ectopic UBs (Figure 4C), yet only the WT cells formed the tips of these UBs. Interestingly, in embryos genetically mosaic for *Gata3*, a transcription factor upstream of *Ret*, *Ret* is expressed only in the subset of WD cells retaining *Gata3*, and the WD also forms numerous ectopic buds (Grote et al., 2008). A possible unifying explanation is that RET signaling normally causes a subset of WD cells to attract each other and converge to form a single primary UB tip domain, but random clusters of cells lacking *Ret* can locally disrupt this process, causing the WT cells to form multiple clusters, each generating a UB tip.

Wolffian Duct Cells that Form the UB Tip Domain Are Selected through Competition Based on the Level of RET Signaling

Further insight into the role of *Ret* in formation of the UB tip domain was obtained from studies of $Spry1^{-/-} \leftrightarrow$WT and $Ret^{-/-} \leftrightarrow$*Ret*-hypomorphic chimeras. In these experiments, the behavior of cells with a given level of RET signaling was strongly altered by the genotype of the cells with which they were asked to compete. First, when WT cells were mixed with $Ret^{-/-}$ cells, they preferentially formed the UB tips, but when mixed with $Spry1^{-/-}$ cells (with elevated RET signaling), the WT cells were *depleted* in the tips. Second, when $Ret^{-/-}$ cells were mixed with WT cells, they were almost entirely absent from the UB tips, but when mixed with *Ret*-hypomorphic cells, they contributed more extensively to the tips. The fact that $Ret^{-/-}$ cells can contribute to the UB tip domain in a *Ret*-hypomorphic background suggests that the difference in signaling levels between *Ret* null and hypomorphic cells is not great enough for the hypomorphic cells to fully out-compete the null cells. Thus, there is not an *absolute* requirement for RET signaling for a cell to contribute to the UB tip, and other growth factors and RTKs may also participate in this process (one candidate is FGF10, which synergizes with GDNF to promote UB outgrowth and branching; O.M. and F.C., unpublished data). However, when cells with stronger RET signaling are present, they will generally outperform those with a lower level. These processes have striking parallels with cell movements in the developing *Drosophila* respiratory epithelium, which are governed by FGF-based competition (Cabernard and Affolter, 2005; Ghabrial and Krasnow, 2006). Thus, RTK signaling-based cell competition may be an evolutionarily conserved mechanism of epithelial branching morphogenesis.

While the competitive cell behaviors were revealed in chimeric embryos, where cells with different *Ret* (or *Spry1*) genotypes were purposely juxtaposed, our data suggest that the forced cell competition in a chimera reflects a similar process occurring during normal ureteric budding. This conclusion is based, first, on the heterogeneity in RET signaling among WT WD cells, as revealed by dp-Erk staining, and second, on the apparently independent movements of neighboring WD cells during bud formation. We predict (although it has not yet been possible to prove) that the cells in WT embryos that preferentially contribute to the forming UB tip correspond to those with higher levels of RET signaling, as they do in the chimeric embryos. While the cause of heterogeneous signaling is not clear, it could result from variable expression of signaling components downstream of RET, from some form of lateral inhibition, or from stochastic variation in binding the limited GDNF available (Costantini and Shakya, 2006). Heterogenous dp-Erk staining can also be observed in other developing tissues (e.g., Lunn et al., 2007, Figure 3). Additionally, in developing blood vessels, mosaic activation of the RTK FLK-1 in a subset of endothelial cells is important for vessel branching (Kappas et al., 2008).

We propose that heterogeneity in RET signaling among WD cells serves to refine and focus the site of UB outgrowth. Since the mesenchymal domain of GDNF expression is quite broad, a uniform response by all the WD cells exposed to GDNF might be expected to form a massive swelling rather than a discrete bud. Instead, we suggest that a subset of caudal WD cells preferentially respond to the GDNF signal, migrate together into a narrower cluster adjacent to the center of the GDNF-expressing MM domain, then grow out at the tip of a discrete bud, while cells with lower RET signaling follow and form the UB trunk.

Possible Implications for the Mechanism of UB Branching

While budding from the WD represents the first branching event leading to ureter and kidney development, it differs from later UB branching, which is primarily dichotomous and occurs mainly at the UB termini (Lin et al., 2001; Watanabe and Costantini, 2004). Thus, formation of the primary UB tip domain might use mechanisms that are unique to this initial branching event. However, *Ret* continues to be expressed at the UB tips, and normal renal branching requires the continued function of Ret and *Gdnf* (Costantini and Shakya, 2006). This raises the possibility that *Ret*-dependent cell movements are important beyond their early role in the WD. In particular, the results suggest a model in which an attraction among *Ret*-expressing UB cells may keep them at the UB tips (and thus maintain the tip-specific pattern of *Ret* expression), while those cells in which *Ret* expression is downregulated are out-competed and contribute to the trunks. Studies in which RET signaling is manipulated in individual UB cells should help to test this model.

EXPERIMENTAL PROCEDURES

Embryonic Stem Cell Lines

$Ret^{-/-}$ ES cells carrying *Hoxb7/GFP* were as described (Shakya et al., 2005) and $Spry1^{-/-}$ ES cells carrying *Hoxb7/myr-Venus* (Chi et al., 2009) were derived similarly.

Generation of Chimeras, YFP Mosaics, and Organ Culture

Chimeric embryos generated by blastocyst injection were recovered and urogenital systems were cultured and imaged as described (Srinivas et al., 1999; Watanabe and Costantini, 2004). For injection into $Ret^{tm2(RET)Vpa}$ hosts,

blastocysts were derived from *Ret*$^{tm2(RET)Vpa/+}$ intercrosses; 25% were *Ret*$^{tm2(RET)Vpa/tm2(RET)Vpa}$. The host embryo was genotyped (de Graaff et al., 2001) retrospectively, using yolk sac visceral endoderm (derived only from the host) separated from the chimeric mesoderm (Hogan et al., 1994). *Ret*$^{CreERT2/+}$; *R26R*$^{YFP/+}$ embryos were YFP-labeled by injecting the mother with 1–2 mg Tamoxifen in corn oil. *Ret*^{CreERT2} mice, carrying *CreERT2* inserted at the *Ret* locus, will be described elsewhere.

Immunofluorescence Microscopy

Primary antibodies were against cytokeratin (Sigma), GFP (Molecular Probes), phosphohistone H3 (Ser10) (6G3) (Cell Signaling), and E-cadherin (BD Transduction Laboratories). Embryos were fixed in 4% paraformaldehyde (PFA) (3 hr, 4°C), washed 3× in PBS, equilibrated in 30% sucrose/PBS, embedded in OCT (Tissue-Tek), and dry-ice frozen. Cryosections (7 μM) were post-fixed with 4% PFA (10 min, 4°C), blocked in 2% donkey serum (Sigma) with 1% BSA in PBT at room temperature (RT) for 1 hr, incubated with a mixture of primary antibodies in PBT (overnight, 4°C), washed, and incubated in a mixture of fluorescent secondary antibodies (Jackson ImmunoResearch) (RT, 2 hr). Sections were stained with Hoechst 33342, mounted in VECTASHIELD (Vector Laboratories), and photographed by Zeiss AxioObserver Z1 or Zeiss 510 META confocal microscope. For cell proliferation assays, total, mutant (GFP$^+$), and pH3$^+$ WD cells were counted in sections from the mesonephric tubules to the caudal end in three chimeras and compared by chi-square test. Apoptosis was assayed by TUNEL using in situ cell death detection kit (TMR red, Roche).

Immunohistochemical Staining for dp-Erk

Frozen sections (10 μm) were treated with 3% H_2O_2 for 10 min at RT, then incubated with anti-dp-Erk2 (Sigma, mouse IgG_1, 1:100) overnight at 4°C, followed by biotinylated goat anti-mouse IgG_1 (SouthernBiotech, 1:200) for 3 hr at RT. After PBS washes, the sections were incubated with ABC reagents (Vector Laboratories) for 45 min at RT, followed by color reaction with a peroxidase substrate kit (Vector Laboratories).

SUPPLEMENTAL DATA

Supplemental Data include six movies and can be found with this article online at http://www.cell.com/developmental-cell/supplemental/S1534-5807(09)00297-4.

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