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vHNF1 functions in distinct regulatory circuits to control ureteric bud branching and early nephrogenesis

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SUMMARY

Mouse metanephric kidney development begins with the induction of the ureteric bud (UB) from the caudal portion of the Wolffian duct by metanephric mesenchymal signals. While the UB undergoes branching morphogenesis to generate the entire urinary collecting system and the ureter, factors secreted by the UB tips induce surrounding mesenchymal cells to convert into epithelia and form the nephrons, the functional units of the kidney. Epithelial branching morphogenesis and nephrogenesis are therefore tightly orchestrated; defects in either of these processes lead to severe kidney phenotypes ranging from hypoplasia to complete aplasia. However, the underlying regulatory networks have been only partially elucidated. Here, we identify the transcription factor vHNF1 (HNF1β) as a crucial regulator of these early developmental events. Initially involved in timing outgrowth of the UB and subsequent branching, vHNF1 is also required for nephric duct epithelial maintenance, Müllerian duct formation and early nephrogenesis. Mosaic analyses further suggest a cell-autonomous requirement for vHNF1 in the acquisition of a specialized tip domain and branching morphogenesis. vHNF1 exerts these intricate functions at least in part through the direct control of key regulatory molecules involved in different aspects of early kidney development. Notably, vHNF1 acting directly upstream of *Wnt9b* appears to orchestrate Wnt signaling action in the mesenchymal-epithelial transitions underlying the initiation of nephrogenesis. These results demonstrate that vHNF1 is an essential transcriptional regulator that, in addition to the known later functions in normal duct morphogenesis, plays a crucial role during the earliest stages of urogenital development and provide novel insights into the regulatory circuits controlling events.

KEY WORDS: Early nephrogenesis, Homeodomain transcription factor, Mouse mosaic and tetraploid chimera analysis, Ureteric bud branching, Urogenital tract development

INTRODUCTION

The urogenital system derives from the intermediate mesoderm and gives rise to the three major kidneys: the pronephros, mesonephros and metanephros (the definitive kidney) (Saxen and Sariola, 1987). The development of the definitive kidney begins at approximately E10.5 with the induction of the ureteric bud (UB) at the posterior end of the nephric duct (ND) [also known as the Wolffian duct (WD)] by signals from the adjacent metanephric mesenchyme (MM). The UB invades the MM, elongates and branches to form the renal collecting system. As each branch is formed, a subset of mesenchymal cells aggregate and condense near the UB tips to form the pretubular aggregates. These structures will further differentiate into comma- and S-shaped bodies, and subsequently into the Bowman's capsule of the glomerulus and the nephron tubules that will connect with the collecting duct (reviewed by Dressler, 2006; Vainio and Lin, 2002). This process of branching and differentiation is reiterated until nephrogenesis is completed shortly after birth in mice. Disruption of any of these events results in congenital anomalies of the kidneys and the lower urinary tract (CAKUT); these represent 20-30% of all anomalies identified in the prenatal period (Pope et al., 1999; Schedl, 2007). In addition to its crucial role in meso- and meta-nephric kidney formation, the WD gives rise to the male reproductive tract, including the epididymis, vas deferens and seminal vesicles. The WD is also required for elongation and maintenance of the Müllerian duct (MD), the precursor of the oviduct, upper vagina and uterus of the female genital tract.

Recent work has identified several signaling molecules and transcription factors that regulate renal branching morphogenesis and nephron formation (reviewed by Bouchard, 2004; Bridgewater et al., 2008; Dressler, 2006). The transcription factors *Pax2*, acting redundantly with *Pax8*, and *Lim1* have essential functions in multiple steps of renal epithelial tubular morphogenesis and are all required for ND formation (Bouchard, 2004; Dressler, 2006). The UB outgrowth step is regulated primarily by the crucial regulatory molecule glial cell-line derived neurotrophic factor (Gdnf), which is expressed in the presumptive MM, and its receptor tyrosine kinase, *Ret*, which is localized in the UB (Costantini and Shakya, 2006). Subsequently, *Ret* and *Gdnf* play a role in UB branching through the establishment of a positive regulatory loop with Wnt11, a Wnt protein expressed in the tip domain (Majumdar et al., 2003). An essential role for the *Wnt9b* signaling pathway has recently been demonstrated for the induction of pretubular mesenchymal aggregates and early nephrogenesis (Carroll et al., 2005). However, the specific interactions of these key regulatory molecules with the genomic template have only been partly elucidated.

The *vHnf1* (also known as *Hnf1*β or *Tcf2*) gene encodes a POU homeodomain transcription factor expressed in the developing liver, pancreas and the entire urogenital system. Heterozygous mutations or large deletions of this gene cause, in humans, a complex syndrome known as renal cysts and diabetes (RCAD), characterized by multiple abnormalities of the kidney and of the male and female genital tract, as well as by early-onset diabetes, pancreas hypoplasia and liver dysfunctions (Barbacci et al., 2004; Bellanne-Chantelot et al., 2004; Edghill et al., 2006; Haumaitre et al., 2006; Lindner et al., 1999). In

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the mouse, inactivation of the *vHnf1* gene leads to embryonic lethality before gastrulation due to defective extra-embryonic visceral endoderm formation (Barbacci et al., 1999). By rescuing this early lethality through the use of tetraploid embryo complementation, we have shown that vHNF1 is required for normal pancreas morphogenesis and liver specification (Haumaitre et al., 2005; Lokmane et al., 2008). Moreover, *vHnf1* tissue-specific inactivation in medullar kidney tubules or transgenic expression of dominant negative mutants causes the formation of medullar renal cysts (Gresh et al., 2004; Hiesberger et al., 2004). In addition, overexpression of human *vHNF1* mutations in *Xenopus* results in morphogenetic alterations of pronephros development (Bohn et al., 2003). These studies, together with the observation that zebrafish hypomorphic *vhnf1* mutants exhibit cystic kidneys (Sun and Hopkins, 2001), strongly suggest a conserved role for this transcription factor during vertebrate kidney development. However, the precise role of vHNF1 during the induction of the mammalian kidney and the reproductive system remains essentially unknown. Here, we investigate the role of this factor during early kidney development through the use of tetraploid and diploid embryo complementation (Haumaitre et al., 2005; Lokmane et al., 2008). Our results indicate that vHNF1 is a crucial component of the regulatory circuits involved in WD integrity, UB branching and early nephrogenesis.

MATERIALS AND METHODS

Diploid and tetraploid chimera generation, β-galactosidase staining and kidney explant culture

Tetraploid (4n) chimeric embryos and diploid (2n) mosaics were generated as previously described (Haumaitre et al., 2005; Lokmane et al., 2008). The genotype of 4n embryos was always confirmed by PCR analysis of genomic DNA: only *vHnf1*$^{-/-}$ embryonic stem (ES)-cell-derived embryos in which no wild-type (WT) allele was detected were analyzed. Control embryos were obtained by parallel matings. Heterozygous knock-in mice expressing the *lacZ* gene into the *vHnf1* locus were used either in organ culture or in expression analysis via X-Gal staining (Barbacci et al., 1999).

Urogenital tracts, microdissected metanephric rudiments or isolated MMs prepared as described (Lin et al., 2003) were cultured on Transwell filters (Millipore, 0.8 μm) in 5% CO_2 at 37°C in DMEM/F12 (1:1) (Invitrogen) with 10% fetal calf serum, 50 μg/ml streptomycin/50 U/ml penicillin. When indicated, cultures were performed on a monolayer of cells expressing *Wnt9b* (L-Wnt9b cells) or *Wnt4* (NIH3T3-Wnt4 cells), kindly provided by A. McMahon (Carroll et al., 2005); the medium was additionally supplemented with half of the conditioned media recovered from a four-day-old culture at the confluence of the corresponding cell line.

Immunohistochemistry, in situ hybridization and RT-PCR analysis

Mouse embryos were fixed, embedded in paraffin, sectioned and used for either in situ hybridization or immunostaining analyses as described (Lokmane et al., 2008). Serial sections from control and mutant embryos were analyzed in parallel and treated under identical conditions. At least three independently prepared embryos were examined. We used rabbit anti-HNF1β at 1:50 (H-85, Santa Cruz Biotechnology), rabbit anti-PAX2 at 1:500 (Covance) and rabbit anti-phosphohistone H3 at 1:250 (Upstate Biotechnology) as primary antibodies. We used biotinylated goat anti-rabbit (Vector) as the secondary antibody, followed by streptavidin-AlexaFluor 488 at 1:500 (Molecular Probes) or the Vectastain ABC kit (Vector laboratories). TUNEL staining was performed using the cell death detection kit (Roche). The following cRNA probes were used: *vHnf1*, *Pax2* (G. Dressler), *Lim1* (R. R. Behringer), *Gdnf*, *Ret* (V. Pachnis), *Eya1* (P. Xu), *Bmp7*, *Bmp4* (J. Collignon), *Wnt11* (A. Kispert), *Fgf8* (G. Martin), *Pax8*, *Wnt4* (S. Vainio). *Wnt9b*, *Wt1*, *Gata3* and *Emx2* probes were generated by PCR.

RNA from microdissected metanephroi was extracted and subjected to semiquantitative RT-PCR as described (Lokmane et al., 2008). Primer sequences used were: *Gapdh* (For: 5′-TCCAGTATGACTCCACTCAC-3′; Rev: 5′-ACCTTGCCCACAGCCTTG-3′); Pax2 (For: 5′-CAGCCTT-TCCACCCAACG-3′; Rev: 5′-GTGGCGGTCATAGGCAGC-3′); *Lim1* (For: 5′-CGGGAAGGCAAGCTCTACT-3′; Rev: 5′-AACCAGATCG-CTTGGAGAGA-3′); *Wnt9b* (For: 5′-CGAGGAGATGCGAGAGTGC-3′; Rev: 5′-GGAAGGGTGTCAGGACCTC-3′). PCR products were analyzed on agarose gels and quantified by QuantityOne 4.3.1 software (BioRad).

Cell culture, transfection, electrophoretic mobility shift assays and ChIP

Human embryonic kidney (HEK) 293 cells and human epithelial carcinoma C33 cells were maintained and transiently transfected as described (Barbacci et al., 2004). Gel shift assays were performed using protein extracts overexpressing vHNF1 and the double-stranded oligonucleotides indicated in Table S1 in the supplementary material (Barbacci et al., 2004). The genomic sequences –7946 to –7529, –7946 to –7809 and +13979 to 14601 of *Wnt9b*, –7310 to –6884 and –5762 to –5572 of *Pax2*, and –7934 to –7711 of *Lim1* were PCR amplified and subcloned upstream to the thymidine kinase (*TK*) promoter (–51 to +10) in the Δ*TK*CAT6 vector. Sites were mutated using the QuikChange site-directed mutagenesis kit using the primers listed in Table S2 in the supplementary material.

Chromatin immunoprecipitation (ChIP) assays were performed using isolated E14.5 metanephros essentially as described (Wiebe et al., 2007). Chromatin was sheared to a range of 0.3 to 0.7 kb, and assays were performed using the ChIP assay kit from Upstate (ref 17-295). The equivalent of 50 μg of chromatin was used in each ChIP experiment and immunoprecipitated with rabbit anti-HNF1β (H85, Santa Cruz), anti-acetylated histone H4 (Upstate) or rabbit pre-immune serum. Purified immunoprecipitated DNA was resuspended into 30 μl of distilled water. The equivalent of 10% of input chromatin was DNA purified in parallel and resuspended in an equal volume. 3 μl were subjected to semiquantitative PCR. Each assay was repeated three to five times using different pools of E14 metanephros chromatin. Pair primers are provided in Table S3 in the supplementary material.

RESULTS

vHnf1 is expressed in the ND epithelium and its derivatives

We performed a detailed analysis of the expression pattern of *vHnf1* at both the transcript and protein levels from the first steps of kidney development. Consistent with previous X-gal staining of *vHnf1* heterozygous embryos (Barbacci et al., 1999), at E9.5, *vHnf1* transcripts were detected in the WD epithelium and cranial mesonephric tubules (Fig. 1A). This expression pattern is maintained at later stages, with high expression in the entire ND and the emerging UB (Fig. 1B). At E11.5, when the UB has undergone the first branching event to generate the T-stage, *vHnf1* was expressed homogeneously in the epithelium of the UB and the WD (Fig. 1C). In situ hybridization and immunostaining analysis revealed that *vHnf1* transcripts (not shown) and protein were present in the epithelium of the UB branches and the pretubular aggregate derivatives, the comma- and S-shaped bodies (Fig. 1D,E). At E17.5, the expression pattern was similar to that of the adult (Fig. 1E,F), with expression in the entire collecting system and all segments of the nephron. Expression was absent in the glomeruli. In addition to the WD, *vHnf1* was expressed in the MD and genital tract derivative tissues (not shown, see below). In adults, *vHnf1* is expressed in the epididymis, vas deferens, seminal vesicle, prostate, uterus and oviduct (Haumaitre et al., 2006; Reber and Cereghini, 2001). Thus, the expression pattern of *vHnf1* is consistent with the function of this factor during the earliest stages of UB branching and nephrogenesis.

Lack of *vHnf1* leads to defective urogenital development

To directly address the role of vHNF1 during early kidney development, we generated *vHnf1*$^{-/-}$ ES-cell-derived embryos (further denoted as *vHnf1*$^{-/-}$) by tetraploid aggregation (Lokmane et

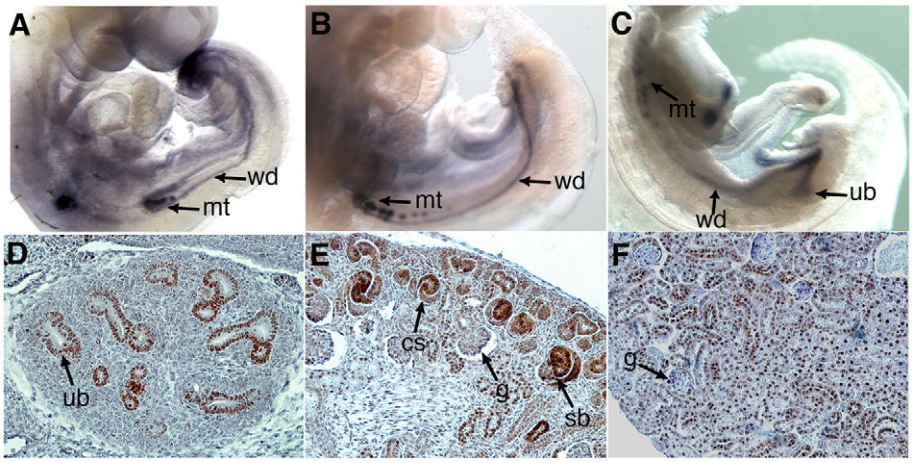

Fig.1. Expression pattern of vHnf1 during kidney development. Whole-mount in situ hybridization of vHnf1 in wild-type (WT) embryos at E9.5 (**A**), E10.5 (**B**) and E11.5 (**C**). Immunostaining of vHNF1 on sagittal sections of kidney at E13 (**D**), E17.5 (**E**) and in the adult (**F**). g, glomeruli; cs, comma-shaped bodies; mt, mesonephric tubules; sb, S-shaped bodies; ub, ureteric bud; wd, Wolffian duct.

al., 2008). We initially compared X-Gal-stained urogenital tracts from mutant and heterozygous embryos microdissected at different stages (Fig. 2A-H). At E10.5, the WD duct was apparently normal and reached the caudal end of mutant embryos (not shown) (Haumaitre et al., 2005). At E11.5, when the UB reached the T-stage, the UB was not detectable in mutant embryos (Fig. 2A,B). Only a rudimentary UB was observed at around E12.5 (Fig. 2C,D). Furthermore, subsequent branching after the T-stage was disrupted, and the UB had a Y shape rather than a T shape, indicating abnormal branching (Fig. 2F, arrowhead). Histological analysis of E13.5 *vHnf1*$^{-/-}$ embryos confirmed severe kidney hypoplasia (approximately 70% smaller than WT), the defective UB branching and a paucity of MM condensations, whereas the adrenal glands were normal (Fig. 2I,J). We also noted premature degeneration of the WD in different regions and the complete absence of cranial mesonephric tubules (Fig. 2C,D). In the mutants, the MD was not observed at any stage, whereas it was clearly observed in heterozygous embryos (Fig. 2E-H).

Thus, vHNF1 is required for different aspects of early urogenital development, including the maintenance of WD integrity, formation of mesonephric tubules and MDs, as well as timed emergence and subsequent branching of the UB.

ND and UB growth defects in *vHnf1* mutants

We subsequently examined a number of regulatory molecules expressed by either the UB or the MM cells, and implicated in UB branching morphogenesis and ND formation (Fig. 3A-C).

The initial step of kidney development is the outgrowth of the UB from the WD in the adjacent MM. *Gdnf*, which is expressed in the MM, and its receptor, *Ret*, which is restricted to the WD epithelium, are both required for UB formation and the branching process (Costantini and Shakya, 2006; Shakya et al., 2005). At E11.5, *Ret* and *Gdnf* were apparently normally induced in mutant UB rudiments (Fig. 3B), despite the important delay in their formation. However, analysis of tissue sections revealed that they were both expressed at relatively lower levels in *vHnf1*$^{-/-}$ embryos compared to expression in the WT (Fig. 3B). At E12.5, the expression of *Ret* and *Gdnf* was severely reduced in mutant metanephros (Fig. 3C). Interestingly, *Wnt11*, a downstream target of GDNF/Ret signaling (Majumdar et al., 2003) that is normally expressed by the tips of the branched UB and in the ND, was specifically absent in the *vHnf1*$^{-/-}$ UB tips, but remained unaffected in the mutant ND (Fig. 3C; see Fig. S1 in the supplementary material).

Pax2, expressed in the ND, the UB and the induced MM, plays a crucial role during early kidney development: in *Pax2* null embryos, the ND initially forms but degenerates prematurely and therefore the kidneys and genital tracts are never formed (Dressler, 2006; Torres et al., 1995). At E11.5, *Pax2* was expressed in the mutant MM and a reduced number of caudal mesonephric tubules, but was specifically downregulated in the ND (Fig. 3B). *Pax2* downregulation in the ND (particularly in its caudal portion) was stronger at E11.5 (Fig. 3B) than at E10 (Fig. 3A). In addition, the discontinuous expression of *Pax2* reflected

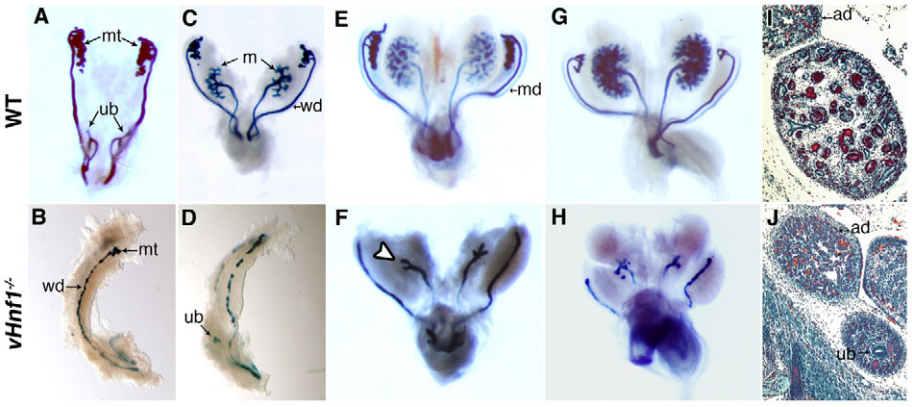

Fig. 2. Urogenital phenotypes in *vHnf1*-deficient embryos. (**A-H**) X-gal staining of WT *vHnf1*$^{+/-}$ (A,C,E,G) and *vHnf1*$^{-/-}$ (B,D,F,H) urogenital tracts dissected at E11.5 (A,B), E12.5 (C,D), E13.5 (E,F) and E14.5 (G,H). The white arrowhead in F shows the strong reduction in UB branching in mutants relative to WT (E). (**I**,**J**) Histological staining of sagittal sections of WT (I) and *vHnf1*$^{-/-}$ (J) embryos at E13.5 shows severe defects in UB branching (J). m, metanephric kidney; md, Müllerian duct; Ad, adrenal gland.

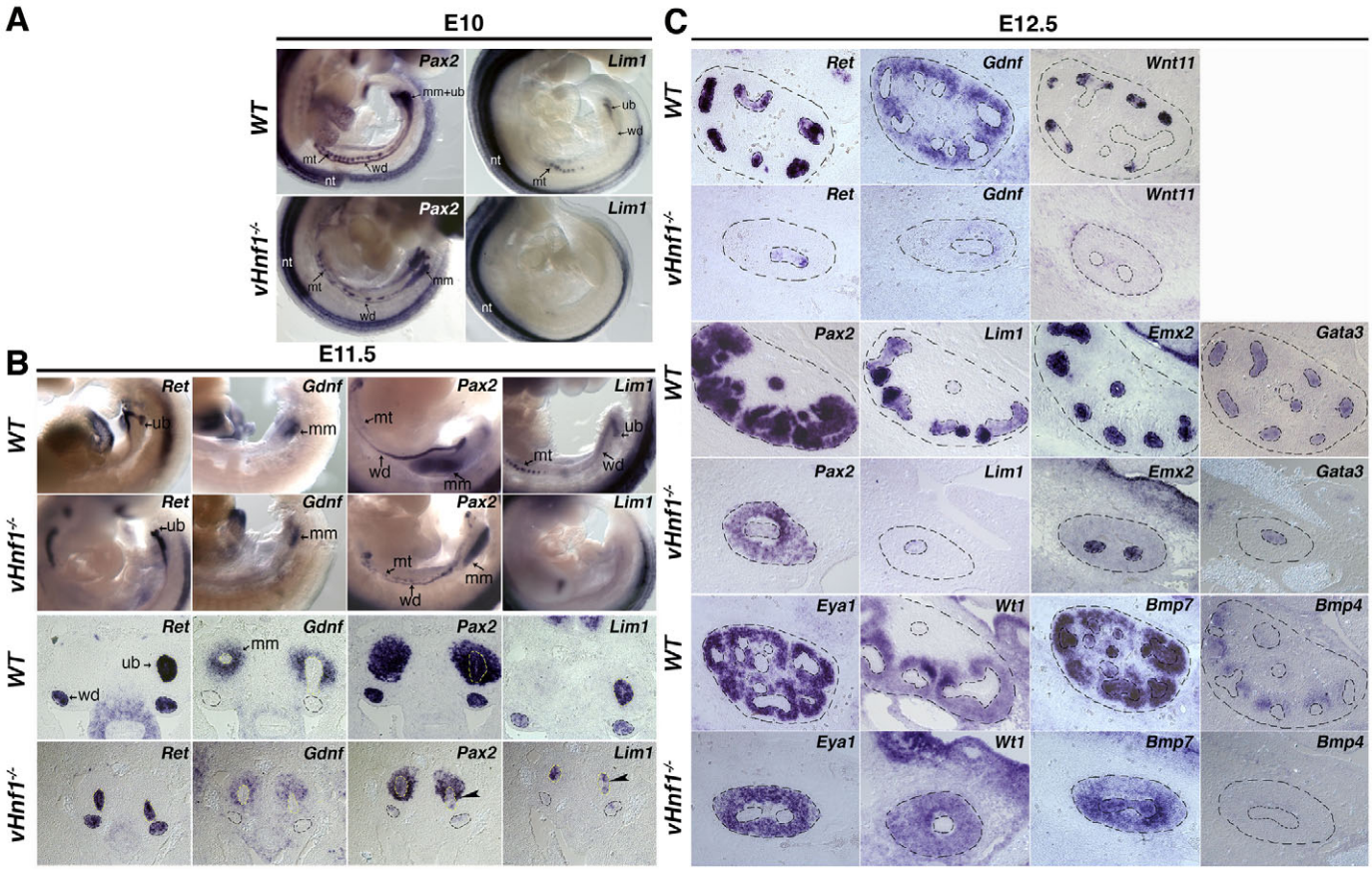

Fig. 3. Molecular analysis of NB and ND genes. (**A**) Whole-mount in situ hybridization at E10 of WT and *vHnf1*$^{-/-}$ embryos with the indicated markers. (**B**) Whole-mount in situ hybridization and transverse sections at E11.5 of WT and *vHnf1*$^{-/-}$ embryos with the indicated markers. (**C**) In situ hybridization at E12.5 of sections of WT and *vHnf1*$^{-/-}$ embryos with the indicated epithelial and mesenchymal markers. The metanephroi and inside epithelial UB branches are outlined (dashed line). nt (neural tube); mm, metanephric mesenchyme.

probably premature WD degeneration. Analysis of serial sections confirmed strong downregulation of *Pax2* transcripts in the mutant WD and UB; the *Pax2* MM expression domain was also reduced compared to that in WT embryos (Fig. 3B). By contrast, the weak expression of *Pax8* in the ND appeared unchanged in the mutants (not shown). Interestingly, the expression of *Lim1*, a transcription factor involved in ND elongation (Pedersen et al., 2005) and normally expressed in the ND, mesonephric tubules and UB, was barely detected in the mutant ND epithelium by E10.5 (Fig. 3A,B) and was lost at later stages (Fig. 3C). However, the strong downregulation of *Pax2* and *Lim1* was not due to a generalized differentiation defect of the mutant ND, as indicated by the analysis of additional epithelial markers. The expression of *Emx2*, a transcription factor required for normal UB formation and growth that displays a strikingly similar phenotype to that of the *vHnf1*$^{-/-}$ embryo (Miyamoto et al., 1997), was, however, only modestly reduced (Fig. 3C). Likewise, the expression of *Gata3*, another transcription factor expressed in the ND and UB epithelium and required for normal ND morphogenesis (Grote et al., 2008; Lim et al., 2000), was not altered in mutant embryos (Fig. 3C).

At E12.5, the expression of several essential mesenchymal genes, including *Eya1*, *Wt1* (Donovan et al., 1999; Xu et al., 1999) and *Bmp7* (Dudley et al., 1995), was unchanged in the mutant MM, although their expression domains were reduced compared that in the WT (Fig. 3C). By contrast, the expression of *Bmp4*, normally restricted to a subpopulation of mesenchymal cells surrounding the ND and UB branches (Miyazaki et al., 2000), was strongly reduced in the mutant mesenchyme (Fig. 3C).

These data confirmed the requirement for vHNF1 in multiple steps of early kidney development. First, vHNF1 is involved in ND differentiation, very probably by controlling the expression of both *Lim1* and *Pax2* in this structure. Although vHNF1 is not required for initial outgrowth of the UB or initial condensation of the MM, it appears essential for UB branching from the T-stage, probably through the control of *Ret* and *Gdnf* signaling (see also below, Fig. 6).

We next examined whether decreased proliferation or increased apoptosis affected the UB and the surrounding mesenchyme. Remarkably, using the mitosis marker phosphorylated histone H3, we found comparable numbers of proliferating cells in the entire UB epithelium of E12.5 mutant and control embryos (see Fig. S2A,B in the supplementary material) and only a moderate reduction in the percentage of proliferating cells in the mutant mesenchymal population (see Fig. S2 in the supplementary material). By contrast, TUNEL experiments performed at the same stage indicated a large increase in the number of apoptotic cells in both the mutant UB epithelium and mesenchymal cells (see Fig. S2 in the supplementary material). Thus, the precise balance between proliferation and apoptosis that characterizes normal kidney morphogenesis appears

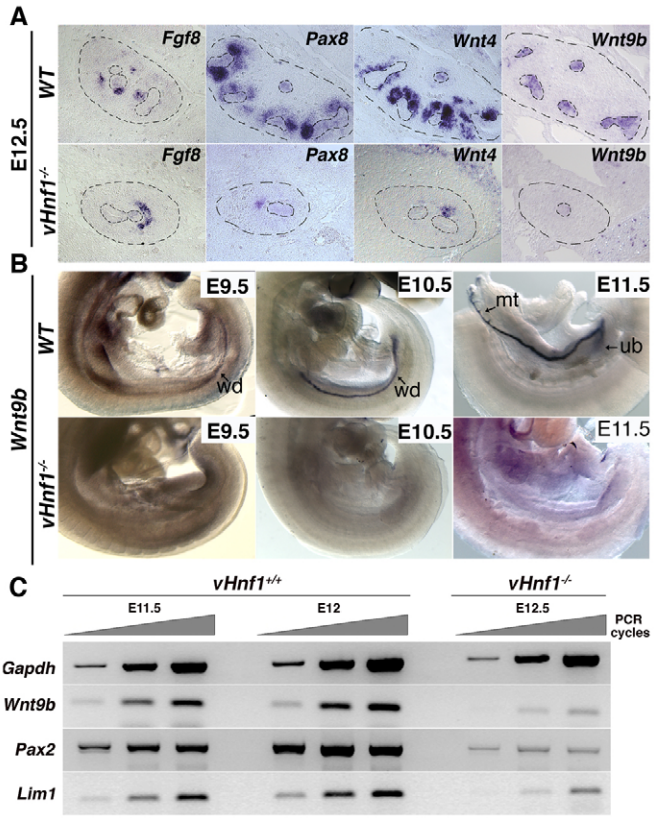

Fig. 4. Analysis of nephrogenic markers in the *vHnf1*$^{-/-}$ MM. (**A**) In situ hybridization at E12.5 of WT and *vHnf1*$^{-/-}$ embryos with *Fgf8*, *Pax8*, *Wnt4* and *Wnt9b*. (**B**) Whole-mount in situ hybridization of *Wnt9b* in WT and mutant embryos at the indicated stages. (**C**) Semiquantitative RT-PCR analysis of metanephric kidney mRNAs isolated from WT and mutant embryos. Given the delay in UB formation in mutants, WT metanephroi at both E11.5 and E12 were compared with E12.5 *vHnf1*$^{-/-}$ metanephroi. *Gapdh* was used for normalization.

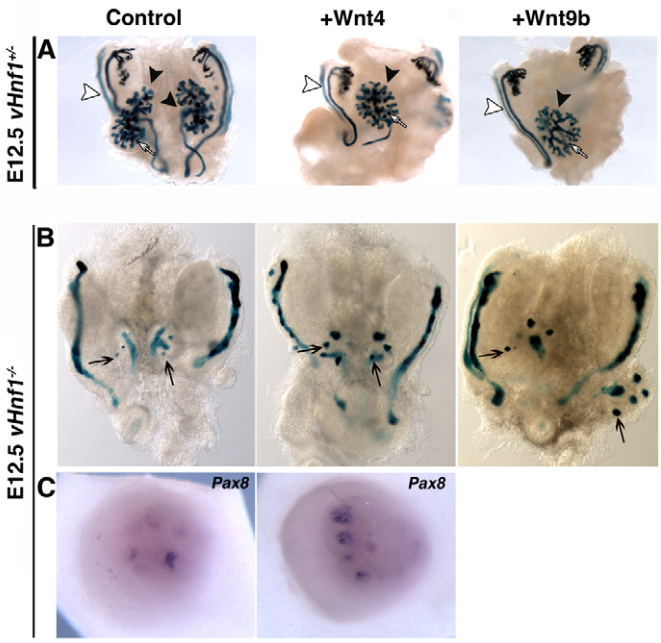

Fig. 5. *In vitro* cultures of WT and *vHnf1*$^{-/-}$ urogenital tracts. (**A**) 48-hour culture of E12.5 *vHnf1*$^{+/-}$ urogenital tracts, followed by X-gal staining to visualize the *vHnf1*^{lacZ}-expressing structures, in the absence or presence of cells expressing Wnt9b and Wnt4, together with the addition of complemented media. Extensive UB branching (black arrowheads), pretubular aggregates (white arrows) and MD formation (white arrowheads) are observed under these different conditions. (**B**) Cultures of E12.5 *vHnf1*$^{-/-}$ urogenital tracts, followed by X-gal staining, in the absence or presence of Wnt9b- or Wnt4-expressing cells and the corresponding complemented media. Wnt9b or Wnt4 induced the formation of mesenchymal aggregates (black arrows). In some experiments, Wnt9b, but not Wnt4, appeared to induce an MD-like structure. (**C**) Culture of E12.5 *vHnf1*$^{-/-}$ metanephros, followed by whole-mount in situ hybridization, showing a significant increase in *Pax8*-positive structures in the presence of Wnt4.

disturbed in mutant kidneys, with a global increase in apoptosis. As *vHnf1* expression is initially restricted to the UB epithelium, these findings suggest that vHNF1 plays a role in sustaining the survival of epithelial UB cells, whereas the increased apoptosis of mesenchymal cells might reflect defective signaling from the UB to these cells.

vHNF1 is required for early nephrogenesis

Early nephrogenesis begins with the formation of mesenchymal pretubular aggregates close to the ureteric branches. *Fgf8*, *Pax8*, *Wnt4* and *Lim1* are all expressed in the pretubular aggregates and their derivatives. Loss of either *Fgf8* or *Wnt4* signaling results in complete failure of renal vesicle formation (Grieshammer et al., 2005; Perantoni et al., 2005; Stark et al., 1994). By contrast, *Lim1* is not required for the initial formation of renal vesicles, but is necessary for their proper patterning (Kobayashi et al., 2005). In E12.5 *vHnf1*-deficient embryos, the expression of *Fgf8*, *Pax8* and *Wnt4* was strongly reduced (Fig. 4A). Furthermore, mesenchymal expression of *Lim1* was undetectable (Fig. 3C). Thus, in mutant embryos, the expression of the characteristic markers of the early nephron structures is severely impaired. Remarkably, at E12.5, the expression of *Wnt9b*, a factor that acts as a paracrine signal to induce nephrogenesis via *Wnt4* (Carroll et al., 2005), was completely lost in the *vHnf1*$^{-/-}$ embryos (Fig. 4A). Furthermore, whole-mount in situ hybridization at earlier stages showed that, in our mutants, *Wnt9b* was not detected in either the ND or the UB (Fig. 4B), indicating that vHNF1 activity was required for *Wnt9b* induction. Thus, lack of *vHnf1* leads to the absence of early nephrogenesis, very probably due to disruption of the regulatory pathway involved in this process, which is mediated by *Wnt9b* and its targets, *Fgf8*, *Pax8 and Wnt4*.

Consistent with the results shown in Figs 3 and 4, semiquantitative RT-PCR analysis from control and *vHnf1*$^{-/-}$ microdissected metanephroi confirmed the strong reduction of *Wnt9b* in E12.5 mutant metanephroi, as well as of *Pax2* and *Lim* transcript levels, compared to either E11.5 or E12 control metanephroi (Fig. 4C).

To examine further the potential involvement of vHNF1 in nephrogenesis, we performed in vitro cultures of mutant urogenital tracts using *vHnf1*$^{+/-}$ urogenital tracts as controls (Fig. 5A). Given the considerable morphogenesis observed upon in vitro culture of entire urogenital tracts and the fact that *vHnf1*$^{-/-}$ embryos die around E14.5, these experiments enabled us to also examine the potential rescue of UB branching and nephrogenesis defects upon further culture under different conditions. In particular, because previous

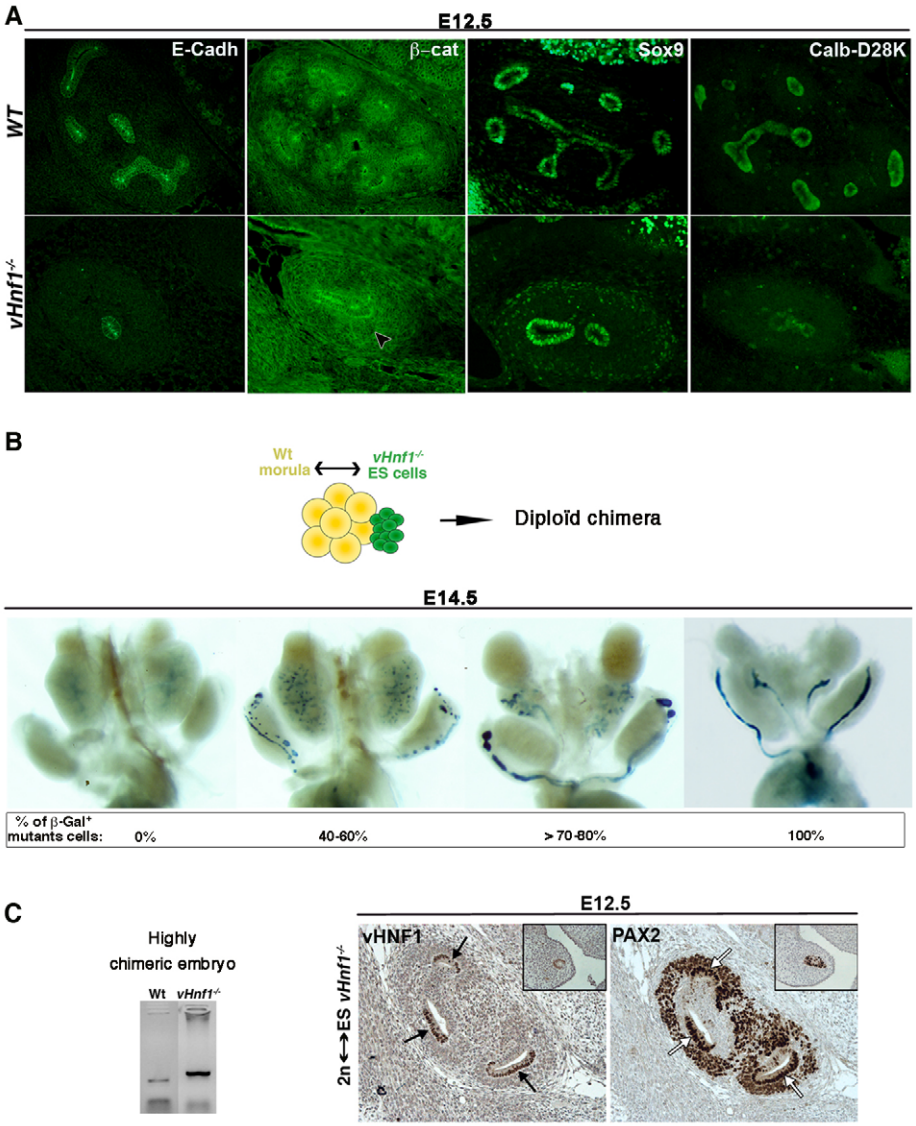

Fig. 6. *vHnf1*$^{-/-}$ *cells* are excluded from the UB tips in 2*n*↔ES *vHnf1*$^{-/-}$ mosaic embryos. (**A**) Immunostaining of sagittal sections of WT and mutant embryos at E12.5. Note the severely decreased calbinin-D28K expression in the mutant UB branches and the rare mesenchymal aggregates in the mutant mesenchyme (black arrowhead). (**B**) E14.5 2*n* mosaic embryos with a variable contribution from *vHnf1*$^{-/-}$ cells, ranging from approximately 50% to fully derived from mutant cells. (**C**) Immunostaining analysis of 2*n* mosaic embryos with a high contribution of *vHnf1*$^{-/-}$ cells. Genotyping was performed from pooled regions of the embryos. Serial sagittal sections of a chimeric embryo composed roughly of the indicated ratio of WT and *vHnf1*$^{-/-}$ cells by PCR analysis were analyzed by immunohistochemistry using antibodies against vHNF1 and PAX2. WT cells (vHNF1-postive cells) are localized at the UB tip domain (black arrows) and co-express Pax2 (white arrows). The insets show the WD, composed of a majority of WT cells (vHNF1 and PAX2 positives).

studies have shown that cells expressing Wnt ligands, including Wnt9b and its direct target Wnt4, can induce isolated MM to undergo tubulogenesis (Carroll et al., 2005; Kispert et al., 1998), we initially tested these ligands. As shown in Fig. 5B, culture of *vHnf1*$^{-/-}$ urogenital tracts with either Wnt9b or Wnt4 had no effect on either the UB branching defect or the defective formation of mesonephric tubules. However, it did result in the formation of several mesenchymal aggregates, as suggested by whole-mount X-gal (Fig. 5B) and E-cadherin stainings (not shown). Additional studies involving cultures, in the presence of Wnt4, of either mutant metanephric rudiments (Fig. 5C) or isolated mutant MM, derived from epiblast *vHnf1*-specific inactivation using the Sox2-cre driver line (our unpublished data) (Hayashi et al., 2002), confirmed the formation of pretubular aggregates positive for *Pax8* (Fig. 5C and data not shown). In these experiments, we used cells expressing Wnt4, because these cells induce pretubular aggregates both more efficiently and reproducibly than Wnt9b-expressing cells (not shown).

These results show that mutant MM appears to be competent to condense and express early nephron markers, further suggesting that defective nephrogenesis in mutant embryos reflects impaired *Wnt9b* signaling from the UB epithelium.

vHnf1 acts cell autonomously in UB branching

The observation that, in *vHnf1*$^{-/-}$ embryos, *Wnt11*, normally confined to the UB tips, was not detected (Fig. 3C and see Fig. S1 in the supplementary material), together with the observed downregulation of *Ret* in the tip domain by E12.5 (Fig. 3C), suggested defective formation and/or maintenance of a specialized tip domain. A number of markers, including the cell adhesion proteins E-cadherin and β-catenin and the tight junction protein ZO-1, were, however, correctly localized in the mutant epithelium (Fig. 6A and data not shown). Similarly, Sox9, a transcription factor expressed by E11.5 to E12.5 throughout the entire UB branches and subsequently enriched in the UB tips, was also correctly induced in the mutant UB (Fig. 6A). By contrast, the expression of calbindin-D28K, an intracellular protein with a high affinity for calcium and expressed at this stage by UB cells, was strongly reduced (Fig. 6A). These data suggest that defective UB branching is apparently not caused by abnormal cell polarity of the mutant epithelium. However, the strong reduction in calbindin-D28K expression might imply that the UB mutant epithelium is not fully differentiated.

To obtain further information on vHNF1 function during UB branching and ND formation, we performed diploid mosaic chimeras. WT diploid embryos were aggregated with *vHnf1*-

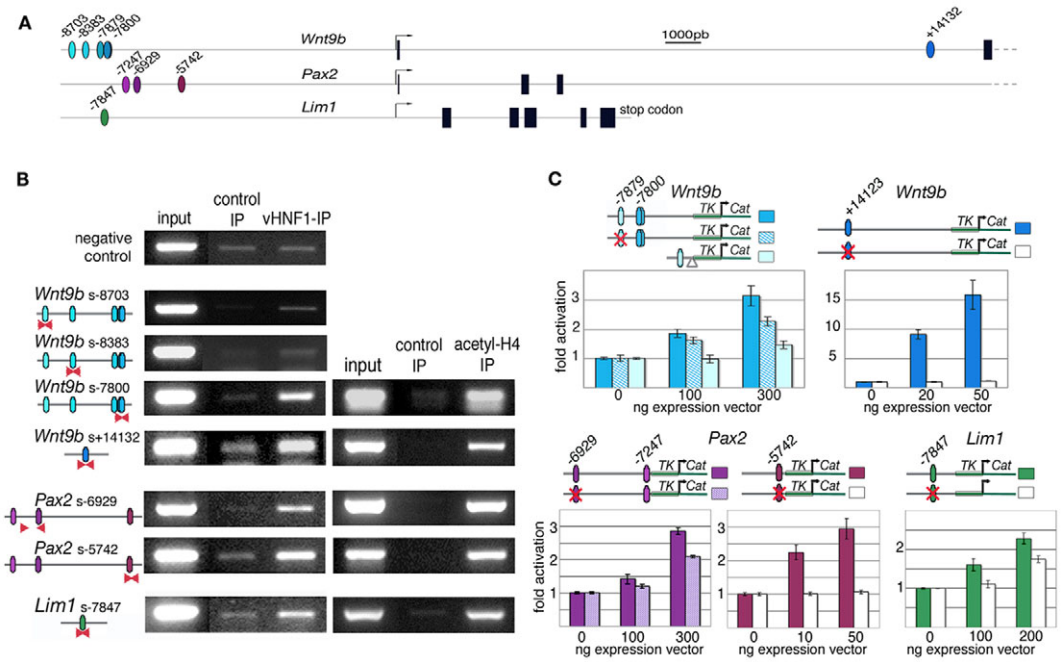

Fig. 7. vHNF1 recruitment in vivo to the regulatory sequences of *Wnt9b*, *Pax2* and *Lim1*. (**A**) Diagrammatic representation of vHNF1-binding sites within the non-coding sequences of the indicated genes. Numbers denote the distance in base pairs (pb) from the transcription start site (arrows). Black boxes denote exons. (**B**) vHNF1 is recruited in vivo to the identified binding sites. ChIP analyses were performed on freshly isolated E14.5 metanephros. Target sequences were amplified by semiquantitative PCR using the pair primers indicated in Table S1 in the supplementary material and denoted by red arrowheads. Representative ChIP experiments are shown. (**C**) Transactivation of CAT-reporter constructs by vHNF1. Cells were transiently transfected with the indicated amounts of vHNF1 expression vector, together with a reporter construct containing the indicated enhancer sequences, depicted above the corresponding histograms. CAT activity was normalized to transfection efficiency by measuring β-galactosidase activity and measured as fold activation above the activity obtained by the CAT-reporter alone. CAT activation is strongly reduced or abolished by site-directed mutagenesis (indicated by red crosses) or deletion of the identified binding sites. The values and standard deviations plotted are the mean of at least five independent experiments.

deficient ES cells; in such chimeras, the embryonic tissues are derived from a variable mix of WT and mutant cells (Fig. 6B), thus enabling the definition of a cell-autonomous function for vHNF1. Diploid chimeras using ES *vHnf1*$^{+/-}$ were normal and exhibited an apparently random distribution of WT and *vHnf1*$^{+/-}$ (not shown). Mosaic urogenital tracts derived from embryos with variable contributions of mutant cells were initially dissected at E13.5 (n=27) and E14.5 (n=9), and X-gal stained. A representative set of E14.5 urogenital tracts exhibiting an increasing number of mutant cells (β-gal positives) is shown in Fig. 6B. Chimeric embryos were indistinguishable from WT embryos when the percentage of mutant cells ranged between 40% and 60%. However, when this percentage was higher than 70-80%, chimeric metanephroi were hypoplastic and exhibited reduced and abnormal branching, and even, in one case, a duplex kidney (Fig. 6B). In these mosaics, mutant cells were able to contribute extensively to the WD, to the primary UB and to early nephron structures. There was, however, a lower proportion of mutant cells in the UB branches, ureter and mesonephric tubules compared with early nephron structures or the WD. Note also that mutant cells remained in clusters rather than being intermingled.

To obtain a better definition of the fate of mutant cells, we then focused our analysis on E12.5, because at this stage the individual UB tips can easily be examined and the branching pattern is well defined (Majumdar et al., 2003). The fate of WT versus mutant cells was next examined in different sections using an anti-vHNF1 antibody; adjacent sections were analyzed for the expression of *Pax*2. In E12.5 WT embryos, vHNF1 is normally present in the epithelium of UB branches, whereas PAX2 is expressed in the MM as well as in the epithelium of the UB. A fraction of E12.5 2n↔ES *vHnf1*$^{-/-}$ chimeras exhibiting a significantly higher contribution of mutant cells relative to WT displayed a partial phenotype: UB branching was significantly reduced when compared to WT (i.e. 3 tips instead of the 6-8 normally observed in WT embryos; Fig. 6C). Remarkably, in these mosaics, vHNF1-positive cells, and therefore WT, co-expressed with PAX2 and were strictly localized to the tip domain of UB branches (Fig. 6C, black and white arrows). Moreover, mutant cells (vHNF1 and PAX2 negatives) were able to contribute to the epithelium of the stalks (Fig. 6C). Interestingly, ES cells lacking vHNF1 behave like *Ret*$^{-/-}$ cells (Shakya et al., 2005), both being excluded from the UB tips. This further suggests that vHNF1 might be required for the branching process through its action at the tip domain. These studies suggest a cell-autonomous function for vHNF1 in UB branching morphogenesis and further confirm that its activity is required for *Pax2* expression in the UB.

vHNF1 recruitment in vivo to the regulatory sequences of key factors involved in urogenital development

To obtain a more comprehensive picture of the putative vHNF1-mediated transcriptional programs, we searched for conserved core vHNF1-binding sites within the non-coding sequences of the regulatory genes whose expression was severely affected by *vHnf1* deficiency. We identified several highly conserved sites, in either the 5′ or intronic sequences, for a subset of these genes, including

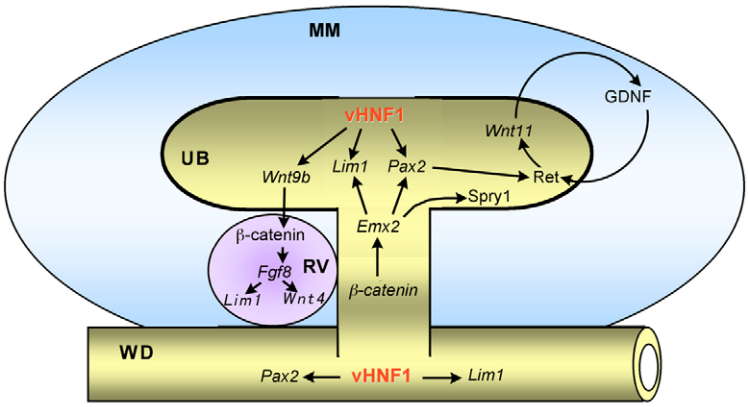

Fig. 8. Proposed model of vHNF1 action in the regulatory network involved in early UB branching and nephrogenesis. Arrows indicate epistatic relationships and/or direct control. Although not required for *Pax2* and *Lim1* initial induction in the intermediate mesoderm, vHNF1 is essential for maintenance of their expression in the epithelium of the WD (Wolffian duct) and UB (ureteric bud), as well as for the maintenance of *Gdnf* and *Ret* expression. vHNF1 is, in addition, required for *Wnt9b* induction and consequently for activation of its targets (*Wnt4*, *Fgf8*) and the initiation of nephrogenesis. β-catenin signaling was recently shown to be required for *Emx2* expression. Emx2, similar to vHNF1, regulates a genetic program involving *Lim1*, *Pax 2*, *Ret* and *Wnt11* (Bridgewater et al., 2008; Marose et al., 2008). Emx2 and vNHF1 very probably act in parallel or in concert to maintain ureteric tip identity and branching morphogenesis. RN, renal vesicle.

Wnt9b, *Pax2*, *Lim*, *Pax8* and *Wnt4*. Among these potential targets, *Wnt9b*, *Pax2* and *Lim1* attracted our attention because their defective regulation appeared to contribute to the *vHnf1* mutant phenotype. Interestingly, we identified a cluster of four partially conserved binding sites in the upstream sequence of *Wnt9b*, and a single site in the *Wnt9b* first intron within a region that is highly conserved among mouse, rat and human orthologous sequences (Fig. 7A; see Fig. S3A in the supplementary material). We also found several vHNF1-binding sites within conserved regions of the already characterized *Pax2* regulatory sequences (Kuschert et al., 2001) (Fig. 7A; see Fig. S3B in the supplementary material) and within a site in the 5′ upstream sequence of *Lim* (Fig. 7A; see Fig. S3C in the supplementary material). The ability of vHNF1 to bind to all of these sites was confirmed in vitro by electrophoretic gel mobility assays (see Fig. S3D in the supplementary material; data not shown). Interestingly, the identified vHNF1-binding sites are clustered with the consensus binding sites of other key transcription factors, notably Pax2/8, Sox-9, HNF4 and FoxA2.

To examine whether vHNF1 was recruited to these sites in vivo, we performed ChIP assays on freshly isolated E14.5 metanephros. Soluble cross-linked chromatin was immunoprecipitated with antibodies against vHNF1. The presence of sequences corresponding to the identified binding sites in the immunoprecipitated material was then assessed by semiquantitative PCR. As shown in Fig. 7B, for the samples immunoprecipitated with the anti-vHNF1 antibody, we were able to specifically amplify the regions encompassing the different consensus binding sites identified. Primers designed to amplify a sequence located 30 kb upstream of *Wnt9b*, which does not contain HNF1-binding motifs, did not yield any enrichment product relative to the control immunoprecipitates, further confirming the specificity of the ChIP assay. Among the four binding sites located within the *Wnt9b* upstream region, we found strong and significant enrichment compared to control immunoprecipitates, only with pair primers surrounding the sites at position –7800 and –7878. Because these sites lie immediately adjacent one another, it most likely represents enrichment from a single target site, probably that at –7800, as suggested by CAT reporter assays (see below). Strong enrichments compared to control immunoprecipitates were also observed for the intronic *Wnt9b* site, as well as for the regions containing the sites found in the *Pax2* (–5742, –6929 and –7247) and *Lim1* (–7847) promoter sequences (Fig. 7B). As expected, ChIP assays using an anti-acetylated histone H4 demonstrated that the genomic regions of *Wnt9b*, *Pax2* or *Lim1* to which vHNF1 was recruited in vivo exhibited H4 acetylation, a hallmark of active transcription and euchromatin. Thus, vHNF1 is recruited in vivo to the promoter-enhancer elements of key regulators involved in early kidney development and nephrogenesis, which very probably results in their direct transcriptional activation.

We then assessed the ability of vHNF1 to activate transcription through the sequences identified by ChIP analysis. Selected genomic fragments were cloned upstream of the Δ*TK*-CAT reporter. The effect of co-expressing increasing amounts of vHNF1 was assayed by transient expression assays. The results show that vHNF1 was able to activate these different promoter-enhancer constructs in a dose-dependent manner (Fig. 7C). Although vHNF1 often behaves as a weak transactivator (Barbacci et al., 2004), it is interesting to note that, among the various enhancer-promoter elements examined, the element located in the *Wnt9b* first intron was specifically activated by vHNF1 at one of the highest known levels. By contrast, the *Lim1* enhancer region was modestly but reproducibly transactivated by vHNF1 (n=6). Further studies are required to define whether the genomic fragment examined lacked additional sequences containing an essential co-activator-binding site or encompassed an unidentified repressor-binding element. Site-directed mutagenesis or selective deletion suggests that vHNF1 activates the *Wnt9b* upstream region through the site located at –7800, whereas it appears to use the two close binding sites present at –6929 and –7247 in the Pax2 promoter (Fig. 7C).

Remarkably, the vHNF1-binding sites identified in the *Pax2* promoter are all located within an 8.5 kb region that drives specific expression, in transgenic mice, in the WD and derivatives but not in the induced MM (Kuschert et al., 2001). This expression profile corresponds precisely to the expression pattern of *vHnf1*, further confirming the possibility that vHNF1 directly controls the expression of *Pax2* in these structures. These observations provide strong support for the hypothesis that different regulatory circuits operate in the ND versus the induced MM, with vHNF1 being a central component of the ND core regulatory circuit, together with *Pax2* and *Lim1*.

DISCUSSION

During kidney organogenesis, the epithelium of the UB undergoes repetitive dichotomous branching events, leading to the formation of novel UB tips. Reciprocal inductive interactions between the branched ureteric tips and the MM lead to mesenchymal epithelial transition (MET) and tubular morphogenesis to form the nephrons. UB branching morphogenesis is therefore a fundamental process that defines the normal architecture of the kidney and the number of nephrons.

In this study, we uncovered novel developmental roles for the transcription factor vHNF1 using tetraploid and diploid chimera analysis. Initially involved in timing outgrowth of the UB and subsequent branching, it is also required for ND epithelial maintenance and MD duct formation, as well as for early nephrogenesis. Molecular analysis suggests that vHNF1 exerts these functions, at least in part, through the direct control of several key regulatory molecules. This enables us to propose a model in which vHNF1 plays a predominant role in the distinct regulatory networks controlling ND morphogenesis, UB branching and early nephrogenesis (Fig. 8).

Nephric lineage specification and maintenance

The initial step of kidney development, the conversion of mesenchymal cells in the intermediate mesoderm to epithelial cells of the ND, is mediated primarily by the transcription factors *Pax2*, acting redundantly with *Pax8* (Bouchard et al., 2002; Torres et al., 1995), and *Lim1* (Kobayashi et al., 2005; Pedersen et al., 2005). Unlike *Pax2/8* and *Lim1*, *vHnf1* is not expressed in the intermediate mesoderm, but in the epithelium of the entire ND as it differentiates from the intermediate mesoderm and in cranial mesonephric tubules. In *vHnf1*$^{-/-}$ embryos, the ND forms normally, but subsequently looses its integrity; cranial mesonephric tubules are absent and only rare caudal mesonephric tubules are formed. Furthermore, formation of the MD is disrupted. This phenotype is highly reminiscent of that reported when *Lim1* is inactivated in the ND (Kobayashi et al., 2005; Pedersen et al., 2005) or in *Pax2* mutants (Torres et al., 1995). Although not involved in the initial induction of *Pax2* and *Lim1* in the intermediate mesoderm, we show that vHNF1 is required for maintenance of their expression specifically in the ND, but not in the MM. These observations, together with the results of our ChIP analysis, led us to propose that vHNF1 is a central component of the ND core regulatory circuit, along with *Pax2/8* and *Lim1* (Fig. 8).

During embryogenesis, the WD is essential for both elongation and maintenance of the MD, the anlagen of the female reproductive tract. In *vHnf1*$^{-/-}$ mutants, formation of the MD is disrupted. Cranial mesonephric tubules, which give rise to the epididymis, are also absent. Lack of *Lim1*, *Wnt9b* or *Pax2* expression (or, alternatively, their combined strong downregulation) very probably explains this phenotype, because all these genes have been shown to be required for the extension of the MDs (Carroll et al., 2005; Kobayashi et al., 2005; Torres et al., 1995) and for mesonephric tubule formation (Carroll et al., 2005). Furthermore, at E11-11.5, the expected expression of *Lim1*, *Wnt9b* or *Pax8* in an anterior epithelial structure corresponding to the initial coelomic invagination of the developing MD was not observed (data not shown). Thus, in *vHnf1* mutants, there is either no MD formation or the anterior-most anlagen of the MD is formed, but it does not express these factors. Regardless of the potential requirement for this early event, our results indicate that vHNF1, in addition to being a crucial regulator of ND morphogenesis, is essential for the generation of a functional male and female reproductive tract, providing a mechanistic explanation for the reported genital tract abnormalities in humans carrying heterozygous mutations in *vHNF1* (*HNF1*β) (Edghill et al., 2006; Haumaitre et al., 2006; Lindner et al., 1999).

Early UB branching and acquisition of a specialized tip domain

During the process of UB branching, the tip cells exhibit a highly distinctive gene expression profile (Schmidt-Ott et al., 2005) and a strong proliferative activity (Michael and Davies, 2004; Bridgewater et al., 2008). UB tip cells, which are in close proximity to the induced mesenchymal cells, control multiple processes, including EMT and subsequent formation of the nephrons.

In *vHnf1*-deficient embryos, formation of the UB is delayed and branching of the UB is disrupted after the first branching event. Whereas *Ret* and *Gdnf* expression are apparently normally induced, their expression is strongly downregulated at later stages and *Wnt11*, a component of the *Ret-Gdnf-Wnt11* feedback signaling loop required for UB outgrowth (Majumdar et al., 2003), is not detected at the mutant bud tips, suggesting an abnormal UB tip specification. Although these phenotypes are closely related to those observed upon conditional inactivation of *Lim1* in the ND (Kobayashi et al., 2005; Pedersen et al., 2005) or in *Wnt9b* mutants (Carroll et al., 2005), there are some differences that suggest additional functions of vHNF1 other than just maintenance of the expression of *Lim1* and/or induction of *Wnt9b* in the ND. As in *vHnf1*$^{-/-}$ embryos, in *Lim1* mutants, formation of the UB is delayed. In contrast to *vHnf1*$^{-/-}$ embryos, *Wnt11* is normally induced in the swollen UB tips of *Lim1*$^{-/-}$ embryos, albeit the strongly reduced levels of *Ret* (Kobayashi et al., 2005; Pedersen et al., 2005). Furthermore, although in *Wnt9b*$^{-/-}$ embryos subsequent branching after the T-stage is disrupted, neither the timing of UB outgrowth nor the size of the UB are affected (Carroll et al., 2005).

These observations, together with the results of our diploid chimera analysis, suggest a cell-autonomous requirement for vHNF1 to establish a specialized tip domain. This appears to be, at least in part, independent of *Lim1* and *Wnt9b* activities. Interestingly, in these mosaic experiments, cells lacking *vHnf1* behave similarly to *Ret* null cells (Costantini and Shakya, 2006). It is uncertain whether *vHnf1* functions upstream of Ret or in parallel pathways; however, it is probable that the behavior of *vHnf1* mutant cells is related to the observed decreased levels of *Ret* signaling (Fig. 3C) (Chi et al., 2009). It is currently unknown how the boundary between tip and stalk cells is established, but recent studies suggest that WD cells undergo extensive movement and the subsequent rearrangements appear to result from competition between cells based on the levels of Ret signaling (Chi et al., 2009). It is therefore tempting to speculate that the dynamic social interactions among epithelial cells reported to occur during *Drosophila* tracheal branching (Ghabrial and Krasnow, 2006) might also be operative in our chimera mosaic analysis (Fig. 6C). In this respect, *vHnf1*$^{-/-}$ cells may be unable both to compete with WT cells and to cooperate with themselves to shape a specialized tip domain.

vHNF1 and Wnt9b and the initiation of the tubulogenic program

The nephrons of the amniote kidney originate from induced mesenchymal cells that aggregate next to the invading UB epithelium and then undergo MET transition and tubulogenesis. *Wnt9b* secreted by the UB has been shown to provide a primary inductive signal to a subset of cells within the adjacent mesenchyme to epithelialize, establishing the renal vesicle and initiating the process of nephrogenesis through the canonical Wnt signaling pathway (Carroll et al., 2005; Park et al., 2007). Remarkably, *vHnf1*-deficient embryos present a similar phenotype to that of *Wnt9b*$^{-/-}$ embryos (Carroll et al., 2005), in regard to the defective initiation of the tubulogenic program. Our results show that vHNF1 acting directly on Wnt9b orchestrates Wnt signaling action in the MET process underlying early nephrogenesis. Consistent with this, culture of urogenital tracts in the presence of either Wnt9b or its direct target, Wnt4, induces the formation of pretubular aggregates by the mutant mesenchyme (Fig. 5B,C). Further studies involving specific

inactivation of *vHnf1* in the MM are, however, required to define whether vHNF1 has additional functions at later stages in the epithelialization of pretubular aggregates and their further differentiation into mature nephrons. The potential involvement of this factor during nephrogenesis and proximal tubule differentiation is suggested both by previous analysis of cystic dysplastic kidneys of human fetuses carrying mutations in the *vHNF1* (*HNF1*β) gene (Haumaitre et al., 2006) and by recent detailed transcriptional profiling of the developing mouse kidney. These studies show, in particular, a strong statistical association between the expression of vHNF1 in the developing proximal tubules and the presence of well-conserved HNF1-binding sites in the promoters of many genes highly enriched in this nephron structure (Brunskill et al., 2008).

Together, our findings demonstrate that vHNF1 is an essential transcriptional regulator that, in addition to its later functions in normal renal tubule morphogenesis (Gresh et al., 2004; Hiesberger et al., 2004) and its involvement in human RCAD syndrome, plays a crucial role during the earliest stages of urogenital development. This has provided new insights into the regulatory circuits controlling these processes. At later stages, renal-specific inactivation of *vHnf1* resulted in the formation of renal cysts accompanied by a reduced expression of genes involved in cystogenesis, including *Pkd2*, *Pkhd1* and *Umod* (Gresh et al, 2004), thus potentially linking the vHNF1 transcriptional regulatory network to ciliogenes. The expression of the cystoproteins encoded by these genes is, however, not altered in the multicystic kidneys of two fetuses carrying heterozygous *vHNF1* mutations (Haumaitre et al, 2006), suggesting that, in humans, impaired gene dosage causes cystic kidneys by additional mechanisms. Notably, and strikingly similar to vHNF1, recent studies show that, in addition to its initial role in nephrogenesis, Wnt9b plays a later role in renal tubule morphogenesis. Specifically, attenuation of Wnt9b signaling during kidney morphogenesis affects planar cell polarity (PCP) of the tubule epithelium and leads to cyst formation (Karner et al., 2009). Collectively, these results suggest that the vHNF1 to Wnt9b cascade may be a major regulatory pathway involved in the development of dysplastic/cystic kidneys in *vHNF1* (*HNF1β*) mutant carriers.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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References

Barbacci, E., Reber, M., Ott, M. O., Breillat, C., Huetz, F. and Cereghini, S. (1999). Variant hepatocyte nuclear factor 1 is required for visceral endoderm specification. *Development* **126**, 4795-4805.

Barbacci, E., Chalkiadaki, A., Masdeu, C., Haumaitre, C., Lokmane, L., Loirat, C., Cloarec, S., Talianidis, I., Bellanne-Chantelot, C. and Cereghini, S. (2004). HNF1beta/TCF2 mutations impair transactivation potential through altered co-regulator recruitment. *Hum. Mol. Genet.* **13**, 3139-3149.

Bellanne-Chantelot, C., Chauveau, D., Gautier, J. F., Dubois-Laforgue, D., Clauin, S., Beaufils, S., Wilhelm, J. M., Boitard, C., Noel, L. H., Velho, G. et al. (2004). Clinical spectrum associated with hepatocyte nuclear factor-1beta mutations. *Ann. Intern. Med.* **140**, 510-517.

Bohn, S., Thomas, H., Turan, G., Ellard, S., Bingham, C., Hattersley, A. T. and Ryffel, G. U. (2003). Distinct molecular and morphogenetic properties of mutations in the human HNF1beta gene that lead to defective kidney development. *J. Am. Soc. Nephrol.* **14**, 2033-2041.

Bouchard, M. (2004). Transcriptional control of kidney development. *Differentiation* **72**, 295-306.

Bouchard, M., Souabni, A., Mandler, M., Neubuser, A. and Busslinger, M. (2002). Nephric lineage specification by Pax2 and Pax8. *Genes Dev.* **16**, 2958-2970.

Bridgewater, D., Cox, B., Cain, J., Lau, A., Athaide, V., Gill, P. S., Kuure, S., Sainio, K. and Rosenblum, N. D. (2008). Canonical WNT/beta-catenin signaling is required for ureteric branching. *Dev. Biol.* **317**, 83-94.

Brunskill, E. W., Aronow, B. J., Georgas, K., Rumballe, B., Valerius, M. T., Aronow, J., Kaimal, V., Jegga, A. G., Yu, J., Grimmond, S. et al. (2008). Atlas of gene expression in the developing kidney at microanatomic resolution. *Dev. Cell* **15**, 781-791.

Carroll, T. J., Park, J. S., Hayashi, S., Majumdar, A. and McMahon, A. P. (2005). Wnt9b plays a central role in the regulation of mesenchymal to epithelial transitions underlying organogenesis of the mammalian urogenital system. *Dev. Cell* **9**, 283-292.

Chi, X., Michos, O., Shakya, R., Riccio, P., Enomoto, H., Licht, J. D., Asai, N., Takahashi, M., Ohgami, N., Kato, M. et al. (2009). Ret-dependent cell rearrangements in the Wolffian duct epithelium initiate ureteric bud morphogenesis. *Dev. Cell* **17**, 199-209.

Costantini, F. and Shakya, R. (2006). GDNF/Ret signaling and the development of the kidney. *BioEssays* **28**, 117-127.

Donovan, M. J., Natoli, T. A., Sainio, K., Amstutz, A., Jaenisch, R., Sariola, H. and Kreidberg, J. A. (1999). Initial differentiation of the metanephric mesenchyme is independent of WT1 and the ureteric bud. *Dev. Genet.* **24**, 252-262.

Dressler, G. R. (2006). The cellular basis of kidney development. *Annu. Rev. Cell Dev. Biol.* **22**, 509-529.

Dudley, A. T., Lyons, K. M. and Robertson, E. J. (1995). A requirement for bone morphogenetic protein-7 during development of the mammalian kidney and eye. *Genes Dev.* **9**, 2795-2807.

Edghill, E. L., Bingham, C., Ellard, S. and Hattersley, A. T. (2006). Mutations in hepatocyte nuclear factor-1beta and their related phenotypes. *J. Med. Genet.* **43**, 84-90.

Ghabrial, A. S. and Krasnow, M. A. (2006). Social interactions among epithelial cells during tracheal branching morphogenesis. *Nature* **441**, 746-749.

Gresh, L., Fischer, E., Reimann, A., Tanguy, M., Garbay, S., Shao, X., Hiesberger, T., Fiette, L., Igarashi, P., Yaniv, M. et al. (2004). A transcriptional network in polycystic kidney disease. *EMBO J.* **23**, 1657-1668.

Grieshammer, U., Cebrian, C., Ilagan, R., Meyers, E., Herzlinger, D. and Martin, G. R. (2005). FGF8 is required for cell survival at distinct stages of nephrogenesis and for regulation of gene expression in nascent nephrons. *Development* **132**, 3847-3857.

Grote, D., Boualia, S. K., Souabni, A., Merkel, C., Chi, X., Costantini, F., Carroll, T. and Bouchard, M. (2008). Gata3 acts downstream of beta-catenin signaling to prevent ectopic metanephric kidney induction. *PLoS Genet.* **4**, e1000316.

Haumaitre, C., Barbacci, E., Jenny, M., Ott, M. O., Gradwohl, G. and Cereghini, S. (2005). Lack of TCF2/vHNF1 in mice leads to pancreas agenesis. *Proc. Natl. Acad. Sci. USA* **102**, 1490-1495.

Haumaitre, C., Fabre, M., Cormier, S., Baumann, C., Delezoide, A. L. and Cereghini, S. (2006). Severe pancreas hypoplasia and multicystic renal dysplasia in two human fetuses carrying novel HNF1beta/MODY5 mutations. *Hum. Mol. Genet.* **15**, 2363-2375.

Hayashi, S., Lewis, P., Pevny, L. and McMahon, A. P. (2002). Efficient gene modulation in mouse epiblast using a Sox2Cre transgenic mouse strain. *Mech. Dev.* 119 **Suppl. 1**, S97-S101.

Hiesberger, T., Bai, Y., Shao, X., McNally, B. T., Sinclair, A. M., Tian, X., Somlo, S. and Igarashi, P. (2004). Mutation of hepatocyte nuclear factor-1beta inhibits Pkhd1 gene expression and produces renal cysts in mice. *J. Clin. Invest.* **113**, 814-825.

Karner, C. M., Chirumamilla, R., Aoki, S., Igarashi, P., Wallingford, J. B. and Carroll, T. J. (2009). Wnt9b signaling regulates planar cell polarity and kidney tubule morphogenesis. *Nat. Genet.* **41**, 793-799.

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.

Kobayashi, A., Kwan, K. M., Carroll, T. J., McMahon, A. P., Mendelsohn, C. L. and Behringer, R. R. (2005). Distinct and sequential tissue-specific activities of the LIM-class homeobox gene Lim1 for tubular morphogenesis during kidney development. *Development* **132**, 2809-2823.

Kuschert, S., Rowitch, D. H., Haenig, B., McMahon, A. P. and Kispert, A. (2001). Characterization of Pax-2 regulatory sequences that direct transgene expression in the Wolffian duct and its derivatives. *Dev. Biol.* **229**, 128-140.
Lim, K. C., Lakshmanan, G., Crawford, S. E., Gu, Y., Grosveld, F. and Engel, J. D. (2000). Gata3 loss leads to embryonic lethality due to noradrenaline deficiency of the sympathetic nervous system. *Nat. Genet.* **25**, 209-212.
Lin, Y., Zhang, S., Tuukkanen, J., Peltoketo, H., Pihlajaniemi, T. and Vainio, S. (2003). Patterning parameters associated with the branching of the ureteric bud regulated by epithelial-mesenchymal interactions. *Int. J. Dev. Biol.* **47**, 3-13.
Lindner, T. H., Njolstad, P. R., Horikawa, Y., Bostad, L., Bell, G. I. and Sovik, O. (1999). A novel syndrome of diabetes mellitus, renal dysfunction and genital malformation associated with a partial deletion of the pseudo-POU domain of hepatocyte nuclear factor-1beta. *Hum. Mol. Genet.* **8**, 2001-2008.
Lokmane, L., Haumaitre, C., Garcia-Villalba, P., Anselme, I., Schneider-Maunoury, S. and Cereghini, S. (2008). Crucial role of vHNF1 in vertebrate hepatic specification. *Development* **135**, 2777-2786.
Majumdar, A., Vainio, S., Kispert, A., McMahon, J. and McMahon, A. P. (2003). Wnt11 and Ret/Gdnf pathways cooperate in regulating ureteric branching during metanephric kidney development. *Development* **130**, 3175-3185.
Marose, T. D., Merkel, C. E., McMahon, A. P. and Carroll, T. J. (2008). Beta-catenin is necessary to keep cells of ureteric bud/Wolffian duct epithelium in a precursor state. *Dev. Biol.* **314**, 112-126.
Michael, L. and Davies, J. A. (2004). Pattern and regulation of cell proliferation during murine ureteric bud development. *J. Anat.* **204**, 241-255.
Miyamoto, N., Yoshida, M., Kuratani, S., Matsuo, I. and Aizawa, S. (1997). Defects of urogenital development in mice lacking Emx2. *Development* **124**, 1653-1664.
Miyazaki, Y., Oshima, K., Fogo, A., Hogan, B. L. and Ichikawa, I. (2000). Bone morphogenetic protein 4 regulates the budding site and elongation of the mouse ureter. *J. Clin. Invest.* **105**, 863-873.
Park, J. S., Valerius, M. T. and McMahon, A. P. (2007). Wnt/beta-catenin signaling regulates nephron induction during mouse kidney development. *Development* **134**, 2533-2539.
Pedersen, A., Skjong, C. and Shawlot, W. (2005). Lim 1 is required for nephric duct extension and ureteric bud morphogenesis. *Dev. Biol.* **288**, 571-581.
Perantoni, A. O., Timofeeva, O., Naillat, F., Richman, C., Pajni-Underwood, S., Wilson, C., Vainio, S., Dove, L. F. and Lewandoski, M. (2005). Inactivation of FGF8 in early mesoderm reveals an essential role in kidney development. *Development* **132**, 3859-3871.
Pope, J. C. t., Brock, J. W., 3rd, Adams, M. C., Stephens, F. D. and Ichikawa, I. (1999). How they begin and how they end: classic and new theories for the development and deterioration of congenital anomalies of the kidney and urinary tract, CAKUT. *J. Am. Soc. Nephrol.* **10**, 2018-2028.
Reber, M. and Cereghini, S. (2001). Variant hepatocyte nuclear factor 1 expression in the mouse genital tract. *Mech. Dev.* **100**, 75-78.
Saxen, L. and Sariola, H. (1987). Early organogenesis of the kidney. *Pediatr. Nephrol.* **1**, 385-392.
Schedl, A. (2007). Renal abnormalities and their developmental origin. *Nat. Rev. Genet.* **8**, 791-802.
Schmidt-Ott, K. M., Yang, J., Chen, X., Wang, H., Paragas, N., Mori, K., Li, J. Y., Lu, B., Costantini, F., Schiffer, M. et al. (2005). Novel regulators of kidney development from the tips of the ureteric bud. *J. Am. Soc. Nephrol.* **16**, 1993-2002.
Shakya, R., Watanabe, T. and Costantini, F. (2005). The role of GDNF/Ret signaling in ureteric bud cell fate and branching morphogenesis. *Dev. Cell* **8**, 65-74.
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.
Sun, Z. and Hopkins, N. (2001). vhnf1, the MODY5 and familial GCKD-associated gene, regulates regional specification of the zebrafish gut, pronephros, and hindbrain. *Genes Dev.* **15**, 3217-3229.
Torres, M., Gomez-Pardo, E., Dressler, G. R. and Gruss, P. (1995). Pax-2 controls multiple steps of urogenital development. *Development* **121**, 4057-4065.
Vainio, S. and Lin, Y. (2002). Coordinating early kidney development: lessons from gene targeting. *Nat. Rev. Genet.* **3**, 533-543.
Xu, P. X., Adams, J., Peters, H., Brown, M. C., Heaney, S. and Maas, R. (1999). Eya1-deficient mice lack ears and kidneys and show abnormal apoptosis of organ primordia. *Nat. Genet.* **23**, 113-117.

Table S1 : Oligonucleotides used for gel shift analysis.

Primer name		Position	Sequence
Wnt9b site -7800	Forward	-7808/-7767	5' taagtatttgttaattattaattaattaattaactaatcaa 3'
	Lower		5' ttgattagttaattaattaattaataattaacaaatactta 3'
Wnt9b site -7879	Forward	-7884 / -7863	5' gaaaaaagttaatatatctaacacatt 3'
	Lower		5' aatgtgttagatatattaacttttttc 3'
Wnt9b site +14132	Forward	+14121/+14149	5' atttgccccctgattaatgtttaacggg 3'
	Lower		5' ggcccgttaaacattaatcaggggggcaaat 3'
Pax2 site -6929	Forward	-6936 / -6906	5' ctcccttcgttaatcagtttcatcaattta 3'
	Lower		5' taaattgatgaaactgattaacgaagggag 3'
Pax2 site -5742	Forward	-5749 / -5719	5' agaggaagttaatcattacttccccaccaa 3'
	Lower		5' ttggtggggaagtaatgattaacttcctct 3'
Lim1 site -7847	Forward	-7853 / -7825	5' ggggtactgaaataattaacttgctaatca 3'
	Lower		5' tgattagcaagttaattatttcagtacc 3'

Table S2 : Primer pairs used for cloning and for site directed mutagenesis.

Primer name		Position	Sequence
Oligonucleotides used for Cloning			
Wnt9b -7946/-7529	Forward	-8038	5' gtt aat act agc tag gct gtg gt 3'
	Reverse	-7529	5' cga ctg ctc ttc cga agg t 3'
Wnt9b -7946/-7809	Forward	-8038	5' gtt aat act agc tag gct gtg gt 3'
	Reverse	-7809	5' tgg cag ctg gca ttt cta t 3'
Wnt9b +13979/14601	Forward	+12948	5' gca aag acc ata cgg caa tc 3'
	Reverse	+14751	5' ggt gat aag gcg tga ctg gt 3'
Pax2 -7310/-6884	Forward	-7310	5' tgg atc cga aga cgc agc ctg aca tct 3'
	Reverse	-6884	5' caa gct tag ata gac ccg tgg cta acg 3'
Pax2 -5762/-5572	Forward	-5762	5' tgg atc cgg ctc cag cag gct aag ag 3'
	Reverse	-5572	5' gaa gct tag gac tca aag tgg gtg ctg 3'
Lim1 -7934/-7711	Forward	-7934	5' caa gct tcc agg tgc tca cca aga aat 3'
	Reverse	-7711	5' tgg atc ctg tgc aga ggg tgt gtt tc 3'
Oligonucleotides used for site directed mutagenesis*			
Wnt9b site -7079	Forward	5’cct gtc tca aaa gga aaa aaa aaG Tgg Ata Tgg CTA aca cat tac3’	
Wnt9b site +14123	Forward	5’gcc ccc tgA Tcc ATG Tcc AAC ggg ccc ctg tgc3’	
Pax2 site -6929	Forward	5’ttc ctc cct tcG TTg gTc AGg gTC atc aat tta ttc gtt ag3’	
Pax2 site -5742	Forward	5’ggc taa gag gaa GTT ccT cAT ccC Ttc ccc acc aac ac3’	
Lim1 site -7847	Forward	5’acc ctt ggg gta cTG ccA TaA ccA ACt tgc taa tca gc3’	

** vHNF1 binding sites are underlined; lower case nucleotides within underlined sequences indicate mutated bases.*

Table S3 : Primer pairs used for ChIP.

Primer name		Position	Sequence
Wnt9b site -8703	Forward	-8782	5' tgc ttc tac ttc cca cag gg 3'
	Reverse	-8610	5' acg tga ccc ttg tgt gtt ca 3'
Wnt9b site -8383	Forward	-8433	5' cta ttg gag aca tgc ggg at 3'
	Reverse	-8310	5' gac aag cac ttt gcc aca ga 3'
Wnt9b site -7800	Forward	-7839	5' ttt atc ctt ata tag aaa tgc cag c 3'
	Reverse	-7701	5' gag tca gag gag gtt aag tc 3'
Wnt9b site +14132	Forward	+13971	5' act gga aac cgt tca agg tg 3'
	Reverse	+14250	5' gtg gag cat atc tcc gaa aca 3'
Pax2 site -6929	Forward	-7140	5' gga ggc gct gga aat gag t 3'
	Reverse	-6884	5' aga tag acc cgt ggc taa cg 3'
Pax2 site -5742	Forward	-5764	5' ggc tcc agc agg cta aga g 3'
	Reverse	-5572	5' agg act caa agt ggg tgc tg 3'
*Lim1*site -7847	Forward	-7932	5' cca ggt gct cac caa gaa at3'
	Reverse	-7711	5' ctg tgc aga ggg tgt gtt tc 3'