Development 130, 3175-3185

doi:10.1242/dev.00520

Wnt11 and *Ret/Gdnf* pathways cooperate in regulating ureteric branching during metanephric kidney development

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Accepted 1 April 2003

SUMMARY

Reciprocal cell-cell interactions between the ureteric epithelium and the metanephric mesenchyme are needed to drive growth and differentiation of the embryonic kidney to completion. Branching morphogenesis of the Wolffian duct derived ureteric bud is integral in the generation of ureteric tips and the elaboration of the collecting duct system. Wnt11, a member of the Wnt superfamily of secreted glycoproteins, which have important regulatory functions during vertebrate embryonic development, is specifically expressed in the tips of the branching ureteric epithelium. In this work, we explore the role of *Wnt11* in ureteric branching and use a targeted mutation of the *Wnt11* locus as an entrance point into investigating the genetic control of collecting duct morphogenesis. Mutation of the *Wnt11* gene results in ureteric branching morphogenesis defects and consequent kidney hypoplasia in newborn mice. *Wnt11* functions, in part, by maintaining normal expression levels of the gene encoding glial cell-derived neurotrophic factor (Gdnf). *Gdnf* encodes a mesenchymally produced ligand for the Ret tyrosine kinase receptor that is crucial for normal ureteric branching. Conversely, *Wnt11* expression is reduced in the absence of *Ret/Gdnf* signaling. Consistent with the idea that reciprocal interaction between *Wnt11* and *Ret/Gdnf* regulates the branching process, *Wnt11* and *Ret* mutations synergistically interact in ureteric branching morphogenesis. Based on these observations, we conclude that *Wnt11* and *Ret/Gdnf* cooperate in a positive autoregulatory feedback loop to coordinate ureteric branching by maintaining an appropriate balance of *Wnt11*-expressing ureteric epithelium and *Gdnf*-expressing mesenchyme to ensure continued metanephric development.

Key words: *Wnt11*, Metanephric kidney, Ureteric branching morphogenesis, *Ret*, *Gdnf*, Epithelial mesenchymal interaction, Mouse

INTRODUCTION

The adult metanephric kidney of mammals is primarily derived from two embryonic tissue sources: the ureteric epithelium and the metanephric mesenchyme. Metanephric development is launched with an outgrowth of the Wolffian duct, termed the ureteric bud, into the neighboring uninduced metanephric mesenchyme (Saxen, 1987). The classical co-culture experiments of Grobstein have demonstrated that the arborization of ureteric epithelium into the mature collecting duct system, and the terminal differentiation of mesenchyme into functional nephrons, is dependent upon continued cell-cell interactions between the component ureteric epithelium and mesenchyme (Grobstein, 1953). Genetically or chemically induced perturbation of either component tissue or of signaling between these tissues obstructs metanephric growth and differentiation (Davies and Bard, 1998; Davies and Davey, 1999; Lechner and Dressler, 1997).

Epithelial branching morphogenesis is common to the development of the kidney, lung, pancreas and other ductal organs, and involves the regulated growth and branching of an epithelial primordium within a mesenchymal environment. The *Ret*/*Gdnf* signaling pathway is a major regulator of ureteric branching in the metanephric kidney (Airaksinen and Saarma, 2002; Davies and Bard, 1998; Lechner and Dressler, 1997; Manie et al., 2001). Glial cell-derived neurotrophic factor (Gdnf), a member of the TGFβ superfamily, functions as a ligand secreted by the metanephric mesenchyme that binds to the Ret tyrosine kinase receptor and GFRα1 co-receptor, both of which are expressed within the ureteric epithelium (Durbec et al., 1996; Pachnis et al., 1993; Sariola and Saarma, 1999; Vega et al., 1996). Targeted mutagenesis of *Gdnf*, *Ret* or *Gfra1* results in failed ureteric bud morphogenesis and consequently kidney agenesis (Schuchardt et al., 1994; Sanchez et al., 1996; Schuchardt et al., 1996; Cacalano et al., 1998; Enomoto et al., 1998). Conversely, ectopic activation of the *Ret/Gdnf* pathway induces the appearance of supernumary ureteric tips. Implantation of Gdnf-coated beads into kidney explant cultures stimulates ectopic ureteric tip formation from the Wolffian duct (Brophy et al., 2001; Pepicelli et al., 1997; Sainio et al., 1997).

Similarly, in the *Foxc1* mutant, an expanded mesenchymal *Gdnf* expression domain is the target of ectopic ureteric bud invasion from the Wolffian duct resulting in multi-lobular kidneys (Kume et al., 2000). Based on these and cell migration studies using MDCK cells, *Gdnf* has been proposed as a mesenchymally localized chemoattractant that promotes Wolffian duct derived ureteric bud outgrowth (Tang et al., 1998).

Several members of the Wnt gene family are expressed in the developing kidney. Wnt genes encode secreted glycoproteins with important roles in regulating cell proliferation, tissue patterning and morphogenesis during vertebrate embryogenesis (Wodarz and Nusse, 1998). The Wnt ligands are thought to elicit their cellular responses by binding to transmembrane Frizzled receptors (Bhanot et al., 1996). Among the Wnt members, *Wnt11*, *Wnt7b*, *Wnt6*, *Wnt2b* and *Wn-4* have been reported to be in unique domains within the embryonic mouse kidney (Kispert et al., 1996; Lin et al., 2001; Stark et al., 1994). *Wnt11* is unique in that it shows a striking expression pattern in the branching ureteric tips suggesting a possible function in regulating ureteric branching morphogenesis (Kispert et al., 1996). In addition to its kidney expression, *Wnt11* is expressed in multiple embryonic tissues, including the node, heart primordium, somites, branchial arches and limb buds (Kispert et al., 1996). Analysis of zebrafish *silberblick* (*slb*), a mutation in zebrafish *wnt11*, and experiments in Xenopus suggest that *Wnt11* signals through the planar cell polarity (PCP), and not the canonical β-catenin pathway, to regulate convergence and extension movements during gastrulation that elongate the axis (Heisenberg et al., 2000; Tada and Smith, 2000). Recently, *Wnt11* has been implicated in the regulation of cardiogenesis in *Xenopus* (Pandur et al., 2002).

In the kidney, *Wnt11* is expressed in the tips of the branching ureter at all stages of ureteric development (Kispert et al., 1996). In addition, the implantation of *Gdnf* coated beads causes induction of ectopic ureteric tips and upregulation of *Wnt11* at these sites (Pepicelli et al., 1997; Sainio et al., 1997). Furthermore, genetic and chemical perturbation of sulfated proteoglycan synthesis blocks ureteric branching and simultaneously results in loss of *Wnt11* expression (Bullock et al., 1998; Kispert et al., 1996). These experiments indicate a correlation between the formation of ureteric tips, the appearance of *Wnt11* expression and the initiation of ureteric branching.

In order to determine the function of *Wnt11* during metanephric kidney development, we generated a targeted knockout mutation of the *Wnt11* locus. We report here the phenotypic analysis of the *Wnt11* mutant mice and show that *Wnt11* is required for embryonic viability and also for normal ureteric branching morphogenesis. In the absence of *Wnt11* function, branching morphogenesis is abnormal resulting in kidney hypoplasia. We show that *Wnt11* regulates ureteric branching, at least in part, by regulating mesenchymal *Gdnf* expression. Ureteric *Wnt11* expression is reciprocally dependent upon *Ret*/*Gdnf* signaling. *Wnt11* and *Ret* mutants genetically interact in the branching morphogenesis process. We propose that the *Wnt11* and *Ret*/*Gdnf* signals may participate in a positive, autoregulatory feedback loop to coordinate branching of the ureteric epithelium and hence normal morphogenesis of the normal kidney.

MATERIALS AND METHODS

Construction of the targeting vector, gene targeting and generation of *Wnt11* knockout mice

The targeting construct, containing 4.8 kb of 5′ and a 4.0 kb of 3′ homology regions (Fig. 1A), was transfected into 5×10^7 R1 ES cells derived from 129Sv strain (Nagy et al., 1993) using a BioRad gene pulser. ES cell clones were selected positively with G418 for presence of the PGK-neo cassette (Swiatek et al., 1994) and negatively with FIAU for absence of the MC1TK cassette (Mansour et al., 1988; Stark et al., 1994). Surviving colonies were isolated, trypsinized and seeded onto mouse embryonic feeder cells or onto non-coated 24-well plates for DNA isolation. Southern blot analysis was used to detect a restriction fragment length polymorphism (RFLP) on DNA purified from 240 colonies by using a 2 kb *Eco*RV genomic fragment as a 3′ probe (Fig. 1A). *Spe*I digestion leads to generation of a 20 kb fragment in the wild-type allele, whereas replacement of exons IV and V of the *Wnt11* gene with *PGK-neo* will introduce additional *Spe*I sites and leads to the generation of a 10 kb mutant *Spe*I fragment (Fig. 1A). One out of 240 screened ES cell clone showed homologous recombination in the *Wnt11* locus. The clone was subjected to Southern blot analysis using the 5′ probe and a *neo* probe to confirm the targeting event (Fig. 1A). The correctly targeted ES cell line was used to generate chimeras by routine blastocyst injection. Germline transmission of the targeted allele was monitored by RFLP analysis using the 5′ probe and *Spe*I digestion on a Southern blot. All subsequent genotyping was carried out by Southern blotting or PCR. Southern blotting was performed using Amersham Pharmacia Biotech Hybond N^+ membranes according to the manufacturer's guidelines. Radioactive probes were labeled using Random Primers DNA Labeling System (Invitrogen). For PCR, genotyping, the wild-type allele was identified using primers 5′CTGGCACTGTCCAAGACTCC3′ and 5′AGCTCGATGGAGGAGCAGT3′, which amplify a 220 bp fragment. The mutant allele was identified using primers 5′GGATCGCAGGCATGTGTCAC3′ and 5′TACCGGTGGATGTGGAATGTGTGCG3′ which amplify a 250 bp fragment. The PCR conditions are 40 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 1 minute.

The *Ret* mutant allele has been described (Schuchardt et al., 1996). The *Ret* mutant embryos were genotyped by PCR as described in Schuchardt et al. (Schuchardt et al., 1996). In double mutant crosses to examine genetic interactions, Southern blot genotyping with *Bam*HI digestion and probing with the *neo* gene identified a 1.1 kb band unique to the *Ret* mutation and a 3.8 kb band unique to the *Wnt11* mutation.

Mouse crosses

All *Wnt11* phenotypic analysis was performed with mice maintained in the 129/Sv background. The *Ret* mutant allele was obtained from F. Costantini and maintained in the 129/Sv background. To visualize ureteric branching morphogenesis, male mice expressing the *Cre* recombinase under control of the *HoxB7* enhancer were crossed to *Rosa26 YFP* females (Srinivas et al., 2001; Yu et al., 2002). Kidneys from *HoxB7 Cre*; *Rosa26 YFP* embryos were dissected and examined under fluorescence using a GFP filter set on a Nikon SMZ1500 stereoscope.

RNA isolation and RT-PCR

Total RNA was isolated from P1 kidneys using TRI reagent (Sigma) and treated with RNase free DNase (Gibco BRL). RT-PCR was performed using the SuperScript Plasmid System (Invitrogen) with the *Wnt11* forward primer 5′GAATTCCGAGGAGAGAGCTCCGGAGA3′ and the *Wnt11* reverse primer 5′TCTAGAGAGCCACCCCAAAGAAAAAG3′. PCR products were digested with *Eco*RI and *Xba*I and cloned into pCR2.1 (Invitrogen). PCR products were sequenced using ABI BigDye cycle sequencing. Wild-type and mutant *Wnt11* cDNA sequences were compared to genomic sequence obtained from the Celera database.

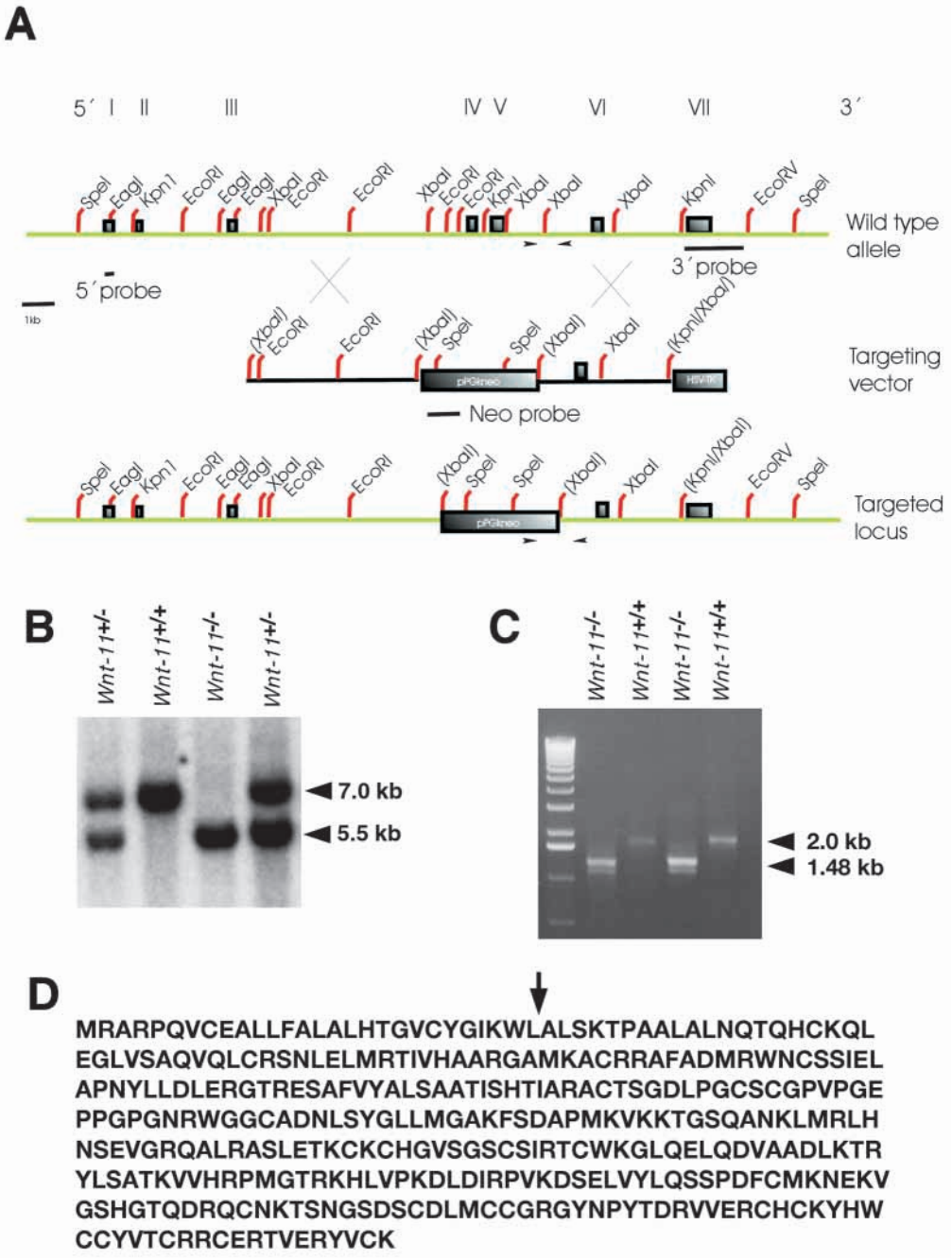

Fig. 1. Gene targeting of the murine *Wnt11* locus. (A) Targeting strategy. Genomic sequences spanning the *Wnt11* locus were cloned and subjected to restriction mapping and sequencing to locate the intron-exon boundaries. The homologous recombination events lead to deletion of exons 4 and 5 and around 1.5 kb of intron 5 and lead to generation of a truncated transcript at amino acid 28 onwards in the corresponding Wnt11 protein. The 5′, 3′and *neo* probes used to screen for gene targeting with Southern blot are indicated, and *Spe*I digestion was used as a diagnostic enzyme to screen the targeting event. Cutting of the wild-type locus with *Spe*I was expected to generate around a 20 kb fragment where the *PGKneo* introduces additional *Spe*I in the targeted allele and was expected to generate a 10 kb fragment. Primers to monitor the wild-type and mutant allele are also indicated with arrowheads. (B) Genotyping the *Wnt11* knock out allele. Genomic DNAs from *Wnt11* wild-type, heterozygote and homozygous mutant alleles were digested with *Afl*II, Southern blotted and probed with the *Wnt11* cDNA 240 bp kb Nco fragment within Exon VI. The *Wnt11* allele specific polymorphism is shown whereby the *Wnt11* wild-type allele is associated with a 7.0 kb band, while the *Wnt11* mutant allele is associated with a 5.5 kb band. (C) *Wnt11* homozygous mutant kidneys produce a shortened *Wnt11* mRNA. Gene-specific primers were used to RT-PCR wild-type and mutant P1 kidney mRNA. The wild-type product is 1.8 kb, while the *Wnt11* mutant product is 1.3 kb, in agreement with the expected size resulting from deletion of Wnt11 exons IV and V. (D) The first 28 amino acids of the mutant *Wnt11* protein match the wild-type sequence. Downstream of the exon IV/V deletion (arrow), the reading frame is out of frame resulting in a null allele.

Histology and quantitating kidney size

Kidneys were fixed in 4% paraformaldehyde and taken through a graded alcohol series in preparation for paraffin wax sectioning. Sections were cut at 6 μm and stained with Hematoxylin/Eosin. Kidney size was quantified throughout the whole kidney by counting absolute numbers of glomeruli in Hematoxylin/Eosin stained sections. Glomeruli were identified by the presence of a Bowmann's capsule and capillary tuft.

Immunohistochemistry

For whole-mount immunocytochemistry, same stage E12.5 kidneys were fixed in methanol prior to antibody staining. Kidneys were re-hydrated, blocked in PBS/0.1% Triton X-100/1% dry milk/2% BSA and stained with a 1:20 dilution of α pan-cytokeratin mAb (Sigma) at 4°C overnight. After washes in PBS/0.1% Triton X-100, staining was visualized with a 1:2000 dilution of Alexa 568 goat-anti-mouse secondary antibody (Molecular Probes). Confocal images were taken on a Zeiss LSM510 Axioplan confocal microscope.

In situ hybridization

Whole-mount in situ hybridization was performed based on the method described by Wilkinson (Wilkinson and Nieto, 1993). Digoxygenin-UTP labeled antisense riboprobes were prepared from the following templates *Wnt11* (*Xho*I/T3), *Ret* (*Bam*HI/T7), *Pax2* (*Xba*I/T3), *Emx2*

(*Eco*RI/T7). The *Gdnf* antisense probe (*Hin*dIII/SP6) was made from pcDNA3/Gdnf originally cloned by Andreas Zimmer.

In all hybridization experiments, only kidneys from same stage embryos were used. Embryos were staged according to the lung branching pattern and only embryos with the same stage of lung branching were used. At this stage of lung development, the medial, caudal and accessory bronchi of the right lobe, and the left lobe main bronchus are clearly visible. Wild-type and mutant kidneys were pooled together during all steps of the protocol to ensure that they were exposed to identical experimental conditions. Wild-type and mutant kidneys were distinguished based on attachment to entire gonad (wild-type kidney) or half a gonad (mutant kidney). After color development, kidneys were washed in PBT (PBS + 0.1% Tween-20), fixed in 4% paraformaldehyde, dehydrated in methanol and photographed in benzyl alcohol:benzyl benzoate (1:1). Images were captured with a Nikon DXM1200 digital camera on a Nikon SMZ1500 stereoscope and assembled using Photoshop 7.0.

RESULTS

Wnt11 targeting

The *Wnt11* gene is spread over seven exons (Fig. 1A). The *Wnt11* locus was mutated by targeted deletion of exons IV and V in mouse 129 ES cells (Fig. 1A). Homologous recombinant 129 ES cells were isolated and used to generate chimeric mice. Germline integrants were identified and bred to homozygosity. *Wnt11* homozygous mutant mice (*Wnt11*$^{-/-}$) were identified by Southern blot and genomic PCR analyses (Fig. 1B). Southern blot analysis was also used to confirm the absence of exons IV and V in the targeted allele.

Analysis of the *Wnt11* allele

RT-PCR analysis on RNA from *Wnt11*$^{-/-}$ P1 kidneys identified a cDNA of ~1.4 kb in agreement with the predicted size of a transcript resulting from deletion of exons IV and V (Fig. 1C). In *Wnt11* mutants, a stable transcript is made containing exons I-III upstream and exons VI and VII downstream of the targeted deletion. Sequence analysis of the *Wnt11* mutant cDNA demonstrated the fusion of exon III to exon VI, and conceptual translation of the open reading frame predicts that only the N-terminal 28 amino acids, including the signal peptide sequence, matches the wild-type sequence while the reading frame downstream of the deletion is out of frame (Fig. 1D). Thus, the targeted allele is expected to eliminate wild-type *Wnt11* function.

Wnt11$^{-/-}$ mutants show lethality in utero

All *Wnt11*$^{-/-}$ mutant pups died by 2 days post-partum (pp). In addition, of 152 genotyped pups, only 13% were *Wnt11*$^{-/-}$ indicating an earlier lethality (Table 1). Analysis of E12.5 embryos revealed a statistically significant (χ^2 test, P<0.001) deviation from expected Mendelian ratios. The cause of the early lethality was not investigated but could correlate with potential roles for *Wnt11* in node and cardiac signaling that has been associated with axis elongation and cardiac morphogenesis in zebrafish (Heisenberg et al., 2000) and *Xenopus* (Pandur et al., 2002) embryogenesis, respectively.

Smaller kidneys in *Wnt11* mutants

Examination of *Wnt11*$^{-/-}$ genitourinary systems from newborn pups revealed that *Wnt11*$^{-/-}$ mutant kidneys have 64% the number of glomeruli compared with their wild-type littermates (P=0.0001) whereas *Wnt11*$^{+/-}$ kidneys were normal (Fig. 2 and Table 2). The smaller kidney phenotype was completely penetrant. Despite the size difference, *Wnt11*$^{-/-}$ kidneys were histologically normal with normal nephron organization along the corticomedullary axis (Fig. 2C,D). The smaller, but otherwise normal, kidneys suggested that *Wnt11* signaling at

Table 1. In utero lethality occurs in *Wnt11*$^{+/-}$ intercrosses

	Genotype		
Age	+/+ (%)	+/– (%)	–/– (%)
E9.5 (*n*=38)	18	63	18
E12.5 (*n*=361)	24	59	17
E18.5/P1 (*n*=152)	32	55	13

The frequencies of *Wnt11* genotypically wild type (*Wnt11*$^{+/+}$), heterozygous (*Wnt11*$^{+/-}$) and mutant homozygous (*Wnt11*$^{-/-}$) embryos at E9.5, E12.5 and combined E18.5/P1 pups are shown. Frequencies were determined by Southern blot and PCR genotyping. *Wnt11*$^{-/-}$ are first recovered in non-Mendelian ratios beginning at E12.5 (χ^2 test, P<0.001). The frequencies of *Wnt11*$^{-/-}$ continue to decline by P1. Homozygous newborn *Wnt11*$^{-/-}$ animals all die within 48 hours of birth for unknown reasons.

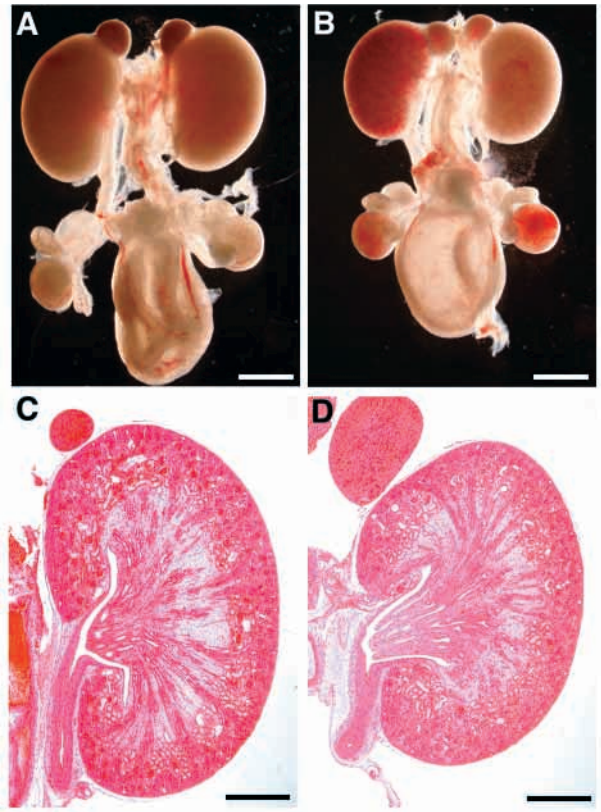

Fig. 2. *Wnt11*$^{-/-}$ mutants have smaller kidneys. Comparison of urogenital systems from wild-type (A) and *Wnt11*$^{-/-}$ mutant (B) P1 pups shows *Wnt11*$^{-/-}$ mutants have reduced kidney size. Hematoxylin/Eosin staining of coronal 6 μm sections from wild-type (C) and *Wnt11*$^{-/-}$ mutant (D) kidneys is shown. In *Wnt11*$^{-/-}$ kidneys, gross cortico-medullary patterning and epithelial integrity appears normal and the ureteric epithelium has undergone extensive branching. Sections are made at the level of the pelvis. Scale bars: 1 mm in A,B; 500 μm in C,D.

Table 2. *Wnt11*$^{-/-}$ kidneys have reduced numbers of nephrons

Genotype	Number of glomeruli (±s.d.)	*n*
Wnt11$^{+/+}$	1934±148	6
Wnt11$^{+/-}$	1804±52	4
Wnt11$^{-/-}$	1237±135	6

In order to quantify kidney size, the numbers of nephron glomeruli in *Wnt11*$^{+/+}$, *Wnt11*$^{+/-}$ and *Wnt11*$^{-/-}$ P1 kidneys were counted from 6 μm Hematoxylin and Eosin stained sections. *Wnt11*$^{-/-}$ kidneys contain ~64% (P=0.0001) the number of glomeruli compared with *Wnt11*$^{+/+}$. *Wnt11*$^{+/-}$ kidneys are not statistically significantly different in size from genotypically wild-type kidneys.

the tips of ureteric branches may be required for normal branching.

Wild-type branching pattern

The early stages of ureteric branching morphogenesis in vivo have not been previously described in detail (for reviews, see Sariola and Sainio, 1997; al-Awqati and Goldberg, 1998; Davies and Davey, 1999). In order to better understand ureteric branching morphogenesis and the role of *Wnt11* in this process, we visualized the time course of ureteric branching morphogenesis using whole-mount in situ hybridization with a *Ret* antisense probe (Fig. 3A-F), a marker strongly upregulated in the ureteric tips (Pachnis et al., 1993), and a YFP reporter protein that was specifically expressed within the ureteric epithelium in response to a ureteric epithelial specific *HoxB7 Cre* transgene (Fig. 3G-I) (Srinivas et al., 1999; Yu et al., 2002).

In the mouse, the Wolffian duct-derived ureteric bud invades the metanephric mesenchyme on E10.5. By E11.5, the ureteric bud has undergone a single branching event giving rise to two ampullae or the 'T stage'. By E12.0, each ampulla has a triangular shape with *Ret* and *Wnt11* expression increased at the tip vertices (arrowheads in Fig. 3A,D,G). The regions of increased *Ret* expression pre-figure the appearance of new ureteric tips. By E12.25, each ampulla is undergoing a trifurcation to give rise to a total of six new ureteric buds (Fig. 3B,E,H). The trifurcation is a stereotyped branching event. These trifurcations appear to give rise to three ureteric tips simultaneously and do not appear to result from rapid sequential bifurcations. In 42% of E12.5 kidneys, a seventh ampulla emerges from the bifurcation point of the T (arrowheads in insets in Fig. 3; Table 3). *Ret* and *Wnt11* are expressed at high levels in the six newly forming ureteric tips and are downregulated in the stems. By E12.5, each of these six morphologically distinct tips start to undergo a round of dichotomous branching (Fig. 3C,F,I). *Ret* and *Wnt11* continue to be highly expressed in the six ureteric tips. We have concentrated our analysis of kidney branching morphogenesis between the E11.5 and E12.5 stages because at this time the individual ureteric tips are easily identified and the kidney branching pattern is readily discernable.

Defects in the *Wnt11*$^{-/-}$ ureteric branching pattern

The α-cytokeratin antibody stains renal epithelia and, prior to the formation of mature nephrons, visualizes the early

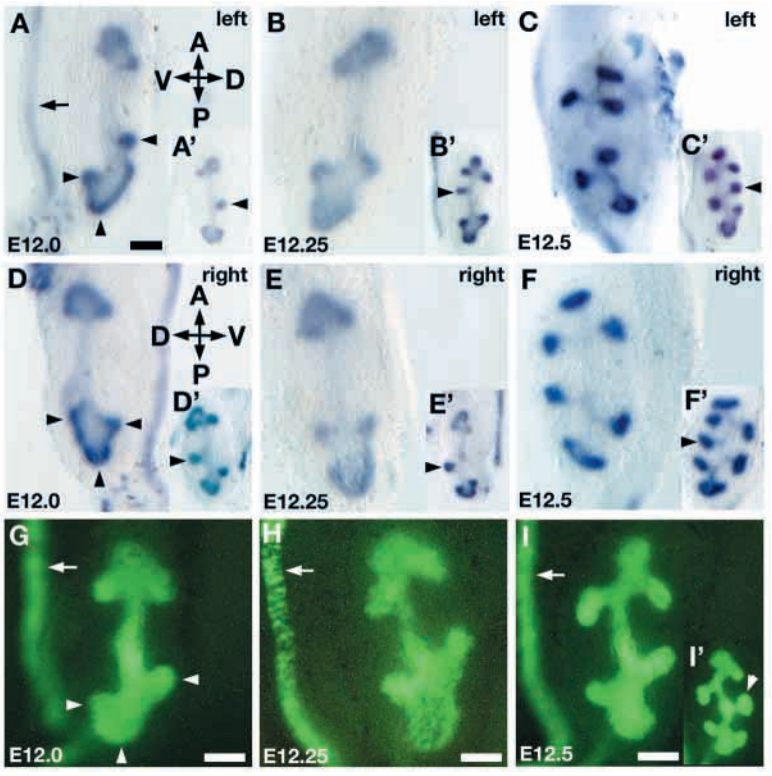

Fig. 3. Timecourse of ureteric branching during wild-type development. Wild-type left (A,A′,B,B′,C,C′) and right (D,D′,E,E′,F,F′) E12.0, E12.25 and E12.5 kidneys were stained with *Ret* antisense in situ probe. *Ret* is expressed specifically in the ureteric epithelium and is used here to visualize the collecting duct system. *Ret* is expressed at higher levels in forming ureteric tips and at lower levels in stems. In A, arrow indicates the Wolffian duct. *Ret* staining at these stages visualizes the trifurcation event that gives rise to new ureteric tips. At E12.0 (A,D), *Ret* is expressed in the two ampullae at the T stage. *Ret* expression appears pronounced in cells at the vertices of the triangle-shaped ampullae (arrowheads). At E12.5 (B,E), *Ret* is expressed most strongly at each of the three vertices of the emerging in the trifurcation. By E12.5 (C,F), *Ret* remains strongly expressed in the new morphologically distinct tips. In ~42% of kidneys, a seventh ureteric tip emerges (arrowheads in insets A′-F′,I′) and undergoes morphogenesis to become a distinct tip. The entire branching ureteric epithelium is visualized in kidneys from *Hoxb7 Cre*; *Rosa26 YFP* embryos (G-I,I′). Arrows indicate the Wolffian duct. Ureteric specific *YFP* expression visualizes the emerging tips of the trifurcation and their morphogenesis into distinct tips. In I′, an example is show where a seventh tip emerges from the original point of bifurcation during the T stage (arrowhead). In addition, the metanephric kidney has undergone a rotation during these stages such that the mediolateral axis present at E11.5 has translated to a dorsoventral axis by E12.5.Both left and right kidneys show similar patterns of branching. Double-headed arrows in A and B indicate orientation [anterior (A), posterior (P), dorsal (D) and ventral (V)]. Scale bars: 100 μm.

Table 3. Quantification of ureteric tips loss in *Wnt11*$^{-/-}$ kidneys

Number of ureteric tips	Wild type (%) (n=57)	*Wnt11*$^{-/-}$ (%) (n=11)
8	5	0
7	42	0
6	49	0
5	4	64
4	0	36

Ureteric tips from E12.5 wild-type and Wnt11$^{-/-}$ kidneys hybridized with *Ret* antisense probe were counted. The percentage of kidneys (n=57 for wild type, 11 for mutant) with eight, seven, six, five or four tips is shown. *Wnt11*$^{-/-}$ kidneys show a statistically significant (P<0.001) difference from wild-type frequencies for the distribution of ureteric tips.

architecture of the branching ureteric duct network. Importantly, α-cytokeratin antibody stained kidneys from E12.5 *Wnt11*$^{-/-}$ embryos show loss of ureteric tips and some ampullae with abnormal morphology where ampullae do not appear to be well separated and continue to share a common lumen (data not shown). Quantification of ureteric tips at this stage in *Wnt11*$^{-/-}$ kidneys shows a statistically significant difference from wild type (χ^2 test, P<0.001; Table 3).

To address the branching phenotype more thoroughly, we examined expression of *Ret* and *Wnt11* at several stages. In all experiments, only kidneys from equivalent stage wild-type and mutant embryos were compared. Embryos were staged according to lung branching pattern whereby only embryos with the identical pattern of lung branching were used (see Materials and Methods). In E11.5 *Wnt11*$^{-/-}$ kidneys, the ureteric bud has undergone one round of branching giving rise to two ampullae by the T-stage of E11.5 suggesting that the timing of ureteric bud invasion into the mesenchyme and first branching event occur on schedule (Fig. 4A-D). At E12.0, the two ampullae of the T-stage appear smaller than those of wild-type kidneys possibly reflecting retarded growth (compare Fig. 4F with 4E). Each trifurcation is retarded in tip formation by E12.25 (Fig. 4I-L). *Wnt11* expression levels are markedly reduced in the tips of mutant kidneys, despite the fact that the *Wnt11* antisense probe is identical to sequences common to wild-type and *Wnt11* mutant transcripts 3′ of the targeted deletion. The early defects result in the loss of ureteric branches when these have clearly resolved from the trifurcation at E12.5, though *Ret* and *Wnt11* are strongly expressed at the branch points (Fig. 4M-P). Thus, the timecourse analysis of the *Wnt11*$^{-/-}$ kidney phenotype shows a retarded morphogenesis that results in a defect in branching trifurcation resulting in loss of ureteric tips. The loss of ureteric tips at these early stages is a likely explanation for the small kidney phenotype observed in *Wnt11*$^{-/-}$ newborns. Nevertheless, some ureteric tips do form in *Wnt11*$^{-/-}$ kidneys and continue to grow and branch during later kidney development, suggesting other signals may be operating to support continued ureteric branching (see Discussion).

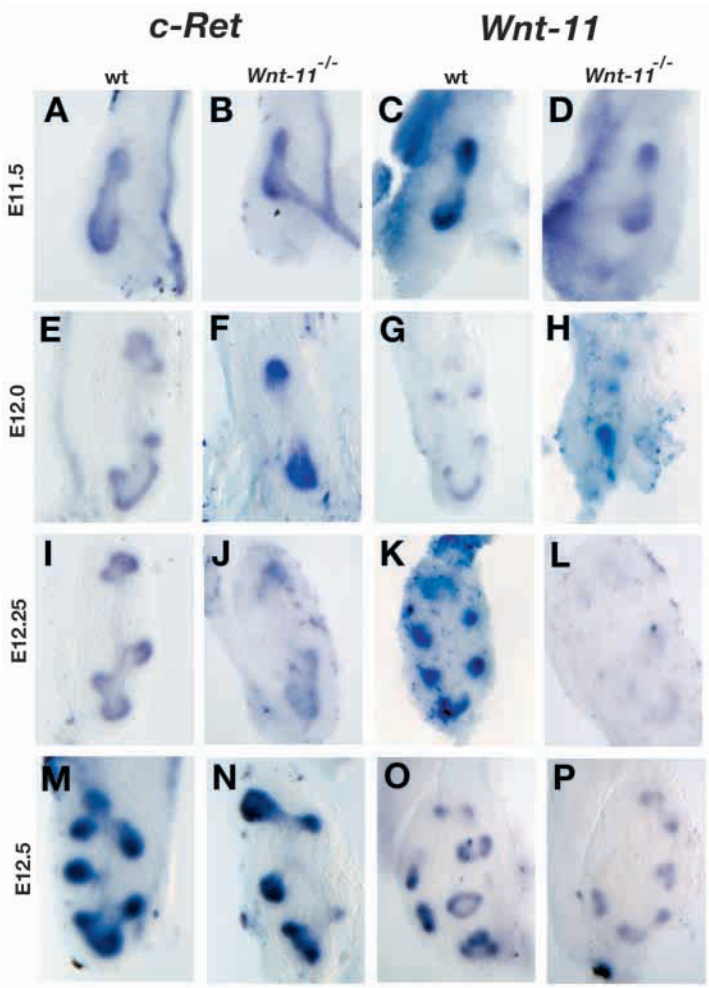

Fig. 4. Branching defects occur during the trifurcation event in *Wnt11*$^{-/-}$ kidneys. Wild-type (A,C,E,G,I,K,M,O) and *Wnt11*$^{-/-}$ mutant (B,D,F,H,J,L,N,P) kidneys at E11.5 (A-D), E12.0 (E-H), E12.25 (I-L) and E12.5 (M-P) were stained with *Ret* (A,B,E,F,I,J,M,N) or *Wnt11* (C,D,G,H,K,L,O,P) antisense in situ probes to visualize the branching process. By E11.5, ureteric bud invasion of mesenchyme and one round of branching have occurred in wild-type to generate the T stage. Ureteric invasion of mesenchyme occurs on schedule in *Wnt11*$^{-/-}$ kidneys (C,D). In wild type, both *Ret* and *Wnt11* are expressed strongly in the ureteric tips emerging from the trifurcation (E,I,G,K). In *Wnt11*$^{-/-}$ kidneys, *Ret* is expressed in the ureteric epithelium; however, the branching process appears retarded. E12.0 and E12.25 *Wnt11*$^{-/-}$ kidneys appear to be lagging behind in making new ureteric tips. In wild type E12.5 kidneys, *Ret* and *Wnt11* are expressed strongly in the new ureteric tips (M,O). In *Wnt11*$^{-/-}$ E12.5 kidneys, *Ret* and *Wnt11* expression patterns indicate loss of tips (compare N with M and P with O). For all these experiments, kidneys are taken from same stage embryos with identical lung branching pattern (see Materials and Methods). Kidneys are oriented anterior towards the top and posterior towards the bottom.

Wnt11 and *Ret*/*Gdnf* signaling in ureteric branching morphogenesis

As *Gdnf* is an important regulator of ureteric branching, we sought to determine whether *Gdnf* expression was normal in *Wnt11*$^{-/-}$ kidneys. In wild-type E12.5 kidneys, *Gdnf* is intensely expressed in mesenchymal cells surrounding the branching ureteric epithelium (Fig. 5A). Importantly, *Gdnf* expression is downregulated in *Wnt11*$^{-/-}$ kidneys at this time, suggesting that *Wnt11* expression in the ureteric epithelium is required for normal *Gdnf* expression in the

adjacent mesenchyme (Fig. 5B). *Gdnf* is expressed normally in earlier E12.0 *Wnt11*$^{-/-}$ kidneys (data not shown). By contrast, mesenchymal expression of *Pax2*, an important regulator of mesenchymal differentiation (Dressler et al., 1990; Torres et al., 1995), is relatively unaffected in E12.5 *Wnt11*$^{-/-}$ kidneys (compare Fig. 5C with 5D), suggesting that the reduction in Gdnf expression is specific.

We next determined whether *Wnt11* expression might be dependent upon *Ret*/*Gdnf* signaling in the developing collecting duct. The *Ret*/*Gdnf* pathway has been shown to play a crucial role in ureteric epithelial branching. The *Ret* targeted mutation, therefore, eliminates signaling in the kidney by all Gdnf family ligands. Ureteric bud invasion into the metanephric mesenchyme fails in most *Ret* and *Gdnf* mutants, resulting in kidney agenesis (Schuchardt et al., 1994; Schuchardt et al., 1996). However, in some *Ret*$^{-/-}$ mutants, the ureteric bud enters the metanephric mesenchyme and undergoes a single bifurcation event to generate the T-stage kidney by E12.0 (Schuchardt et al., 1996). Interestingly, *Wnt11* expression is dramatically reduced in these mutants (compare Fig. 5E with 5F), suggesting that ureteric *Wnt11* expression is dependent upon *Ret*/*Gdnf* signaling within the ureteric epithelium. By contrast, *Emx2* is expressed at comparable levels throughout the entire branching ureteric epithelium in wild-type and *Ret*$^{-/-}$ kidneys (compare Fig. 5G with 5H) (Miyamoto et al., 1997; Pellegrini et al., 1997). Thus, *Wnt11* is specifically downregulated in *Ret*$^{-/-}$ kidneys.

Fig. 5. *Wnt11* and *Ret/Gdnf* signals are mutually dependent. *Gdnf* is downregulated in *Wnt11*$^{-/-}$ kidneys. *Gdnf* expression in E12.5 wild-type (A) kidneys is found in the mesenchyme surrounding the non-staining ureteric epithelium. Mesenchymal *Gdnf* expression is reduced in E12.5 *Wnt11*$^{-/-}$ kidneys (B). By contrast, *Pax2* continues to be expressed in *Wnt11*$^{-/-}$ kidney mesenchyme at E12.5 (compare D with C). *Wnt11* expression is reduced in *Ret*$^{-/-}$ kidneys. *Wnt11* expression in wild-type (E) E12.0 kidneys marks the forming ureteric tips during the trifurcation stage. *Wnt11* expression is dramatically reduced in E12.0 *Ret*$^{-/-}$ kidneys (arrows in F). However, *Emx2* continues to be expressed in *Ret*$^{-/-}$ ureteric epithelium comparable with wild type (compare H with G). Kidneys are oriented anterior towards the top and posterior towards the bottom.

Genetic interactions between *Wnt11* and *Ret*

The observation that *Wnt11* and *Gdnf* expression levels are mutually interdependent in E12.5 kidneys, prompted us to ask whether *Wnt11* genetically interacts with members of the *Ret*/*Gdnf* pathway. We crossed the *Ret* mutation into the *Wnt11* genetic background to generate *Ret*$^{+/-}$; *Wnt11*$^{+/-}$ compound heterozygotes and *Ret*$^{+/-}$; *Wnt11*$^{-/-}$ mutant mice. As shown in Fig. 6 and Table 4, *Wnt11*$^{+/-}$; *Ret*$^{+/-}$ E18.5 kidneys are 52% (P=0.007) the size of same stage wild-type kidneys, indicating a genetic interaction between the *Wnt11* and *Ret* mutations in the compound heterozygote state (Fig. 6E,F). Removal of another copy of *Wnt11* demonstrates dose-dependent interactions between *Wnt11* and *Ret*. *Ret*$^{+/-}$; *Wnt11*$^{-/-}$ kidneys are 67% (P=0.0001) the size of *Ret*$^{+/-}$; *Wnt11*$^{+/-}$ and 44% (P=0.0008) the size of *Wnt11*$^{-/-}$ kidneys, again suggesting a genetic interaction between the *Ret*/*Gdnf* and *Wnt11* pathways (compare Fig. 6H with 6E-G). *Ret*$^{+/-}$ kidneys are not significantly different in size from genotypically wild-type kidneys (P=0.65).

We also investigated the pattern of ureteric branching at E12.5 in these genetic combinations using the *Ret* probe. The observation that ureteric branching in *Ret*$^{+/-}$; *Wnt11*$^{+/-}$ kidneys appears normal at E12.5, yet the E18.5 kidneys are smaller than wild-type controls suggests that both *Ret* and *Wnt11* are also required for branching morphogenesis throughout later embryonic kidney development after E12.5. Analysis of E12.5 ureteric branching morphogenesis using in situ hybridization with a *Ret* antisense probe reveals that ureteric branching is severely affected in *Ret*$^{+/-}$; *Wnt11*$^{-/-}$ kidneys (Fig. 6L). The E12.5 *Ret*$^{+/-}$; *Wnt11*$^{-/-}$ kidneys show branching defects that are more severe than those observed in *Wnt11*$^{-/-}$ kidneys (Fig. 6K). E12.5 kidneys from *Ret*$^{+/-}$; *Wnt11*$^{-/-}$ embryos have two to four ureteric tips compared with the seven found in wild type. Thus, reducing Ret activity appears to enhance the effects of a loss of *Wnt11* signaling on the branching process.

DISCUSSION

Wnt11 is required for normal kidney development

Our analysis reveals a genetic requirement for *Wnt11* in kidney ureteric branching morphogenesis. Previous studies on *Wnt11* during kidney development raised correlative evidence for *Wnt11* function in ureteric branching. First, *Wnt11* is expressed

Table 4. *Wnt11* and *Ret* mutations synergistically interact to yield kidney hypoplasia

Genotype	Number of glomeruli (±s.d.)	*n*
Ret$^{+/+}$; *Wnt11*$^{+/+}$	1358±258	4
Ret$^{+/-}$; *Wnt11*$^{+/+}$	1447±278	4
Ret$^{+/+}$; *Wnt11*$^{-/-}$	1055±161	4
Ret$^{+/-}$; *Wnt11*$^{+/-}$	702±40	5
Ret$^{+/-}$; *Wnt11*$^{-/-}$	469±131	10

Average total numbers of glomeruli in E18.5 kidneys from *Ret*;*Wnt11* double mutant combinations are shown. *Ret*$^{+/-}$;*Wnt11*$^{+/-}$ kidneys contain fewer glomeruli than wild-type controls. *Ret*$^{+/-}$;*Wnt11*$^{-/-}$ kidneys are much smaller than either *Ret*$^{+/-}$ or *Wnt11*$^{-/-}$ kidneys by themselves. *Ret*$^{+/-}$ kidneys are not statistically significantly different in size from genotypically wild-type controls. Glomeruli were counted from 6 μm coronal Hematoxylin/Eosin stained kidney sections.

at the tips of the ureteric epithelium where branching morphogenesis is occurring, suggesting that *Wnt11* may participate in regulating branching events (Kispert et al., 1996). Second, *Wnt11* expression directly correlates with genetically and chemically induced gain and loss of ureteric tips (Kispert et al., 1996; Pepicelli et al., 1997; Sainio et al., 1997). Our observations demonstrate that *Wnt11* is required for collecting duct development and suggest that *Wnt11* signaling is most probably one component of reciprocal signaling mechanisms that act between the ureteric epithelium and metanephric mesenchyme to regulate the *Ret/Gdnf* signaling pathway to control normal ureteric branching.

Initial ingrowth of the ureteric bud into the mesenchyme appears to be independent of *Wnt11* function as all *Wnt11* mutant kidneys examined had progressed to the T-stage on schedule. The progression of ureteric branching to the T-stage in *Wnt11*$^{-/-}$ mutants is associated with normal *Gdnf* expression (data not shown). However, an abnormal branching pattern comprised of retarded morphogenesis and loss or ureteric tips was observed in *Wnt11*$^{-/-}$ kidneys from the T stage. These branching defects are associated with a reduction in mesenchymal *Gdnf* expression. Given that Gdnf can function as a chemoattractant, low Gdnf levels may result in lower outgrowth promoting activity and decreased numbers of ureteric tips as is observed in *Wnt11*$^{-/-}$ mutants. The loss of ureteric tips early in metanephric development results in significantly smaller kidney size by birth.

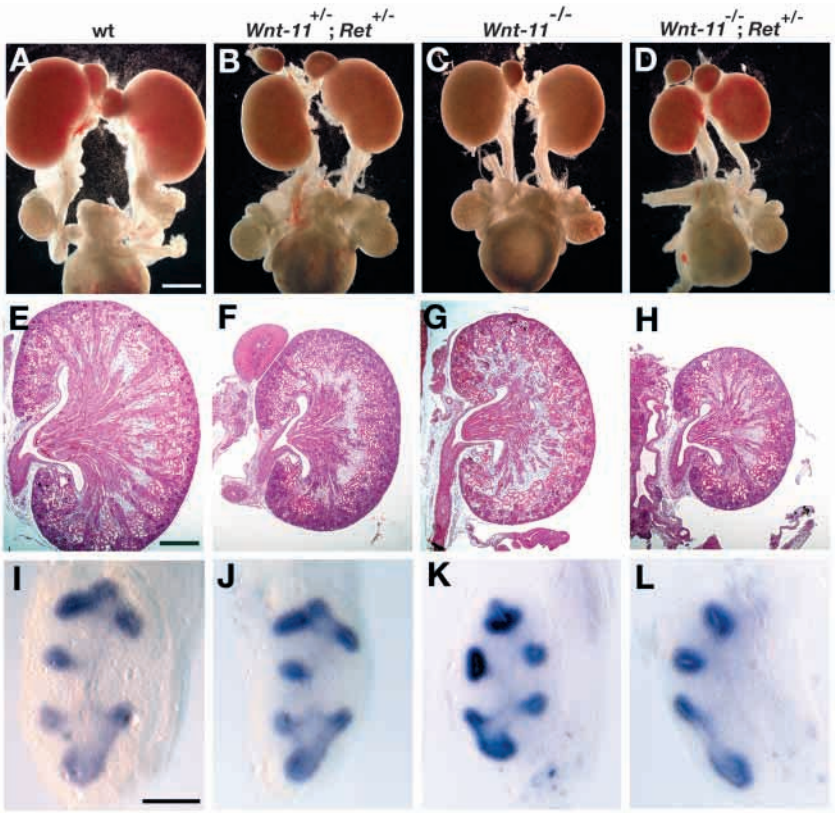

Fig. 6. Synergystic genetic interactions between *Wnt11* and *Ret*. Wild-type (A,E,I), *Ret*$^{+/-}$; *Wnt11*$^{+/-}$ (B,F,J), *Wnt11*$^{-/-}$ (C,G,K) and *Wnt11*$^{-/-}$; *Ret*$^{+/-}$ (D,H,L) kidneys are shown. (A-D) E18.5 urogenital systems. (E-H) Hematoxylin/Eosin staining of E18.5 kidney sections taken at the level of the pelvis. (I-L) E12.5 kidneys in situ hybridized to a *Ret* probe. *Wnt11*$^{+/-}$; *Ret*$^{+/-}$ kidneys are smaller than wild type (compare F with E), despite having a normal ureteric branching pattern at E12.5 (compare J with I). *Wnt11*$^{-/-}$ *Ret*$^{+/-}$ kidneys are smaller than wild type, *Wnt11*$^{-/-}$ or *Wnt11*$^{+/-}$; *Ret*$^{+/-}$ kidneys (compare H with E-G) and show much more severe branching defects at E12.5 (compare L with I-K). *Ret*$^{+/-}$ E12.5 kidneys appear wild type in size and in their branching pattern. In I-L, kidneys are oriented anterior towards the top and posterior towards the bottom, dorsal towards the left and ventral towards the right. Scale bars: in A, 1mm for A-D; in E, 500 μm for E-H; in I, 100 μm for I-L.

In wild type, *Wnt11* is expressed robustly in ureteric tips during all stages of metanephric development, suggesting a potential role for *Wnt11* in branching morphogenesis throughout kidney development. The defects in *Wnt11*$^{-/-}$ kidneys correlate with a trifurcation and trifurcations are observed at later stages of kidney development (A.M., unpublished), again pointing to a larger role for *Wnt11* in branching. Our analysis of *Wnt11* in a genetically sensitized *Ret*$^{+/-}$ background supports this hypothesis, as *Wnt11*$^{+/-}$; *Ret*$^{+/-}$ E18.5 kidneys are significantly reduced in size compared with controls, even though the branching pattern at E12.5 is indistinguishable from wild type. Furthermore, we observe that *Wnt11*$^{+/-}$; *Ret*$^{+/-}$ kidneys are smaller than *Wnt11*$^{-/-}$ kidneys, even though the branching at E12.5 in the compound heterozygotes is unaffected. This may suggest that ureteric branching is differentially sensitive to the level of Wnt11 and Ret/Gdnf signals at different times during kidney development. Thus, the genetic interaction studies in the sensitized *Ret*$^{+/-}$ background reveal a wider requirement for *Wnt11* in ureteric branching beyond the stages analyzed here.

Although it is clear that *Wnt11* is required for normal ureteric branching, considerable branching morphogenesis still occurs in *Wnt11*$^{-/-}$ mutant kidneys. As the targeted allele encodes only the first 28 amino acids of the total 354 amino acid wild-type Wnt11 ligand, our *Wnt11* allele most probably encodes a nonfunctional peptide. Thus, no residual Wnt11 signaling should remain in *Wnt11*$^{-/-}$ kidneys.

A second possibility that might explain the branching in *Wnt11*$^{-/-}$ kidneys is the functional redundancy of another Wnt in the ureteric epithelium. Though *Gdnf* expression is reduced in E12.5 *Wnt11*$^{-/-}$ kidneys, *Gdnf* expression appears normal by E13.5 (data not shown) suggesting that the kidney has invoked a compensatory mechanism to support continued branching in the absence of *Wnt11* activity. Although other Wnt genes, including *Wnt7b*. *Wnt6* and *Wnt15* are expressed in the branching ureter proximal to the *Wnt11* domain, none extend into the ureteric tips themselves nor do any of these Wnt expression domains alter in the ureteric epithelium in *Wnt11*$^{-/-}$ kidneys. Of these, *Wnt6* is weakly expressed throughout the ureteric epithelium at the stages studied here. However, renal tubulogenesis induction assays suggest that *Wnt11* and *Wnt6* have different activities (Kispert et al., 1998; Itaranta et al., 2002).

An alternative explanation for branching in *Wnt11*$^{-/-}$ kidneys is the influence of other functionally redundant signaling pathways regulating branching. Multiple fibroblast growth factor (Fgf) ligands and their receptors are expressed during metanephric development and can modulate ureteric branching (Qiao et al., 2001). Among the Fgfs, mesenchymally expressed Fgf7 has been proposed as a modulator of ureteric growth and branching (Qiao et al., 1999). Like *Wnt11*, *Fgf7* does not appear to be required for ureteric bud invasion into the mesenchyme, but is required for subsequent elaboration of the collecting duct system, as *Fgf7*$^{-/-}$ mutants have normally patterned but smaller kidneys. Whether *Gdnf* expression is dependent upon *Fgf7* is not known. Kidney culture experiments have shown that members of the TGFβ and bone morphogenetic protein families can also modulate ureteric branching (Grisaru et al., 2001; Piscione et al., 1997). Finally, the stroma is known to provide signals promoting ureteric *Ret* expression and ureteric outgrowth (Batourina et al., 2001; Mendelsohn et al., 1999). *Wnt2b* is expressed at sites of epithelial/mesenchymal interaction in multiple organs (Lin et al., 2001). In the kidney, *Wnt2b* is expressed in the presumptive stromal cell population. In kidney explant culture experiments, incubation of ureteric buds with NIH3T3 cells expressing *Wnt2b* results in increased ureteric branching. This result has been interpreted as evidence that *Wnt2b* present in the stroma promotes, either directly or indirectly, branching of the ureteric epithelium. *Wnt2b* mutants have not been reported. Therefore, it is likely that multiple signaling pathways acting from different cellular populations are integrated by the ureteric epithelium and metanephric mesenchyme to maintain appropriate *Ret/Gdnf* signal levels to support collecting duct morphogenesis, and one such signal appears to be *Wnt11*.

The *silberblick* (*slb*) mutation demonstrates a requirement for *Wnt11* in regulating convergence/extension movements during zebrafish gastrulation (Heisenberg et al., 2000) and *Wnt11* appears to have a similar role in *Xenopus* (Djiane et al., 2000; Tada and Smith, 2000). *Wnt11* is thought to signal through a planar cell polarity (PCP) pathway to regulate cytoskeletal rearrangements, thus coordinating polarized cell movement during vertebrate gastrulation. Recently, a role for *Wnt11* PCP signaling has been demonstrated in *Xenopus* cardiogenesis (Pandur et al., 2002). We found no similar absolute requirement for *Wnt11* in either mouse gastrulation or cardiac development. This may reflect a difference in the genetic regulation of gastrulation between mouse and zebrafish or it may reflect a functional redundancy in mouse. Although *Wnt11* is required for viability during the embryonic and post-partum stages, these lethalities do not arise from the kidney defects we describe here.

Although our analysis advances *Wnt11* as a modulator *of Ret/Gdnf* signaling, *Wnt11* may have other roles in the branching process. *Wnt11* PCP signaling employs Rho kinase 2 (Rok2), Rho GTPase and Jun N-terminal kinase (JNK) to effect changes in actin cytoskeleton organization (Marlow et al., 2002; Mlodzik, 2002). In the kidney, *Wnt11* may regulate branching morphogenesis by causing cytoskeletal reorganization within the plane of the ureteric epithelium. These additional roles for *Wnt11* in ureteric branching await further investigation.

Wnt11 and *Ret*/*Gdnf* signals cooperate in a regulatory circuit to control ureteric branching morphogenesis

Three observations suggest that *Wnt11* and *Ret*/*Gdnf* signals cooperate to regulate ureteric branching morphogenesis. First, mesenchymal *Gdnf* expression is dependent upon ureteric *Wnt11* signal. Second, *Wnt11* expression is reciprocally dependent upon *Ret*/*Gdnf* signaling within the ureteric epithelium. Third, *Wnt11* and *Ret* mutants synergistically interact during ureteric branching morphogenesis, suggesting both pathways are functioning cooperatively and inter-dependently in a common branching process.

What is not clear is whether *Wnt11* acts as a paracrine factor to regulate Gdnf expression directly in the metanephric mesenchyme or if *Wnt11* itself is a direct transcriptional target of the *Ret/Gdnf* signaling pathway. Mesenchymal *Gdnf* expression is known to be dependent upon at least two transcription factors, *Pax2* and *Sal1* (Brophy et al., 2001; Miyamoto et al., 1997; Nishinakamura et al., 2001). Indeed, cell culture experiments and analysis of cis-regulatory regions in the *Gdnf* gene indicate that *Pax2* may be a direct regulator of *Gdnf* expression (Brophy et al., 2001). Wnt-mediated regulation of Pax gene expression in the kidney has been reported in *Wnt4* mutants where *Pax8* and *Pax2* expressions are absent in the pre-tubular aggregates (Stark et al., 1994). We failed to observe any obvious alteration in *Pax2* levels in *Wnt11*$^{-/-}$ kidneys.

In addition, our results suggest that *Wnt11* expression is dependent upon *Ret/Gdnf* signaling within the ureteric epithelium and the *Wnt11* locus may therefore be a downstream target of *Ret/Gdnf* signaling, consistent with our earlier observations where implantation of Gdnf-coated beads into kidney explant cultures significantly upregulated *Wnt11* expression (Pepicelli et al., 1997; Sainio et al., 1997). Upon ligand binding, Ret activates multiple downstream signaling pathways (reviewed by Airaksinen and Saarma, 2002; Manie et al., 2001). Inhibition of PI-3 kinase activity with the small molecule LY294002 prevented Gdnf-induced ectopic ureteric outgrowth in kidney explant culture, implicating PI-3 kinase signaling in ureteric morphogenesis (Tang et al., 2002). Whether *Wnt11* expression is altered in these experiments has not been addressed.

The genetic interactions observed in *Ret*$^{+/-}$; *Wnt11*$^{+/-}$ kidneys suggest that the *Wnt11* and *Ret/Gdnf* signaling pathways function serially and not in parallel. The *Wnt11* and *Ret*/*Gdnf* signals may participate in a positive, autoregulatory feedback loop to coordinate ureteric branching by maintaining a balance between appropriate amounts of *Gdnf*-expressing mesenchyme with *Wnt11*-expressing ureteric tips. *Wnt11* levels may inform the mesenchyme as to the number of ureteric buds present. Therefore, this regulatory network may function as a counting mechanism for the developing kidney to determine the extent of branching, convey this information to the mesenchyme and respond with a matching level of outgrowth-promoting *Gdnf*.

Wnt genes and branching morphogenesis

Other Wnt genes have also been proposed to play roles in branching morphogenesis. In addition to *Wnt2b* (discussed earlier), the *Wnt4*$^{-/-}$ knockout mouse has been used to demonstrate a requirement for *Wnt4* function in progesterone

induced mammary epithelium branching morphogenesis during pregnancy (Brisken et al., 2000). However, substantial branching still occurs in grafted *Wnt4*$^{-/-}$ ductal tissue at later stages of pregnancy, implying that *Wnt4* may act in concert with other Wnt genes in this tissue. Although past studies of Wnt genes have focused on their roles in growth and patterning, future investigations may uncover other examples of these genes in morphogenetic processes during vertebrate development.

We are grateful for the technical support of Mike Rule and Diane Faria. In particular, we are grateful to Harvard Kidney Consortium (HKC) members Tom Carroll, Jing Yu and M. Todd Valerius. We thank F. Costantini for the pcDNA3/Gdnf clone and the *Ret* mutant and *Rosa26 YFP* reporter mouse strains. We thank Frank Riikka Knuuti for bioinformatics. We are grateful to the mouse maintenance crew at MCB for upkeep of mice. A.M. is supported by a Charles A. King Fellowship from The Medical Foundation. S.V. was supported by the Academy of Finland (#41001 and 46146), the Sigrid Juselius Foundation, the European Union (project QLRT-2000-01275) and by EMBO. Work in A.P.M.'s laboratory is supported by a grant from the NIH (R01DK54364).

REFERENCES

Airaksinen, M. S. and Saarma, M. (2002). The GDNF family: signalling, biological functions and therapeutic value. *Nat. Rev. Neurosci.* **3**, 383-394.

al-Awqati, Q. and Goldberg, M. R. (1998). Architectural patterns in branching morphogenesis in the kidney. *Kidney Int.* **54**, 1832-1842.

Batourina, E., Gim, S., Bello, N., Shy, M., Clagett-Dame, M., Srinivas, S., Costantini, F. and Mendelsohn, C. (2001). Vitamin A controls epithelial/mesenchymal interactions through Ret expression. *Nat. Genet.* **27**, 74-78.

Bhanot, P., Brink, M., Samos, C. H., Hsieh, J. C., Wang, Y., Macke, J. P., Andrew, D., Nathans, J. and Nusse, R. (1996). A new member of the frizzled family from Drosophila functions as a Wingless receptor. *Nature* **382**, 225-230.

Brisken, C., Heineman, A., Chavarria, T., Elenbaas, B., Tan, J., Dey, S. K., McMahon, J. A., McMahon, A. P. and Weinberg, R. A. (2000). Essential function of Wnt-4 in mammary gland development downstream of progesterone signaling. *Genes Dev.* **14**, 650-654.

Brophy, P. D., Ostrom, L., Lang, K. M. and Dressler, G. R. (2001). Regulation of ureteric bud outgrowth by Pax2-dependent activation of the glial derived neurotrophic factor gene. *Development* **128**, 4747-4756.

Bullock, S. L., Fletcher, J. M., Beddington, R. S. and Wilson, V. A. (1998). Renal agenesis in mice homozygous for a gene trap mutation in the gene encoding heparan sulfate 2-sulfotransferase. *Genes Dev.* **12**, 1894-1906.

Cacalano, G., Farinas, I., Wang, L. C., Hagler, K., Forgie, A., Moore, M., Armanini, M., Phillips, H., Ryan, A. M., Reichardt, L. F. et al. (1998). GFRα1 is an essential receptor component for GDNF in the developing nervous system and kidney. *Neuron* **21**, 53-62.

Davies, J. (2001). Intracellular and extracellular regulation of ureteric bud morphogenesis. *J. Anat.* **198**, 257-264.

Davies, J. A. and Bard, J. B. (1998). The development of the kidney. *Curr. Top. Dev. Biol.* **39**, 245-301.

Davies, J. A. and Davey, M. G. (1999). Collecting duct morphogenesis. *Pediatr. Nephrol.* **13**, 535-541.

Djiane, A., Riou, J., Umbhauer, M., Boucaut, J. and Shi, D. (2000). Role of frizzled 7 in the regulation of convergent extension movements during gastrulation in Xenopus laevis. *Development* **127**, 3091-3100.

Dressler, G. R., Deutsch, U., Chowdhury, K., Nornes, H. O. and Gruss, P. (1990). Pax2, a new murine paired-box-containing gene and its expression in the developing excretory system. *Development* **109**, 787-795.

Durbec, P., Marcos-Gutierrez, C. V., Kilkenny, C., Grigoriou, M., Wartiowaara, K., Suvanto, P., Smith, D., Ponder, B., Costantini, F., Saarma, M. et al. (1996). GDNF signalling through the Ret receptor tyrosine kinase. *Nature* **381**, 789-793.

Enomoto, H., Araki, T., Jackman, A., Heuckeroth, R. O., Snider, W. D., Johnson, E. M., Jr and Milbrandt, J. (1998). GFR α1-deficient mice have deficits in the enteric nervous system and kidneys. *Neuron* **21**, 317-324.

Grisaru, S., Cano-Gauci, D., Tee, J., Filmus, J. and Rosenblum, N. D. (2001). Glypican-3 modulates BMP- and FGF-mediated effects during renal branching morphogenesis. *Dev. Biol.* **231**, 31-46.

Grobstein, C. (1953). Inductive epithelio-mesenchymal interactions in cultured organ rudiments of the mouse. *Science* **118**, 52-55.

Heisenberg, C. P., Tada, M., Rauch, G. J., Saude, L., Concha, M. L., Geisler, R., Stemple, D. L., Smith, J. C. and Wilson, S. W. (2000). Silberblick/Wnt11 mediates convergent extension movements during zebrafish gastrulation. *Nature* **405**, 76-81.

Itaranta, P., Lin, Y., Perasaari, J., Roel, G., Destree, O. and Vainio, S. (2002). Wnt-6 is expressed in the ureter bud and induces kidney tubule development in vitro. *Genesis* **32**, 259-268.

Kispert, A., Vainio, S., Shen, L., Rowitch, D. H. and McMahon, A. P. (1996). Proteoglycans are required for maintenance of Wnt-11 expression in the ureter tips. *Development* **122**, 3627-3637.

Kispert, A., Vainio, S. and McMahon, A. P. (1998). Wnt-4 is a mesenchymal signal for epithelial transformation of metanephric mesenchyme in the developing kidney. *Development* **125**, 4225-4234.

Kume, T., Deng, K. and Hogan, B. L. (2000). Murine forkhead/winged helix genes Foxc1 (Mf1) and Foxc2 (Mfh1) are required for the early organogenesis of the kidney and urinary tract. *Development* **127**, 1387-1395.

Lechner, M. S. and Dressler, G. R. (1997). The molecular basis of embryonic kidney development. *Mech. Dev.* **62**, 105-120.

Lin, Y., Liu, A., Zhang, S., Ruusunen, T., Kreidberg, J. A., Peltoketo, H., Drummond, I. and Vainio, S. (2001). Induction of ureter branching as a response to Wnt-2b signaling during early kidney organogenesis. *Dev. Dyn.* **222**, 26-39.

Manie, S., Santoro, M., Fusco, A. and Billaud, M. (2001). The RET receptor: function in development and dysfunction in congenital malformation. *Trends Genet.* **17**, 580-589.

Mansour, S. L., Thomas, K. R. and Capecchi, M. R. (1988). Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. *Nature* **336**, 348-352.

Marlow, F., Topczewski, J., Sepich, D. and Solnica-Krezel, L. (2002). Zebrafish rho kinase 2 acts downstream of wnt11 to mediate cell polarity and effective convergence and extension movements. *Curr. Biol.* **12**, 876-884.

Mendelsohn, C., Batourina, E., Fung, S., Gilbert, T. and Dodd, J. (1999). Stromal cells mediate retinoid-dependent functions essential for renal development. *Development* **126**, 1139-1148.

Miyamoto, N., Yoshida, M., Kuratani, S., Matsuo, I. and Aizawa, S. (1997). Defects of urogenital development in mice lacking Emx2. *Development* **124**, 1653-1664.

Mlodzik, M. (2002). Planar cell polarization: do the same mechanisms regulate Drosophila tissue polarity and vertebrate gastrulation? *Trends Genet* **18**, 564-571.

Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W. and Roder, J. C. (1993). Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc. Natl. Acad. Sci. USA* **90**, 8424-8428.

Nishinakamura, R., Matsumoto, Y., Nakao, K., Nakamura, K., Sato, A., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Scully, S., Lacey, D. L. et al. (2001). Murine homolog of SALL1 is essential for ureteric bud invasion in kidney development. *Development* **128**, 3105-3115.

Pachnis, V., Mankoo, B. and Costantini, F. (1993). Expression of the c-ret proto-oncogene during mouse embryogenesis. *Development* **119**, 1005-1017.

Pandur, P., Lasche, M., Eisenberg, L. M. and Kuhl, M. (2002). Wnt-11 activation of a non-canonical Wnt signalling pathway is required for cardiogenesis. *Nature* **418**, 636-641.

Pellegrini, M., Pantano, S., Lucchini, F., Fumi, M. and Forabosco, A. (1997). Emx2 developmental expression in the primordia of the reproductive and excretory systems. *Anat. Embryol.* **196**, 427-433.

Pepicelli, C. V., Kispert, A., Rowitch, D. H. and McMahon, A. P. (1997). GDNF induces branching and increased cell proliferation in the ureter of the mouse. *Dev. Biol.* **192**, 193-198.

Piscione, T. D., Yager, T. D., Gupta, I. R., Grinfeld, B., Pei, Y., Attisano, L., Wrana, J. L. and Rosenblum, N. D. (1997). BMP-2 and OP-1 exert direct and opposite effects on renal branching morphogenesis. *Am. J. Physiol.* **273**, F961-F975.

Qiao, J., Bush, K. T., Steer, D. L., Stuart, R. O., Sakurai, H., Wachsman, W. and Nigam, S. K. (2001). Multiple fibroblast growth factors support

growth of the ureteric bud but have different effects on branching morphogenesis. *Mech. Dev.* **109**, 123-135.

Qiao, J., Uzzo, R., Obara-Ishihara, T., Degenstein, L., Fuchs, E. and Herzlinger, D. (1999). FGF-7 modulates ureteric bud growth and nephron number in the developing kidney. *Development* **126**, 547-554.

Sainio, K., Suvanto, P., Davies, J., Wartiovaara, J., Wartiovaara, K., Saarma, M., Arumae, U., Meng, X., Lindahl, M., Pachnis, V. et al. (1997). Glial-cell-line-derived neurotrophic factor is required for bud initiation from ureteric epithelium. *Development* **124**, 4077-4087.

Sanchez, M. P., Silos-Santiago, I., Frisen, J., He, B., Lira, S. A. and Barbacid, M. (1996). Renal agenesis and the absence of enteric neurons in mice lacking GDNF. *Nature* **382**, 70-73.

Sariola, H. and Saarma, M. (1999). GDNF and its receptors in the regulation of the ureteric branching. *Int. J. Dev. Biol.* **43**, 413-418.

Sariola, H. and Sainio, K. (1997). The tip-top branching ureter. *Curr. Opin. Cell Biol.* **9**, 877-884.

Saxen, L. (1987). *Organogenesis of the Kidney*. New York: Cambridge University Press.

Schuchardt, A., D'Agati, V., Larsson-Blomberg, L., Costantini, F. and Pachnis, V. (1994). Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. *Nature* **367**, 380-383.

Schuchardt, A., D'Agati, V., Pachnis, V. and Costantini, F. (1996). Renal agenesis and hypodysplasia in ret-k- mutant mice result from defects in ureteric bud development. *Development* **122**, 1919-1929.

Srinivas, S., Wu, Z., Chen, C. M., D'Agati, V. and Constantini, F. (1999). Expression of green fluorescent protein in the ureteric bud of transgenic mice: a new tool for the analysis of ureteric bud morphogenesis. *Development* **126**, 1375-1386.

Srinivas, S., Watanabe, T., Lin, C. S., William, C. M., Tanabe, Y., Jessell, T. M. and Costantini, F. (2001). Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *Dev. Biol.* **1**, 4.

Stark, K., Vainio, S., Vassileva, G. and McMahon, A. P. (1994). Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by Wnt-4. *Nature* **372**, 679-683.

Stark, K., Vainio, S., Vassileva, G. and McMahon, A. P. (1994). Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by Wnt-4. *Nature* **372**, 679-683.

Swiatek, P. J., Lindsell, C. E., del Amo, F. F., Weinmaster, G. and Gridley, T. (1994). Notch1 is essential for postimplantation development in mice. *Genes Dev.* **8**, 707-719.

Tada, M. and Smith, J. C. (2000). Xwnt11 is a target of Xenopus Brachyury: regulation of gastrulation movements via Dishevelled, but not through the canonical Wnt pathway. *Development* **127**, 2227-2238.

Tang, M. J., Worley, D., Sanicola, M. and Dressler, G. R. (1998). The RET-glial cell-derived neurotrophic factor (GDNF) pathway stimulates migration and chemoattraction of epithelial cells. *J. Cell Biol.* **142**, 1337-1345.

Tang, M. J., Cai, Y., Tsai, S. J., Wang, Y. K. and Dressler, G. R. (2002). Ureteric bud outgrowth in response to RET activation is mediated by phosphatidylinositol 3-kinase. *Dev. Biol.* **243**, 128-136.

Torres, M., Gomez-Pardo, E., Dressler, G. R. and Gruss, P. (1995). Pax-2 controls multiple steps of urogenital development. *Development* **121**, 4057-4065.

Vega, Q. C., Worby, C. A., Lechner, M. S., Dixon, J. E. and Dressler, G. R. (1996). Glial cell line-derived neurotrophic factor activates the receptor tyrosine kinase RET and promotes kidney morphogenesis. *Proc. Natl. Acad. Sci. USA* **93**, 10657-10661.

Wilkinson, D. G. and Nieto, M. A. (1993). Detection of messenger RNA by in situ hybridization to tissue sections and whole mounts. *Methods Enzymol.* **225**, 361-373.

Wodarz, A. and Nusse, R. (1998). Mechanisms of Wnt signaling in development. *Annu. Rev. Cell Dev. Biol.* **14**, 59-88.

Yu, J., Carroll, T. J. and McMahon, A. P. (2002). Sonic hedgehog regulates proliferation and differentiation of mesenchymal cells in the mouse metanephric kidney. *Development* **129**, 5301-5312.