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Notch signaling, wt1 and foxc2 are key regulators of the podocyte gene regulatory network in *Xenopus*

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SUMMARY

Podocytes are highly specialized cells in the vertebrate kidney. They participate in the formation of the size-exclusion barrier of the glomerulus/glomus and recruit mesangial and endothelial cells to form a mature glomerulus. At least six transcription factors (wt1, foxc2, hey1, tcf21, lmx1b and mafb) are known to be involved in podocyte specification, but how they interact to drive the differentiation program is unknown. The *Xenopus* pronephros was used as a paradigm to address this question. All six podocyte transcription factors were systematically eliminated by antisense morpholino oligomers. Changes in the expression of the podocyte transcription factors and of four selected markers of terminal differentiation (*nphs1*, *kirrel*, *ptpru* and *nphs2*) were analyzed by in situ hybridization. The data were assembled into a transcriptional regulatory network for podocyte development. Although eliminating the six transcription factors individually interfered with aspects of podocyte development, no single gene regulated the entire differentiation program. Only the combined knockdown of wt1 and foxc2 resulted in a loss of all podocyte marker gene expression. Gain-of-function studies showed that wt1 and foxc2 were sufficient to increase podocyte gene expression within the glomus proper. However, the combination of wt1, foxc2 and Notch signaling was required for ectopic expression in ventral marginal zone explants. Together, this approach demonstrates how complex interactions are required for the correct spatiotemporal execution of the podocyte gene expression program.

KEY WORDS: Amphibian, Glomus, Glomerulus, Mesangial cells, Notch, Slit diaphragm, *Xenopus*

INTRODUCTION

The kidney is required for water homeostasis and waste excretion (Saxén, 1987; Vize et al., 2003). In vertebrates, three successively more complex kidney structures – the pronephros, mesonephros and metanephros – have evolved. The metanephros is the adult kidney in higher vertebrates, such as humans and mice, whereas the mesonephros is the adult kidney of fish and amphibians. The pronephros is the simplest and earliest kidney form. It is rudimentary in mammals, but is required in aquatic animals for water homeostasis at larval stages (Howland, 1916). Moreover, the development of the pronephros is a prerequisite for the subsequent formation of the mesonephric and metanephric kidney (Torrey, 1965; Bouchard et al., 2002). Despite the differing complexity of the three kidney types, their functional unit, the nephron, is organized very similarly. Many transcription factors, structural proteins and signaling pathways pattern the pronephros and metanephros in an evolutionarily conserved manner (Carroll et al., 1999; McLaughlin et al., 2000; Cheng et al., 2003; Wang et al., 2003; Zhou and Vize, 2004; Cheng et al., 2007; Wingert et al., 2007; Raciti et al., 2008).

Among the different cell types present in the nephron, podocytes are unique in that they link the vasculature to the urinary system (Johnstone and Holzman, 2006; Quaggin and Kreidberg, 2008). They are highly specialized epithelial cells required for kidney filtration. Podocytes form the slit diaphragm, a sieve-like structure with a 4-nm pore size that restricts the transport of molecules from the blood to the ultrafiltrate (Rodewald and Karnovsky, 1974; Edwards et al., 1999; Takahashi-Iwanaga, 2002). The slit diaphragm contains proteins that are commonly found in adherens junctions (e.g. α-, β-, γ-Catenin, Vinculin and α-Actinin-4) and tight junctions [e.g. Tjp1 (ZO1)], as well as a set of slit diaphragm-specific proteins [e.g. Nphs1 (Nephrin), Kirrel (Neph1) and Nphs2 (Podocin)] (Pavenstadt et al., 2003; Johnstone and Holzman, 2006; Quaggin and Kreidberg, 2008). Although the importance of the slit diaphragm complex is well established, the details of its formation during podocyte differentiation are still unknown.

Several transcription factors and signaling pathways have been implicated in this process (Quaggin and Kreidberg, 2008). Among these, Wilms tumor 1 (Wt1) is regarded as the key regulator of podocyte development. Although *Wt1* mutant mice do not form kidneys (Kreidberg et al., 1993), mice lacking the transcriptionally active *Wt1* splice variant *Wt1-KTS* develop kidneys with very few immature glomeruli (Hammes et al., 2001). The role of Wt1 is evolutionarily conserved. Studies in zebrafish and *Xenopus* show that Wt1 is also important for glomerulogenesis in the pronephros (Majumdar et al., 2000; Taelman et al., 2006; Perner et al., 2007). Other podocyte transcription factors develop weaker podocyte phenotypes in mouse loss-of-function studies. *Foxc2* homozygous mouse mutants have hypoplastic kidneys with few glomeruli, exhibiting dilated capillary loops and expressing decreased levels of Nphs2 and Mafb (Takemoto et al., 2006). *Mafb*, *Tcf21* and *Lmx1b* mouse mutants develop glomeruli, but the podocytes show defects at the ultrastructural level (Quaggin et al., 1999; Miner et al., 2002; Quaggin, 2002; Rohr et al., 2002; Sadl et al., 2002; Cui et al., 2003; Moriguchi et al., 2006). Notch is one of the best-documented signaling pathways in the kidney. It is involved in the initial patterning of the nephron, determining proximal (i.e. glomerulus and

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proximal tubules) versus distal cell fates in both mouse and *Xenopus* (McLaughlin et al., 2000; Cheng et al., 2003; Wang et al., 2003; Taelman et al., 2006; Cheng et al., 2007; Naylor and Jones, 2009).

Although glomerular transcription factors have been studied extensively, it is still not known how they cooperate to form a functional podocyte and regulate podocyte-specific transcription. To address this question, we have performed a systematic knockdown of six transcription factors (wt1, foxc2, hey1, mafb, tcf21 and lmx1b) using the *Xenopus* pronephros as a model for podocyte development. This analysis identified a specific role for each transcription factor in podocyte specification. More importantly, the data were assembled into a gene regulatory network to visualize interactions between the transcription factors. This revealed that wt1 and foxc2 together are required, but not sufficient, for podocyte development. A third input, activated Notch signaling, was necessary to induce ectopic podocyte gene expression. This suggested that the correct spatiotemporal execution of the podocyte program relies on the concerted action of at least three independent inputs: wt1, foxc2 and Notch signaling.

MATERIALS AND METHODS

Embryo manipulations and RT-PCR analyses

Xenopus embryos obtained by in vitro fertilization were maintained in 0.1× modified Barth medium (Sive et al., 2000) and staged following Nieuwkoop and Faber (Nieuwkoop and Faber, 1994). For antisense morpholino oligomer (MO) injections, a total of 3.2 pmol of MO was injected radially into *Xenopus* embryos at the 2- to 4-cell stage. MOs were dissolved to 1 mM stock solutions and were used at a final concentration of 200 μM. The following MOs were obtained from Gene Tools: 5′-ATTCATATCCCGCACATCAGATCCC-3′ (*wt1-MO1*), 5′-CATATCCCGGACATCAGACCCCATC-3′ (*wt1-MO2*) (Taelman et al., 2006), 5′-ACGCGCCTGCATCATTAGTGCTGAA-3′ (*foxc2-MO*), 5′-TAGTCGTGTCCCCGCTTCATGGCTG-3′ (*hey1-MO*) (Taelman et al., 2006), 5′-CATCACTGAGAGAACCGGTGGACAT-3′ (*tcf21-MO*), 5′-CGGGACCTGTTGCAATATCCATGCC-3′ (*lmx1b-MO*), 5′-GCCACTCTCCAAAACTCACTTCAGT-3′ (*lmx1b-MO2*) (Haldin et al., 2008), 5′-AATGGGCAACTCTCCAGCCATACTG-3′ (*mafb-MO*) and a standard control MO (*Std-MO*).

For synthetic mRNA, plasmids were linearized as indicated and transcribed with SP6 RNA polymerase using the mMessage mMachine (Applied Biosystems) as follows: *pCS2-foxc2** (*Apa*I), *pCS2-GFP-foxc2* (*Not*I), *pCS2-lmx1b** (*Not*I), *pCS2-GFP-lmx1b* (*Not*I), *pCS2-mafb** (*Not*I), *pCS2-GFP-mafb* (*Not*I), *pCS2-NICD* (*Not*I) (Coffman et al., 1990), *pCS2-GFP-tcf21* (*Not*I), *pCS2-wt1** (*Ksp*I), *pCS2-wt1-GFP* (*Not*I), *pCS2-wt1(mut)-GFP* (*Not*I); *pXEX-β-Gal* was linearized with *Asp*718 and transcribed with T7 RNA polymerase (detailed information about the individual constructs is available upon request).

Ectodermal explants were excised at stage 9; marginal zone explants were excised at stage 10. Explants were cultured in 0.5× MMR (Sive et al., 2000) until sibling embryos reached stage 35. For Activin A treatment, explants were incubated for 3 hours with 10 ng/ml Activin A (Cell Sciences) in 0.1% BSA low Ca^{2+}/low Mg^{2+} Ringer's Solution (Sive et al., 2000). Explants and whole control embryos were processed for RT-PCR analysis as described (Sasai et al., 1995). The sequences of the primers are listed in Table S1 in the supplementary material. RT-PCR products were analyzed by polyacrylamide gel electrophoresis. In some cases results were quantified using a GelDoc-It Imaging System (UVP). The percentage change in gene expression in injected embryos compared with controls was calculated and averaged between three experiments. The F-statistic was used to verify equal variance between the experimental and control groups so that statistical significance could be calculated using a one-tailed Student's *t*-test.

GFP reporter assays

For the GFP reporter assays, 250 pg of synthetic mRNA was injected into the animal pole of each blastomere at the 4-cell stage, alone or preceding animal injection of 3.2 pmol of the corresponding MO at the 2-cell stage. Once sibling uninjected embryos reached stage 10, embryos or dissected animal poles were analyzed for fluorescence on a Zeiss Axio Imager A1 with an Axiocam digital camera. Fluorescence intensity was quantified using ImageJ (NIH). MO-injected embryos and transcription factor-GFP + MO co-injected embryos were compared using Student's *t*-test.

Whole-mount in situ hybridization

Whole-mount in situ hybridization and in situ hybridization on Paraplast sections were performed as described (Tran et al., 2007; Agrawal et al., 2009). The following plasmids were linearized and transcribed for antisense probes: *pSK-aplnr*, *Not*I/T7 (Devic et al., 1996); *pSK-foxc2*, *Eco*RI/T7 (GenBank accession AJ249225); *pCS2-hey1*, *Eco*RI/T7 (DC130331); *pCS2p-kirrel*, *Cla*I/T7 (BC057728); *pSK-lmx1b*, *Bam*HI/T7 (AF414086); *pCS2p-mafb*, *Eco*RI/T7 (BC077255); *pCMV-SPORT6-nphs1*, *Eco*RI/T7 (Tran et al., 2007); *pGEM-T-Easy-nphs2*, *Apa*I/SP6 (GQ370808); *pCMV-SPORT6-ptpru*, *Eco*RI/T7 (CD300952); *pCMV-SPORT6-tcf21*, *Eco*RI/T7 (BC073597); *pSK-wt1*, *Bam*HI/T7 (Carroll and Vize, 1996).

All experiments were performed in triplicate and analyzed by comparing the expression patterns of the podocyte genes at stage 35 between morphants and sibling control embryos of the same experiment. Embryos were embedded in Paraplast and sectioned transversely at 25 μm. For each gene at least six control and six morphant embryos were evaluated by examining every section of the embryo. The effects of knockdowns on the expression of podocyte genes were recorded as increased, decreased, absent or unchanged. Sectioning of whole-mount embryos was essential to accurately judge the individual expression patterns as the analysis of whole-mount in situ hybridizations tended to underestimate the effects. Moreover, the podocyte expression of *foxc2* and *mafb* was obscured by other expression domains and could not be evaluated in whole-mounts.

Histology and immunohistochemistry

For histological staining, *Xenopus* embryos were fixed in Bouin's Fixative, dehydrated, embedded in Paraplast, sectioned at 7 μm, dewaxed, and stained with Hematoxylin and Eosin. For immunohistochemistry, embryos were fixed in Dent's Fixative. For whole-mount immunostaining, embryos were incubated overnight with Vimentin antiserum [14h7 (Dent et al., 1989)] followed by incubation with a horseradish peroxidase-coupled anti-mouse IgG and developed using the ImmPACT DAB Kit (Vector Laboratories). Embryos were subsequently embedded in Paraplast and sectioned coronally at 25 μm to visualize the glomus. Immunohistochemistry for β1-Integrin [8C8 (Gawantka et al., 1992)] was performed on Paraplast sections using an Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (Invitrogen).

RESULTS

Temporal expression pattern of podocyte genes

As a first step to understand podocyte development in *Xenopus*, the expression patterns of six podocyte transcription factors [wt1, foxc2, hey1 (xhrt1), tcf21, mafb and lmx1b] were compared with the expression of four markers of terminal differentiation [nphs1 (Nephrin), Kirrel (neph1) and nphs2 (Podocin)] that are expressed in the slit diaphragm (Ruotsalainen et al., 1999; Schwarz et al., 2001; Barletta et al., 2003) and with ptpru (glepp1) as a non-slit diaphragm protein (Thomas et al., 1994). *wt1*, *hey1*, *mafb*, *lmx1b*, *tcf21* and *nphs1* have been studied previously in *Xenopus* whole-mounts (Carroll and Vize, 1996; Ishibashi and Yasuda, 2001; Haldin et al., 2003; Coolen et al., 2005; Gerth et al., 2005; Simrick et al., 2005; Taelman et al., 2006; Haldin et al., 2008), but to precisely compare the glomus expression domains it was important to perform a side-by-side analysis of all ten genes. We initially analyzed embryos at stage 35 because *Xenopus* podocytes are functional at stage 38 and vascularization of the glomus occurs around stage 32 (Nieuwkoop and Faber, 1994; Vize et al., 2003; Doherty et al., 2007). Both whole-mount in situ hybridizations and Paraplast-embedded sections thereof revealed distinct expression

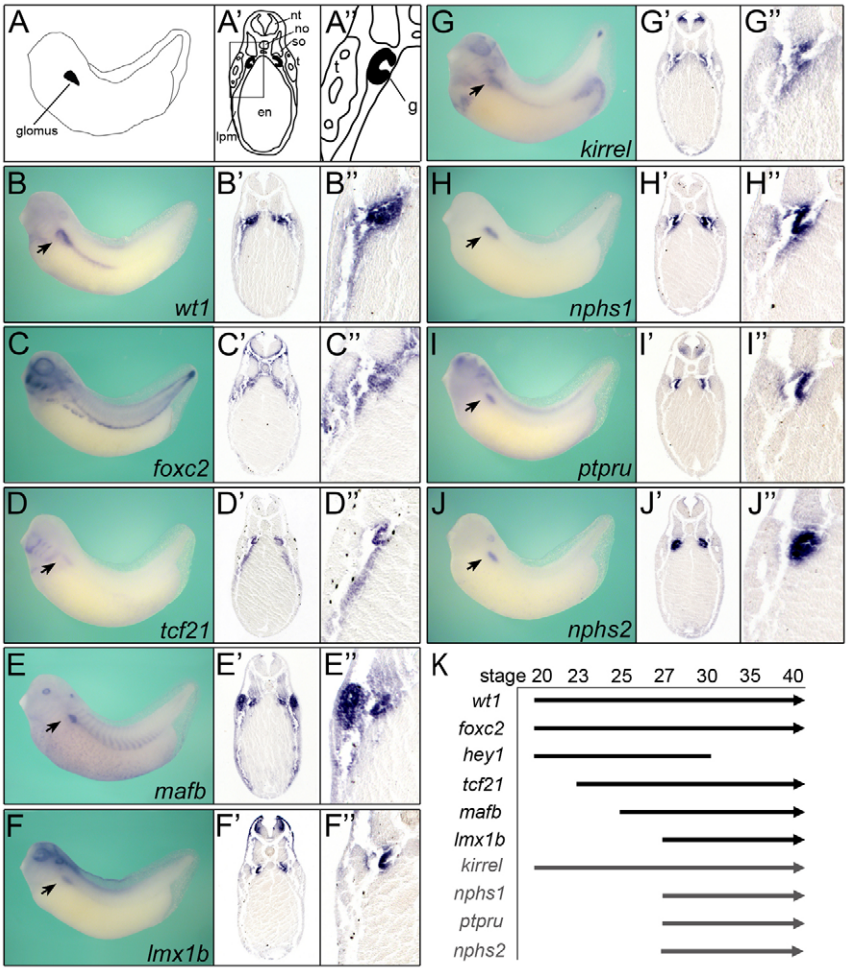

Fig. 1. Spatiotemporal expression of podocyte genes. (**A-A″**) Schematics depicting the glomus (black) in whole *Xenopus* embryos (A) and in transverse section at low (A′) and high (A″) magnification at stage 35. (**B-J″**) In situ hybridization of podocyte transcription factors *wt1* (B-B″), *foxc2* (C-C″), *tcf21* (D-D″), *mafb* (E-E″) and *lmx1b* (F-F″) and of markers of podocyte differentiation *kirrel* (G-G″), *nphs1* (H-H″), *ptpru* (I-I″) and *nphs2* (J-J″) at stage 35 in whole-mounts (B-J) and transverse sections (B′-J″). (**K**) Temporal profile of podocyte gene expression in the glomus. en, endoderm; g, pronephric glomus; lpm, lateral plate mesoderm; no, notochord; nt, neural tube; so, somites; t, pronephric tubules.

patterns. *wt1* mRNA was detected in the glomus, the intermediate mesoderm surrounding the pronephric duct and the heart (Fig. 1B-B″ and data not shown). *foxc2* was expressed in the glomus, the eyes, the branchial arches, hypaxial muscles and the tailbud, as well as in the mesenchyme surrounding the notochord and neural tube (Fig. 1C-C″). *tcf21* was expressed in the glomus, lateral plate mesoderm and branchial arches (Fig. 1D-D″). *mafb* mRNA was detected in the glomus, proximal tubules, somites, ventral blood islands, eyes and hindbrain (Fig. 1E-E″). *lmx1b* was detected in the glomus, eyes, otic placodes, dorsal ectoderm and the dopaminergic neurons of the neural tube (Fig. 1F-F″). *kirrel* was expressed in the glomus, eyes, brain, branchial arches, heart, tailbud and the ventral pancreatic bud (Fig. 1G-G″). *nphs1* was exclusively expressed in the glomus (Fig. 1H-H″). *ptpru* was detected in the glomus, eyes, brain and neural tube (Fig. 1I-I″). *nphs2* was detected in the glomus and the inner lining of the neural tube (Fig. 1J-J″). As previously reported (Taelman et al., 2006), *hey1* was no longer expressed in the glomus region at stage 35 (data not shown). Examining in situ hybridizations at progressively earlier stages also established a temporal profile of glomus gene expression (Fig. 1K and data not shown). Based on this analysis, *wt1*, *foxc2* and *hey1* are the earliest expressed transcription factors at stage 20, followed by *tcf21* at stage 23, *mafb* at stage 25 and *lmx1b* at stage 27. Interestingly, of the terminal differentiation genes, *kirrel* was already detected at stage 20, whereas the other three were not detected until stage 27.

Together, these data showed that the podocyte genes analyzed here are present in the glomus by stage 27, even though the pronephros is not functional until stage 38 (Nieuwkoop and Faber, 1994). Interestingly, none of the podocyte transcription factors and only one terminal differentiation marker is glomus specific. Thus, combinatorial inputs most likely play an important role in restricting podocyte development to the glomus anlage.

Knockdown of wt1

The expression analysis suggested that *wt1* is among the earliest transcription factors expressed in the future glomus. Work by others has suggested that wt1 is a key regulator in podocyte development (Hammes et al., 2001; Taelman et al., 2006; Perner et al., 2007). Thus, to better understand the role of wt1 in podocyte specification, the protein was eliminated using two antisense morpholino oligomers (*wt1-MO1*, *wt1-MO2*) that target the ATG of the two pseudo-alleles of *wt1* mRNA (Fig. 2A). In the absence of an antibody recognizing *Xenopus* wt1 protein, a reporter assay was developed to test the efficacy of the *wt1-MO*. Part of the 5′UTR and the first 125 amino acids of wt1 were fused in-frame with eGFP (*wt1-GFP*, Fig. 2B). A second construct mimicking the second allele of *wt1* was generated by site-directed mutagenesis [*wt1(mut)-GFP*, Fig. 2B]. Synthetic *wt1-GFP* or *wt1(mut)-GFP* mRNA (1 ng) was injected into the animal pole of *Xenopus* embryos at the 4-cell stage in the presence or absence of a mixture of the two *wt1* MOs (3.2 pmol each, *wt1-MO1+2*). Embryos were

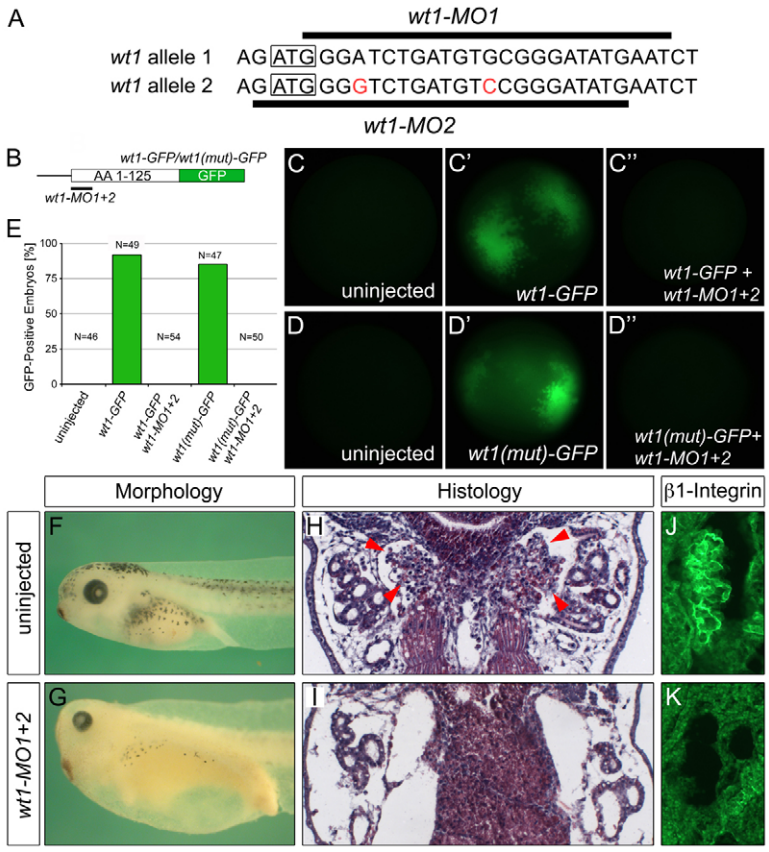

Fig. 2. Knockdown of wt1 results in a glomus phenotype. (**A**) Sequences of the two pseudo-alleles of *Xenopus wt1* and the location of *wt1-MO1* and *wt1-MO2*. Mismatches between *wt1-MO* and the second allele of *wt1* are indicated in red. The translational start site is boxed. (**B**) Schematic of the wt1-GFP reporter constructs. (**C-E**) Fluorescence of uninjected control embryos (C,D), embryos injected with the *wt1-GFP* reporter mRNA (C′), with *wt1-MO1+2* and the *wt1-GFP* reporter (C″), *wt1(mut)-GFP* (D′) or with *wt1-MO1+2* and the *wt1(mut)-GFP* (D″) at stage 10. The results of multiple experiments were quantified (E); the number of embryos analyzed is indicated above the bars. (**F-K**) Phenotype, histology and β1-Integrin immunofluorescence of *wt1-MO1+2*-injected embryos (G,I,K) and sibling controls (F,H,J) at stage 40 (F,G) and stage 42 (H-K). Red arrowheads indicate glomus in H.

cultured until gastrula stage and imaged by fluorescence microscopy (Fig. 2C-E). Whereas fluorescence could be detected in the *wt1-GFP*-injected and *wt1(mut)-GFP*-injected embryos, it was completely abolished upon co-injection of *wt1-MO1+2*.

Next, *Xenopus* embryos were injected radially with *wt1-MO1+2* at the 2- to 4-cell stage, cultured until sibling embryos reached stage 40/42 and analyzed by morphology and histology. *wt1-MO1+2*-injected embryos were characterized by the formation of fluid-filled edema (Fig. 2F-I). This phenotype was most likely caused by a defective pronephros. Histological sections and immunofluorescence for β1-Integrin, which marks the basolateral side of podocytes (Pozzi et al., 2008), indicated a complete absence of the glomus domain in the injected embryos (Fig. 2H-K).

To substantiate this phenotype at the molecular level, whole-mount in situ hybridizations were performed to analyze the expression of the podocyte transcription factors *wt1*, *foxc2*, *tcf21*, *lmx1b* and *mafb* and the terminal differentiation genes *nphs1*, *kirrel*, *ptpru* and *nphs2*. Embryos were analyzed at stage 35 because all the genes, with the exception of *hey1*, were robustly expressed at this stage (Fig. 1K). It is very difficult to dissect the glomus without contamination from the surrounding tissue, and this might influence the results of quantitative RT-PCR analysis. Instead, whole-mounts and Paraplast sections thereof were used to compare gene expression patterns or levels (see Materials and methods for details on the evaluation process). *wt1-MO1+2*-injected embryos displayed a wide range of changes in the

Table 1. Expression of podocyte transcription factors and markers of terminal differentiation after knockdown

	Gene								
Treatment	*wt1*	*foxc2*	*lmx1b*	*mafb*	*tcf21*	*ptpru*	*nphs1*	*kirrel*	*nphs2*
Std-MO	–	–	–	–	–	–	–	–	–
wt1-MO1+2	↑↑*	↑*	ø	↓↓	ø	ø	↓↓	↓†	ø
wt1-MO	↑↑*	↑*	↓↓	↓	–	↓↓	↓↓	↓*	↓↓
foxc2-MO	–*	–	↓*	↓†	↑	↓↓	↓	↓	↓↓
tcf21-MO	↑	–	–*	–	↑	↓	–*	↑	–
lmx1b-MO	–*	–*	–*	↓*	↑†	–*	–*	↑*	–*
mafb-MO	↑↑*	↑*	↓↓	–	–	↓	↓*	↑*	↓↓
hey1-MO	↑↑*	↑↑*	↓	–†	↓	↓↓	–†	↑	↓↓
wt1-MO1 + foxc2-MO	–	↑↑*	ø	ø	ø	ø	ø	ø	ø

↑/↓, increase or decrease in expression.
↑↑/↓↓, large increase or decrease in expression.
ø, Absence of expression.
–, No change in expression.
*Expression pattern extended ventrolaterally into the lateral plate mesoderm.
†Changes in expression were observed in approximately half of the embryos.

podocyte expression domains (Fig. 3A-I′, Table 1; see Fig. S1 and Table S2 in the supplementary material). Expression of *lmx1b*, *tcf21*, *ptpru* and *nphs2* was completely lost (Fig. 3B-C′,F,F′,I,I′) and *mafb* and *nphs1* were markedly reduced (Fig. 3D,D′,G,G′). By contrast, *kirrel* was weakly reduced (Fig. 3H,H′) and *wt1* and *foxc2* were elevated (Fig. 3A,A′,E,E′). In addition to these changes in expression levels, the expression domain of *wt1* was enlarged and those of *foxc2* and *kirrel* were expanded ventrolaterally into the lateral plate mesoderm (Fig. 3A,A′,E,E′,H,H′). Injection of 3.2 pmol of *wt1-MO1* alone resulted in a qualitatively similar, yet less robust, loss-of-function phenotype (Table 1; see Fig. S2A-I′ in the supplementary material). This was noteworthy because the two pseudo-alleles of *wt1* only differ by two nucleotides within the *wt1-MO1* binding site (Fig. 2A).

To document these changes in gene expression a wire diagram was developed, similar to those initially described for sea urchin mesendoderm formation (Davidson et al., 2003). Genes were connected by arrows or inhibitory bars to indicate how a given gene product regulates the transcription of other genes. Since we did not perform promoter studies, such as chromatin immunoprecipitations, the connections do not necessarily reflect a direct interaction between the transcription factor and its target, but might involve a transcriptional relay. In the case of the *wt1-MO1+2* experiment the following connections were made (Fig. 3J): as the expression of *mafb*, *lmx1b*, *tcf21*, *ptpru*, *nphs1* and *nphs2* was lost or strongly reduced, wt1 was connected to these genes with a thick arrow to indicate a positive input; *kirrel* mRNA levels were only weakly reduced and therefore connected by a thin arrow; conversely, *wt1* and *foxc2* expression was increased and so these genes were connected with an inhibitory bar symbolizing transcriptional repression, again using thin and thick lines to reflect the strength of the effect.

The specificity of the *wt1-MO1+2* results was addressed using two assays. First, injections of a standard control MO (*Std-MO*) did not show any reproducible changes in any of the podocyte genes analyzed (Table 1 and see Fig. S3 in the supplementary material). Secondly, *Xenopus* embryos were injected with the *wt1-MO1* in the presence or absence of a one-sided injection of 500 pg *wt1** mRNA, a *wt1-KTS* construct that was mutated in the *wt1-MO1* binding site and was therefore resistant to the *wt1-MO1* activity. Whole-mount in situ hybridizations were performed using *nphs1* expression as readout. *nphs1* was strongly reduced following wt1 knockdown, but was regained on the *wt1**-injected side in 90% of cases (Fig. 3K-L and see Fig. S2J-L in the supplementary material). It is important to note that the *wt1* construct used in the present study differs from the constructs used by others (Wallingford et al., 1998; Perner et al., 2007) as it contains an optimized Kozak sequence and lacks the entire 3′UTR, and single injections did not result in gross morphological defects (data not shown).

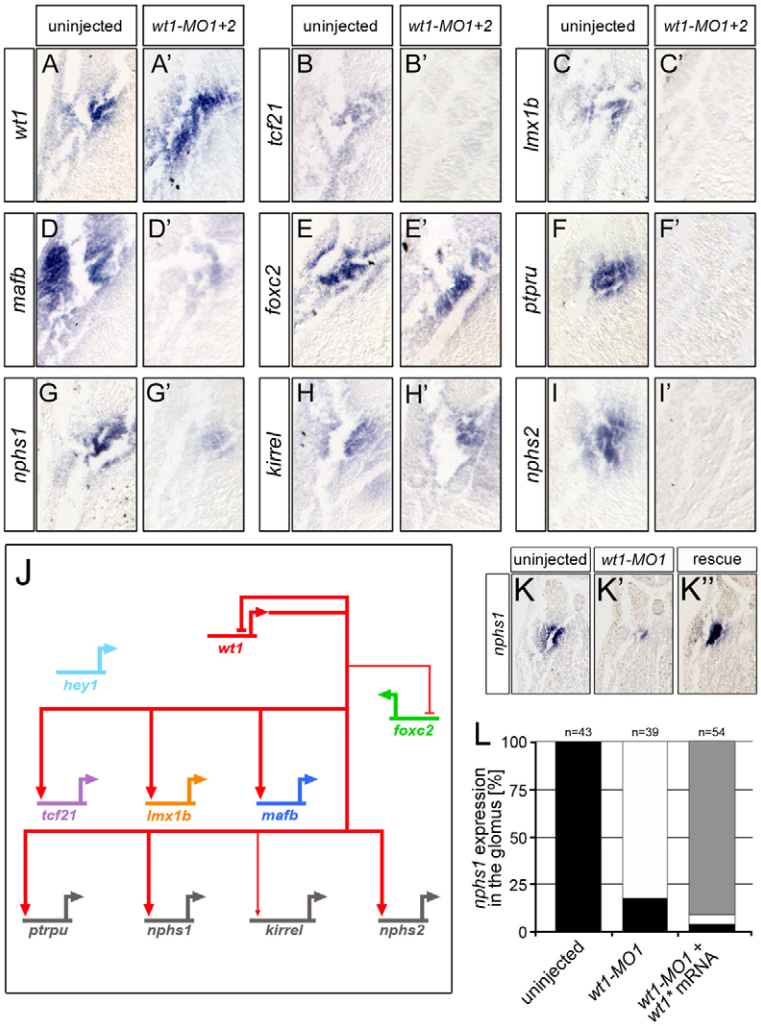

Fig. 3. wt1 regulates podocyte gene expression. (**A-I′**) High magnification of representative transverse sections of whole-mount in situ hybridizations comparing the expression of podocyte genes *wt1* (A,A′), *tcf21* (B,B′), *lmx1b* (C,C′), *mafb* (D,D′), *foxc2* (E,E′), *ptpru* (F,F′), *nphs1* (G,G′), *kirrel* (H,H′) and *nphs2* (I,I′) between *wt1* morphants and sibling control *Xenopus* embryos at stage 35. (**J**) Wire diagram summarizing the in situ hybridization data. Arrows, positive regulation; T-bars, negative regulation; thick lines, strong effects; thin lines, weak effects. Note that none of the interactions is necessarily direct and might involve intermediary players. (**K-L**) In situ hybridization of *nphs1* in uninjected controls (K), embryos injected with *wt1-MO1* (K′) and with *wt1** mRNA and *wt1-MO1* (K″). The results of multiple experiments were quantified (L). Black, strong bilateral expression; white, loss of expression; gray, unilateral expression rescued by *wt1** mRNA.

This analysis showed that wt1 is a very important player in podocyte specification, but is not sufficient to block all aspects of podocyte gene expression.

Knockdown of other podocyte transcription factors

Next, we examined the contribution of the other transcription factors to the podocyte program. foxc2, hey1, tcf21, lmx1b and mafb were eliminated using MOs, following the experimental layout described for wt1 knockdown. With the exception of the previously described MO targeting *hey1* (Taelman et al., 2006), all MOs were tested for efficacy and specificity. Knockdown phenotypes were characterized by morphology and histology, and podocyte gene expression was examined by in situ hybridization and the data assembled into a wire diagram (results are summarized in Table 1, Figs S4-S12 and Table S2 in the supplementary material). There were only two deviations from this scheme. Despite noticeable changes in the glomus architecture of *tcf21* morphants, only weak changes in gene expression were detected (see Figs S6 and S7 in the supplementary material). Since these were not amendable as readouts of the *tcf21-MO* phenotype, no rescue experiment was attempted. Secondly, the results of the lmx1b knockdown were confirmed using a second previously published MO (Haldin et al., 2008) (see Table S2 in the supplementary material). It is also noteworthy that the effect of the *hey1-MO* seemed to be stage dependent. Although *wt1* and *nphs1* were strongly downregulated at stage 28 (data not shown) (Taelman et al., 2006), the expression recovered by stage 35.

Once all the data were obtained, the individual wire diagrams were combined into a single gene regulatory network (Fig. 4A). It is important to note that the connections might not reflect direct transcriptional effects. The analysis did not exclude relays of consecutive gene activations. Despite its complexity, five messages were readily apparent. First, the network displays a high degree of redundancy. All genes have multiple transcriptional inputs. Second, genes are integrated into feedback loops. For example, lmx1b activates *mafb* transcription and mafb activates *lmx1b*. Third, mafb, a downstream transcription factor, negatively regulates its upstream activators, wt1 and foxc2. Fourth, no single transcription factor regulates the entire network. Lastly, wt1 is the most potent podocyte transcription factor.

wt1 and foxc2 are required together for podocyte gene expression

The analysis so far indicated a high redundancy among the podocyte transcription factors. Since knockdown of wt1 had the most dramatic effect, we next asked whether any of the other five transcription factors cooperates with wt1 and whether this cooperation can explain all of the podocyte gene expression patterns. The initial analysis focused on *kirrel*, as it was least affected by the knockdown of wt1 (Fig. 3H,H′ and see Fig. S1G,G′ in the supplementary material). *Xenopus* embryos were injected radially with *wt1-MO1+2* in combination with either *foxc2-MO*, *tcf21-MO*, *mafb-MO* or *lmx1b-MO* at the 2- to 4-cell stage. *hey1* radial injections impaired overall development of the embryos (data not shown), and *wt1-MO1+2* and *hey1-MO* were therefore injected unilaterally into one blastomere at the 2-cell stage. Whole-mount in situ hybridization at stage 35 demonstrated that from all the combinations, only co-injection of *wt1-MO1+2* and *foxc2-MO* resulted in a loss of *kirrel* expression in the glomus region (Fig. 4B-H′).

To address whether this effect of wt1 and foxc2 is restricted to *kirrel*, we extended the analysis to the other genes of the network. Injection of *wt1-MO1+2* alone already strongly reduced the expression of many of the podocyte genes and resulted in the absence of a histologically identifiable glomus (Figs 2 and 3 and see Fig. S1 in the supplementary material). Thus, to achieve a more sensitive assay, only one of the two *wt1* MOs (*wt1-MO1*) was used in this part of the study. Knockdown with *wt1-MO1* indeed resulted in a less pronounced, but qualitatively similar phenotype (see Fig. S2A-I′ in the supplementary material). *Xenopus* embryos were co-injected with 3.2 pmol *wt1-MO1* and 3.2 pmol *foxc2-MO*, cultured until the uninjected control embryos reached stage 35 and then processed for in situ hybridization. The glomus expression of the other three transcription factors (*tcf21*, *lmx1b* and *mafb*) and of all four terminal differentiation markers (*ptpru*, *nphs1*, *kirrel* and *nphs2*) was lost (Fig. 5, Table 1). *wt1* expression was unchanged, whereas *foxc2* mRNA was greatly increased throughout the embryo. In the case of *kirrel*, a new expression domain of unknown function was observed in the dorsal endoderm (Fig. 5H′). These data suggested that wt1 and foxc2 cooperate in the expression of podocyte markers.

Next, we extended our analysis to the morphological differentiation of the glomus. The pronephric glomus is composed of three cell types: podocytes, endothelial and mesangial-like cells

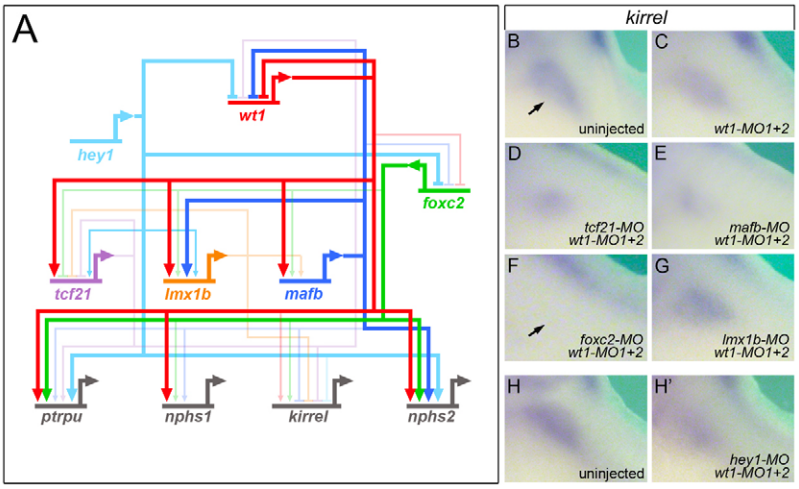

Fig. 4. wt1 and foxc2 are key regulators of the podocyte gene regulatory network. (**A**) Wire diagram combining the data for the individual knockdowns of the six podocyte transcription factors, showing all the connections. Note that none of the interactions is necessarily direct and might involve intermediary players. (**B-G**) Whole-mount in situ hybridization for *kirrel* mRNA expression in uninjected control embryos (B) and *Xenopus* embryos injected radially with *wt1-MO1+2* alone (C) and in the presence of *tcf21-MO* (D), *mafb-MO* (E), *foxc2-MO* (F) or *lmx1b-MO* (G). (**H**,**H′**) *kirrel* expression in a *Xenopus* embryo injected unilaterally with *wt1-MO1+2* and *hey1-MO* showing the uninjected (H) or injected (H′) side.

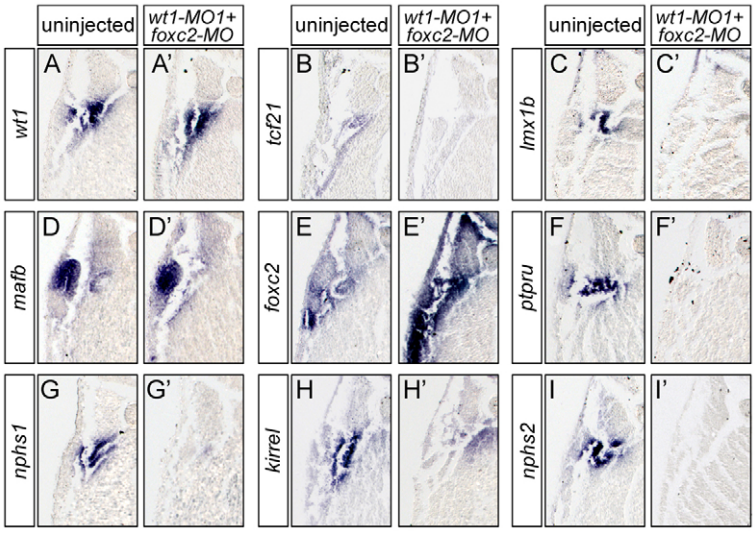

Fig. 5. wt1 and foxc2 are required together for expression of podocyte genes. (**A-I'**) High magnification of transverse sections of whole-mount in situ hybridizations comparing the expression of podocyte genes *wt1* (A,A′), *tcf21* (B,B′), *lmx1b* (C,C′), *mafb* (D,D′), *foxc2* (E,E′), *ptpru* (F,F′), *nphs1* (G,G′), *kirrel* (H,H′) and *nphs2* (I,I′) between *wt1-MO1* plus *foxc2-MO* co-injected *Xenopus* embryos (A′-I′) and sibling controls (A-I) at stage 35.

(Ellis and Youson, 1991; Kramer-Zucker et al., 2005). In *Xenopus*, the detailed organization of the glomus and the existence of mesangial-like cells have not been previously demonstrated. However, all three cell types could be identified in the *Xenopus* pronephric glomus using a combination of histology, immunohistochemistry and in situ hybridization on coronal sections at stage 40 (Fig. 6A-A′′′′): podocytes were marked by β1-Integrin (Pozzi et al., 2008), the mesangial-like cells by the mesenchymal marker Vimentin (Dent et al., 1989; Gonlusen et al., 2001) and the endothelial cells by *apelin receptor* [*aplnr* (*X-msr*)] mRNA (Devic et al., 1996). In contrast to *wt1-MO1+2*-injected embryos, which do not show any glomerular structures (Fig. 2H-K), injection of *wt1-MO1* resulted in a compacted glomus with no capillary loops and a few endothelial and mesangial-like cells present in its center (Fig. 6B-B′′′′). *foxc2* morphants displayed a slightly smaller glomus with enlarged capillary loops. Both endothelial and mesangial-like cells were grouped in the center of the glomus and did not extend towards its lateral edges (Fig. 6C-C′′′′). *wt1-MO1* and *foxc2-MO* co-injected embryos exhibited a more severe phenotype than the *wt1-MO1* or *foxc2-MO* single knockdowns, characterized by the complete absence of the glomus (Fig. 6D-D′′′′). Strong staining of *aplnr* and Vimentin at the midline of the embryos suggested that endothelial and mesangial-like cells were present, but could not assemble into a glomus structure in the absence of podocytes. Together, these experiments suggest that even though wt1 is the most potent podocyte transcription factor, it still cooperates with foxc2 in some aspects of glomus formation.

wt1, foxc2 and Notch signaling induce ectopic podocyte marker gene expression

The experiments so far demonstrated that the combined knockdown of wt1 and foxc2 abolishes podocyte formation, but did not address whether the two transcription factors are also sufficient to do so. To address this point, three approaches were

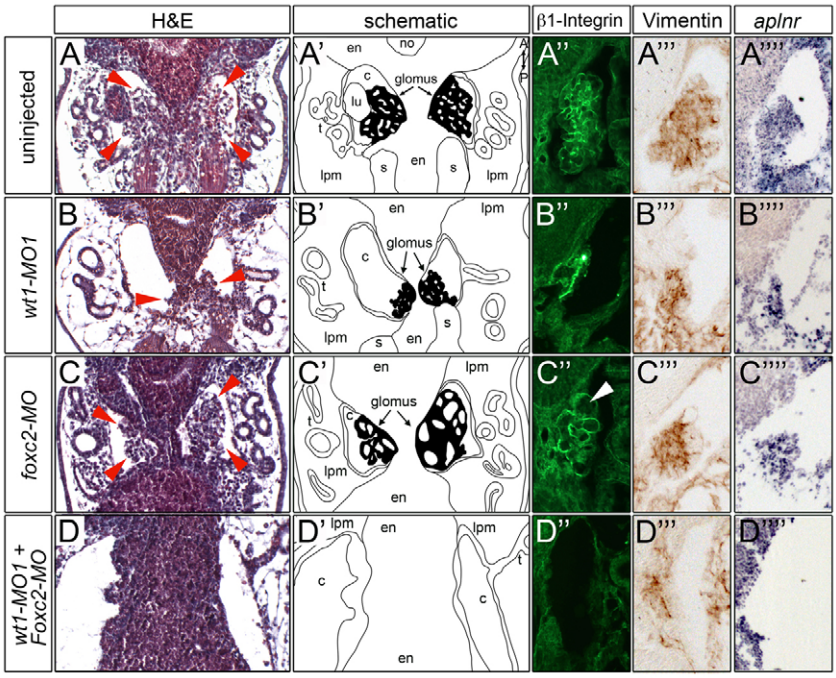

Fig. 6. wt1 and foxc2 regulate glomulogenesis. (**A-D′′′′**) Uninjected control *Xenopus* embryos (A-A′′′′) and embryos injected with *wt1-MO1* (B-B′′′′), *foxc2-MO* (C-C′′′′) or with *wt1-MO1* plus *foxc2-MO* (D-D′′′′) at stage 40 were analyzed by histological staining with Hematoxylin and Eosin (H&E, A-D), immunohistochemistry for β1-Integrin (A′′-D′′) or Vimentin (A′′′D′′′), and in situ hybridization for *aplnr* (A′′′′-D′′′′). (A′-D′) Schematics of the histology, in which the glomus is highlighted in black. The arrowhead in C′′ indicates enlarged capillary loops in *foxc2* morphants. c, coelom; en, endoderm; lpm, lateral plate mesoderm; lu, lung bud; no, notochord; s, somites; t, tubule.

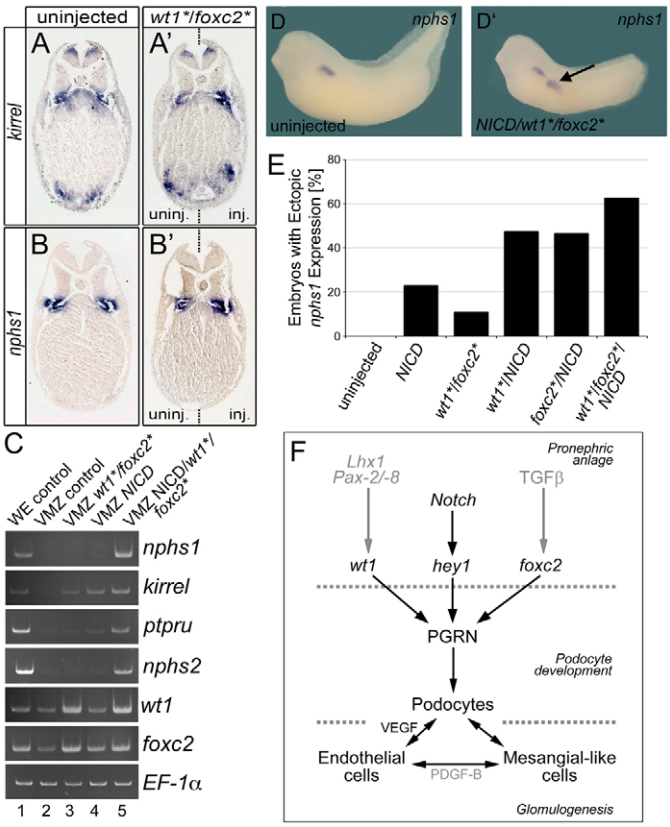

Fig. 7. *wt1, foxc2* and *NICD* are sufficient to activate podocyte gene expression. (**A-B′**) Transverse sections of whole-mount in situ hybridizations comparing *kirrel* (A,A′) and *nphs1* (B,B′) expression in *wt1** plus *foxc2** mRNA-injected *Xenopus* embryos (A′,B′) and uninjected sibling controls (A,B) at stage 35. Injected and uninjected sides are indicated. (**C**) RT-PCR analysis comparing the expression of podocyte terminal differentiation genes *nphs1*, *kirrel*, *ptpru* and *nphs2* and the transcription factors *wt1* and *foxc2* in uninjected whole embryos (WE control, lane 1), uninjected ventral marginal zone explants (VMZ control, lane 2) or ventral marginal zones injected with *wt1** plus *foxc2** mRNA (VMZ *wt1*/foxc2**, lane 3), with *NICD* mRNA (VMZ *NICD*, lane 4), or co-injected with *NICD*, *wt1** and *foxc2** mRNA (VMZ *NICD/wt1*/foxc2**, lane 5). (**D**,**D′**) Whole-mount in situ hybridization of control embryos (D) and embryos co-injected with *NICD*, *wt1** and *foxc2** mRNA (D′) for *nphs1* expression at stage 35. Arrow indicates an ectopic patch of *nphs1* expression. (**E**) Quantification of ectopic *nphs1* patches in embryos injected with *wt1*/foxc2**, *NICD*, *wt1*/NICD*, *foxc2*/NICD* and *wt1*/foxc2*/NICD* mRNA. (**F**) Schematic of *Xenopus* glomus development. The three transcription factors, wt1, foxc2 and hey1, activate the PGRN (podocyte gene regulatory network). Subsequently, differentiated podocytes secrete Vegf to recruit endothelial cells and mesangial cells to form the differentiated glomus (see Discussion for further details).

used. First, *Xenopus* embryos were microinjected marginally into a single blastomere at the 4-cell stage with a mixture of *wt1** and *foxc2** mRNA (1 ng each), as rescue constructs shown to be biologically active (Fig. 3K-L and see Fig. S5K-L in the supplementary material). In situ hybridization at stage 35 showed that the expression domains of the podocyte markers *nphs1* and *kirrel* were increased in size and intensity within the glomus proper in 90% of the embryos (Fig. 7A-B′) (*nphs1*, *n*=12; *kirrel*, *n*=11). *kirrel* was detected in the ventral mesoderm in 63% of the embryos, but this domain was most likely an expansion of the heart-specific expression of *kirrel* and unrelated to its expression in the glomus. These experiments were extended by co-injecting *lacZ* mRNA with *wt1** plus *foxc2**. Although *lacZ* staining could be detected outside of the glomus, these cells did not co-express *nphs1* or *kirrel* (data not shown). In a second approach analogous to experiments spearheaded by Asashima and colleagues (Asashima et al., 2009), ectodermal explants were treated with 10 ng/ml Activin A for 3 hours at stage 9.5. This exposure was sufficient to induce podocyte terminal differentiation genes as assessed by RT-PCR analysis and suggested that ectodermal explants can be transdifferentiated into podocytes (see Fig. S13A, lane 3, in the supplementary material). However, ectodermal explants from embryos injected with 2 ng *wt1** and *foxc2** mRNA did not show any podocyte gene expression (see Fig. S13A, lane 4, in the supplementary material). A similar result was obtained when ventral marginal zone explants were used; this tissue is already committed to mesoderm and should be more easily converted to podocytes. However, RT-PCR analysis of these explants at stage 35 did not detect significant expression of *nphs1*, *kirrel*, *ptpru* or *nphs2* (Fig. 7C, compare lanes 2 and 3, and see Fig. S13B in the supplementary material). Based on these data, we concluded that wt1 and foxc2 are not sufficient to induce podocyte formation by themselves.

The time course of podocyte gene expression (Fig. 1K) demonstrated that *hey1* is co-expressed with *wt1* and *foxc2* as early as stage 20. Moreover, *hey1* is downstream of Notch signaling, a pathway known to be involved in podocyte development (McLaughlin et al., 2000; Cheng et al., 2003; Wang et al., 2003; Taelman et al., 2006; Cheng et al., 2007; Naylor and Jones, 2009). Thus, we next tested whether Notch signaling was the missing input to induce podocyte development outside of the glomus. *Xenopus* embryos were injected with either 4 ng of *wt1** mRNA and 1 ng *foxc2** mRNA, or 4 ng of *NICD* mRNA [a constitutively active Notch receptor (Coffman et al., 1990)], or a mixture of all three mRNAs at the 2- to 4-cell stage. Ventral marginal zone explants were excised at stage 10, cultured until sibling embryos reached stage 35 and processed for RT-PCR analysis. Whereas neither *wt1** plus *foxc2** nor *NICD* induced the podocyte terminal differentiation markers, the combination of *wt1**, *foxc2** and *NICD* strongly upregulated all four (Fig. 7C, compare lanes 2-4 with lane 5). This effect was specific to the presence of all three mRNAs because neither *wt1** plus *NICD* mRNA nor *foxc2** plus *NICD* mRNA induced *nphs1* expression (see Fig. S13C in the supplementary material).

Interestingly, the same combination of mRNAs (1 ng *wt1**, 200 pg *foxc2**, 1 ng *NICD*) injected into a single vegetal blastomere at the 8-cell stage was sufficient to induce ectopic patches of *nphs1* expression in 65% of the embryos (*n*=51) (Fig. 7D-E). These ectopic domains were mostly restricted to the posterior intermediate mesoderm, but isolated cases also showed expression in the head region (data not shown). In contrast to the ventral marginal zone explants (Fig. 7C), injection of *NICD* mRNA alone, *wt1** plus *NICD* or *foxc2** plus *NICD* also resulted in ectopic *nphs1* expression, albeit at lower frequency than when all three mRNAs were co-injected (Fig. 7E). It is noteworthy that *wt1* and *foxc2* are expressed at lower levels throughout the intermediate mesoderm and only become restricted to the podocyte lineage later during development (Fig. 1B,C). Thus, for example, injected *wt1* mRNA can already cooperate with endogenous foxc2, resulting in some ectopic patches of *nphs1* expression, and co-injection with *foxc2* mRNA only augments this.

In summary, these experiments extend reports by others (McLaughlin et al., 2000; Cheng et al., 2003; Wang et al., 2003; Taelman et al., 2006; Cheng et al., 2007; Naylor and Jones, 2009) that Notch signaling is a very important aspect of kidney development. The interplay between the two transcription factors wt1 and foxc2 and Notch signaling specifies the podocyte lineage and highlights the importance of combinatorial signaling.

DISCUSSION

The data presented here provide the first model of a gene regulatory network for cell fate decisions in the kidney. They show that the differentiation of a very specialized cell type, the podocyte, does not rely on a single master gene, but instead requires multiple inputs. These inputs ensure the correct spatiotemporal execution of the program so that podocytes are only found within the glomus and not in other parts of the kidney or elsewhere in the embryo.

The main conclusion of our study is that the two transcription factors wt1 and foxc2 and Notch signaling are not only very important for podocyte development, but also for the assembly of the entire glomus structure (Fig. 7F). Intense midline staining of *aplnr* and Vimentin in the *wt1*/*foxc2* double morphants (Fig. 6D″,D‴) suggested that endothelial and mesangial-like cells were present, but lacked the interaction with podocytes required to form a glomus. In mouse, it has been shown that podocytes secrete Vegf to attract endothelial cells (Jokelainen, 1963; Eremina et al., 2007) and that endothelial cells secrete Pdgfβ to recruit mesangial cells (Bernstein et al., 1981; Lindahl et al., 1998). If Vegf is not present, neither endothelial nor mesangial cells can be recruited to the glomerulus. Similarly, *Xenopus* embryos express *vegfa* mRNA in the developing podocytes (Cleaver et al., 1997) and this expression is lost in embryos injected with *wt1-MO* (our unpublished data). Importantly, the transcriptional network presented here (Fig. 4A) only provides a snapshot and needs to be extended in the future. It will, for example, be worthwhile investigating how the upper-tier transcription factors are induced and what their function is in non-podocyte tissues.

Interestingly, foxc2, wt1 and Notch signaling seem to reflect three different aspects in determining future podocytes (Fig. 7F). *foxc2* is broadly expressed in the mesoderm, is a direct transcriptional target of Activin/Tgfβ signaling (Koster et al., 2000) and probably determines the mesodermal precursor population of the future kidney. Indeed, studies in mouse suggest that Foxc2 and its close relative Foxc1 regulate the decision between paraxial and intermediate mesoderm cell fates (Wilm et al., 2004). Wt1, by contrast, is a more kidney-restricted transcription factor. In the *Xenopus* early nephric mesenchyme, *wt1* is co-expressed with the three kidney-specific transcription factors *lhx1* (*lim1*), *pax2* and *pax8* (Carroll et al., 1999) and its podocyte expression has been shown in several species to be regulated by cross-talk between Wt1 and Pax2 (Ryan et al., 1995; Dehbi et al., 1996; Majumdar et al., 2000). Finally, Notch signaling and its downstream effector hey1 provide the positional information for podocyte development (McLaughlin et al., 2000; Taelman et al., 2006; Naylor and Jones, 2009). As recently demonstrated by Naylor and Jones (Naylor and Jones, 2009), Notch signaling patterns the pronephros along its mediolateral axis. Notch signaling is required for the expression of *wnt4*, a secreted factor that has a central role in kidney development in *Xenopus* and mouse (Stark et al., 1994; Saulnier et al., 2002).

The *Xenopus* pronephros proved to be a very powerful animal model for our study. In mammals, the molecular analysis of podocyte maturation is complicated by the asynchronous development of podocytes. At any given time during kidney development, the metanephros contains mature as well as developing podocytes, making it difficult to follow individual cell fate decisions. In *Xenopus*, by contrast, the bilateral pronephros and its glomus develop synchronously and independently of the tubules and duct (Urban et al., 2006). In addition, loss-of-function studies with MOs circumvented the difficulty in obtaining compound mouse mutants of more than two genes at the same time. In our case, two MOs were sufficient, but other studies have successfully eliminated up to four genes at the same time (Khokha et al., 2005; Reversade and De Robertis, 2005; Eisen and Smith, 2008). Finally, insights into pronephric development are easily applicable to the metanephric kidney. The molecular regulation of nephron development in both kidney types follows an evolutionarily conserved path (Carroll et al., 1999; Zhou and Vize, 2004; Reggiani et al., 2007; Raciti et al., 2008). For example, some of the regulations reported in this study (e.g. the autoregulation of wt1) have also been observed in mouse (Rupprecht et al., 1994; Rohr et al., 2002; Wagner et al., 2004; Moriguchi et al., 2006; Takemoto et al., 2006).

Notably, some of our observations are not in complete agreement with all previously published reports. In contrast to Taelman et al. (Taelman et al., 2006), we still detected low-level expression of *nphs1* upon injection of *wt1-MO1+2*, probably owing to longer staining of the whole-mount in situ hybridizations. Similarly, Haldin et al. (Haldin et al., 2008) showed a marked downregulation of *wt1* and *nphs1* in *Xenopus* lmx1b knockdowns, whereas we only observed small changes, even when using their published *lmx1b* MO (see Figs S8, S9 and Table S2 in the supplementary material). These rather mild phenotypes of *lmx1b* morphants are similar to those observed in mouse. Animal-wide, as well as podocyte-specific, knockouts in mouse did not show strong changes in the transcription of genes such as *wt1* and *nphs1*, and induced rather mild changes in the foot processes of the podocytes (Miner et al., 2002; Rohr et al., 2002; Suleiman et al., 2007).

Our model of a podocyte gene regulatory network is based on six transcription factors and four terminal differentiation genes. This analysis obviously does not provide a global transcription profile of all genes expressed in the podocyte. The six transcription factors studied here (wt1, foxc2, hey1, tcf21, lmx1b and mafb) are the best-characterized regulators of podocyte specification (Rascle et al., 2007; Quaggin and Kreidberg, 2008). Although this is unlikely to be an exhaustive list, only a few other transcription factors, which were not tested here, have been implicated in this process. They include members of the ZHX (zinc-fingers and homeoboxes) family of proteins that regulate podocyte gene expression during disease formation, but have not yet been linked to podocyte development (Liu et al., 2006), and Ldb1 and E47 (Tcf3) heterodimerization partners of Lmx1b. Although E47 is not necessary for podocyte development, mice lacking *Ldb1* in podocytes display a phenotype that is similar, but less severe, than that observed in *Lmx1b* mutants (Suleiman et al., 2007; Haldin et al., 2008).

For the terminal differentiation genes the scenario is even more complex. Hundreds of genes are involved in podocyte function (Takemoto et al., 2006). Of the four genes chosen for this study, the expression of three is localized to the slit diaphragm (*nphs1*, *kirrel* and *nphs2*) and one is expressed outside of this junctional complex (*ptpru*). Interestingly, all had inputs from at least three transcription factors, and no combination of inputs was identical. This suggests a high degree of redundancy in the regulation of terminal differentiation genes, which probably provides robustness to the

podocyte program. However, the type of analysis performed here does not rule out the possibility that some of the observed connections are indirect. To completely understand all the nuances of this regulation, it will be necessary to address in vivo promoter occupancy using high-throughput sequencing of chromatin immunoprecipitation products (Wold and Myers, 2008).

In *Xenopus*, naïve ectoderm can be transdifferentiated into kidney tissue by the addition of Activin and retinoic acid (Moriya et al., 1993; Uochi and Asashima, 1996; Brennan et al., 1999; Osafune et al., 2002). These in vitro induced embryonic kidneys could even restore kidney function upon transplantation into nephrectomized *Xenopus* embryos (Chan et al., 1999). Thus, it will be very interesting to see whether this better-defined gene set (wt1, foxc2 and Notch signaling) will allow the in vitro generation of podocytes from pluripotent cells. Since podocyte malfunction is one of the main causes of kidney failure, the regeneration of podocytes might provide a novel therapeutic approach to restore podocyte and kidney function in humans.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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Table S1. Primers for RT-PCR

Gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
wt1	AAGACTGTGAGAGGCGATTTTCTC	CTTTCGTTGGCAGGTTTTGC
foxc2	CTCAATCCTGGCGCAGACAT	GCCGGTGAGAGTTGAACATTTC
tcf21	CATAGCTCACCTGCGTCAAATT	TCGGCTAGTGCTCACCACTTC
lmx1b	ACATGAATCCTTATGGCAATGACA	CAGTCGGTCAATGGGATTCC
mafb	AGCGACGGACCCTAAAGAACA	TGCTGAACCAGTTGGGTCTTC
nphs1	CGATACGAGTGCGCTGTAGATAA	AGTGCACCCCCTTGTGTATATCA
kirrel	GGGACCCTGTGCATCTTTGT	TCGCTCCCCGACATGTCTAC
ptpru	CCAATTACATTCCGGGCTACA	TTAATCCGGCGCTTCTCATT
nphs2	CATCTCCAGTGCTTTCCAGTTG	GCATCCAGAGCCACCTTTACTT
α-actin	CGTACCACAGGTATCGTTCTTGAC	TCTGTGAGGTCTCTACCAGCTAGGT
Ef-1α	CCTGAACCACCCAGGCCAGATTGGTG	GAGGGTAGTCAGAGAAGCTCTCCACG