

WT1 Activates a Glomerular-Specific Enhancer Identified from the Human Nephin Gene

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Abstract. The glomerular filtration barrier separates the blood from the urinary space. Nephin is a transmembrane protein that belongs to the immunoglobulin superfamily and is localized to the slit diaphragms that are a critical component of this filtration barrier. Mutations in the nephin gene (NPHS1) lead to congenital Finnish nephropathy, whereas alterations in the level of nephin expression have been identified in a wide range of acquired glomerular diseases. A 186-bp fragment from the human NPHS1 promoter is capable of directing

podocyte-specific expression of a β -galactosidase transgene when placed in front of a heterologous minimal promoter in transgenic mice. The Wilms tumor suppressor gene (WT1) is a zinc-finger-containing transcription factor that is coexpressed with NPHS1 in differentiated podocytes; gel shift binding assays demonstrate that a recombinant WT1 protein can bind and activate the 186-bp NPHS1 fragment in a sequence-specific manner. Taken together, these results suggest that WT1 may be required for regulation of the NPHS1 gene *in vivo*.

Kidney disease affects over 20 million individuals in the United States alone and is a major cause of morbidity and mortality worldwide. Although the causes of renal failure are diverse, the glomerulus and renal filtration barrier are often the target of injury (1,2). Despite the significant burden of disease, treatment options are limited. In the majority of cases, damage to the renal filtration barrier leads to progressive glomerular scarring and may result in end-stage kidney failure that requires dialysis or transplantation for survival. The glomerular filtration barrier consists of two individual cell types: glomerular visceral epithelial cells (podocytes), and fenestrated endothelial cells that are separated by a glomerular basement membrane. Approximately 180 L of blood per day passes through the glomerular capillaries in the average adult human kidney. Excess solutes and water are filtered into the urine, while critical blood proteins such as albumin and blood cells remain within the capillaries. Damage to this filtration barrier leads to the loss of protein into the urine (proteinuria).

Nephin belongs to the immunoglobulin superfamily and is localized to the slit diaphragm, a specialized intercellular connection found between podocyte foot processes (3,4). Nephin was originally identified as the gene that is mutated in congenital Finnish nephropathy (5), a rare autosomal recessive disease that results in severe proteinuria and death in infants

unless they receive a renal transplant. Mutant mice that are missing the nephin protein fail to form slit diaphragms and die at birth with massive proteinuria and kidney failure (6). Alterations in the level of nephin expression have also been documented in a variety of acquired renal diseases that include diabetic nephropathy, focal segmental glomerulonephritis, and minimal change nephropathy (7,8). Previous studies have shown that 8.3, 5.4, or 4.125 kb of the murine proximal nephin promoter and 1.125 kb of the human proximal nephin promoter are capable of directing expression of a β -galactosidase transgene to podocytes *in vivo* (9–11). Although the human fragment was exclusively expressed in podocytes, the larger fragments directed expression to the developing hindbrain as well. Recently, an alternate first exon that is required for neural expression of the nephin gene has been identified (12).

By using the NCBI Pairwise-Blast sequence alignment program, we identified an 83-bp region of homology between the 4.125-kb murine and 1.125-kb human promoters. We found that a 186-bp fragment of the human promoter that contains this 83-bp region of homology is capable of directing podocyte-specific expression in transgenic mice. Furthermore, we demonstrate that the Wilms tumor suppressor protein can bind to a putative WT1 consensus sequence from this fragment *in vitro* and activates a nephin-luciferase reporter transgene. By using this enhancer, it will be possible to identify additional trans-acting factors that are required for podocyte-specific gene regulation with the goal of finding new molecular targets for the treatment of renal patients.

Materials and Methods

Identification of Homology between the Human and Murine Promoters

We have previously shown that 4.125-kb of the murine nephin promoter and 1.125-kb of the human nephin promoter are capable of

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directing expression of a reporter transgene (β -galactosidase) specifically to podocytes *in vivo*. To identify regions of homology within these DNA fragments, the sequences from these two promoters were aligned by the NCBI program (Pairwise BLAST; available at <http://www.ncbi.com>).

Generation of the Nephrin-LacZ Transgene

A total of 186 bp of the human nephrin promoter encoding the 83-bp core region of homology was amplified from the 1.125-kb human nephrin promoter fragment with the following primers: 5'-GGCCCTGGGGTACGGAGGCTGGGGA-3' and 5'-CGGGTC-GACCTCGTATTTTAGGGGA-3' (Sigma); and under the following PCR conditions: 95°C \times 30 s; 95°C \times 1', 68°C \times 4' for 30 cycles followed by 72°C for 4'. This 186-bp fragment was subcloned into the PCR 2.1 vector (Invitrogen) according to manufacturer's directions. After digestion with EcoRI, either four copies or one copy of this fragment were subcloned into the hsp-lacZ vector that has been previously described (13). Both one- and four-copy transgenes were constructed to determine whether tandem repeats or a single copy of the enhancer alone were required for expression. This plasmid encodes the minimal promoter from the heat shock protein upstream of a β -galactosidase transgene (Figure 1). Both one-copy and four-copy transgenes were injected into one cell ICR (Charles River) murine embryos as described (14). To identify founder lines, genomic DNA was purified from tails as described (15), and Southern blot analysis was performed. The lacZ gene was used as the hybridization probe as described previously (10).

β -Galactosidase Staining of Kidneys and Extrarenal Tissue

Kidneys or tissues from newborn pups were fixed and stained as described previously (10,16). In addition, 10 μ m cryosections of each of these tissues were prepared and stained as described previously (17).

Electrophoretic Mobility Shift Assays

A double-stranded DNA probe corresponding to the 186-bp human nephrin promoter fragment was released from pKS4 \times 191 plasmid by EcoRI digestion and end-labeled with [α -³²P] dATP by Superscript II (GibcoBRL). Cloning and expression of GST fusion proteins GST-WT1 (A isoform) and GST- Δ ZF (WT1 lacking DNA-binding zinc fingers) was previously described (18). DNA binding reactions were performed with 1 μ g of purified protein in a total volume of 25 μ l containing radiolabeled probe (50,000 cpm), 1 μ g poly (dI-dC), 1 mg/ml BSA, and binding buffer (50 mM Tris (pH 8.8), 60 mM NaCl, 5 mM MgCl₂, 100 μ M ZnCl₂, 12% glycerol, 0.5 mM DTT). The

reaction mixture was preincubated at room temperature for 10 min before addition of probe. Then protein/DNA complexes were allowed to form for 15 min at room temperature and were resolved on a 5% nondenaturing polyacrylamide gel in 0.5 \times TBE at 300 V for 1.5 h. Gel was dried and exposed to film. For competition experiments, 1.2 μ g of anti-WT1 antibody (C-19, Santa Cruz) or rabbit IgG (Zymed) and a 100- to 1000-fold excess of unlabeled Zif3 (TCGAGCCCG-GCGCGGGGGCGAGGGTCTGA) or NF κ B (AGCTT GGCATAG-GTCTCTGGC) double-stranded oligonucleotides were added during the preincubation period.

Plasmid Construction

Construction of WT1-A and WT1-A (F112Y) expression vectors was previously described (18). To make the human nephrin luciferase reporter construct (pGL₂-neph-luc) containing four copies of the human nephrin enhancer, pKS-4 \times 191 was digested with *KpnI*/*BamHI* and the insert cloned into a promoterless luciferase vector pGL₂-Basic (Promega) digested with *KpnI*/*BglII*. The plasmid was confirmed by sequencing.

Luciferase Assay

For transient cotransfection assays, WT1-A wild-type or F112Y mutant expression vectors (150, 495, 1600 ng), pGL₂-neph-luc (150 ng), and an internal Renilla control (5 ng) were transfected into 293T cells with FuGENE6 reagent (Roche) according to manufacturer's instructions. Cells were harvested 48 h later and assayed for luciferase activity with the Dual-Luciferase Reporter Assay System (Promega).

Results

Identification of a Podocyte-Specific Enhancer

To identify a putative glomerular enhancer element, the human and murine promoters that have been shown to direct podocyte-specific expression within transgenic kidneys were compared. By using the NCBI Pairwise BLAST program, an 83-bp region of homology was identified (Figure 2A). At the nucleotide level, there was 83% identity. There were no other conserved regions identified. This region was 743 bp upstream of the glomerular transcription initiation site for the human

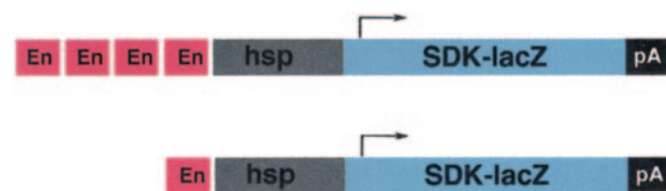
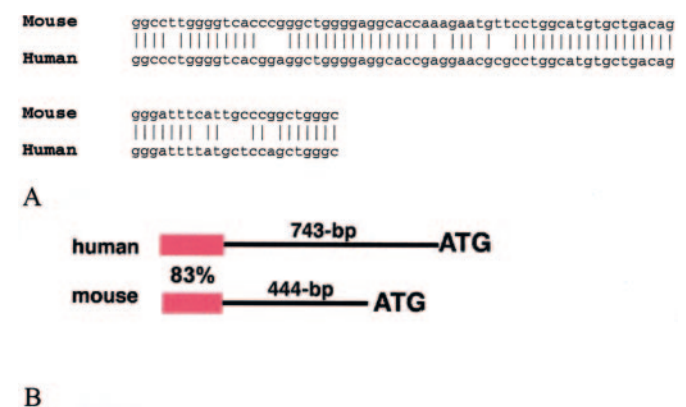


Figure 1. Four copies or one copy of the 186-bp human NPHS1 fragment (En) were subcloned into EcoRI sites upstream of a heterologous minimal promoter from the heat shock promoter (hsp) and a β -galactosidase (lacZ) transgene. The hsp-lacZ transgene has previously been described (13). All of the nephrin enhancer elements were subcloned from the 5' to 3' direction.

Figure 2. (A) An 83-bp region of homology was identified between the human and mouse NPHS1 promoters. There is 83% identity between these two regions. (B) The homologous region resides 743 bp and 444 bp upstream of the initiating ATG in the human (accession no. AC002133) and murine (accession no. AF190638.1) NPHS1 genes, respectively.

nephrin gene and 444 bp upstream of the predicted initiation site for the murine nephrin gene (Figure 2B). The TF TRANSFAC database was used to identify putative consensus binding sites for transcription factors, an E-box site and retinoic acid receptor sites were identified, and a putative WT1-binding site was identified by visual inspection (Figure 3).

Transgenic Founder Lines Exhibit Podocyte-Specific Expression

Four independent founder mice that carried four copies of the nephrin “enhancer” and the hsp-lacZ transgene were identified. LacZ staining confirmed that 50% of these independent founders expressed β -galactosidase specifically in podocytes within the kidney from the late S-shape stage of glomerulogenesis onward (see Figure 4). The other two founder mice did not demonstrate any lacZ staining. In addition, two of four founder mice that carried one copy of the nephrin enhancer demonstrated podocyte-specific expression. One of these founder mice exhibited mosaic expression as only a subset of podocytes demonstrated β -galactosidase activity (Figure 4C). Transgene copy number was estimated by dot blot analysis. There was no correlation between copy number and presence or absence of lacZ staining (data not shown).

To determine whether there was extrarenal expression of the transgene, all tissues from the newborn founder pups were stained with lacZ. Notably, there was no neural expression in any of the mice. In one of the lines that carried four copies of the transgene, there was also staining in whisker follicles and in the epicardium of the heart.

WT1 Binds to the Enhancer Region In Vitro

To demonstrate a direct interaction between WT1 protein and the nephrin promoter, an electrophoretic mobility shift assay was performed (Figure 5). A DNA probe corresponding to the 186-bp nephrin “enhancer” was incubated with bacterial expressed and purified GST proteins, and subjected to electrophoresis. No complex was formed with GST alone or GST Δ ZF (lanes 1 and 3); however, a specific protein-DNA complex was formed when the DNA probe was incubated with GSTWT1 (lane 2). The complex was abolished by incubation with increasing amounts of excess cold competitor probe containing a

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CTGCTGAGCTGGGAGACCACCTTGATCTGACTTCTCCATCTTCCAGCCTAAGCCA
      RAR, RXR           WT1
GGCCCTGGGGTCACGGAGGCTGGGGAGGCACCGAGGAACGCGCCTG
      E-box
GCATGTGCTGACAGGGGATTTATGCTCCAGCTGGGCCAGCTGGGAGGA
GCCTGCTGGGCAGAGGCCAGAGCTGGGGCTCTG

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Figure 3. Consensus binding sites for transacting factors. The TF TRANSFAC database was used to identify putative consensus binding sites for transcription factors. An E-box (single underline) and retinoic acid receptor binding sites (double underline) were identified. In addition, a putative WT1-binding site was identified (bold letters). The core 83-bp region of homology is shown in larger type; the flanking nucleotides were present in the transgene.

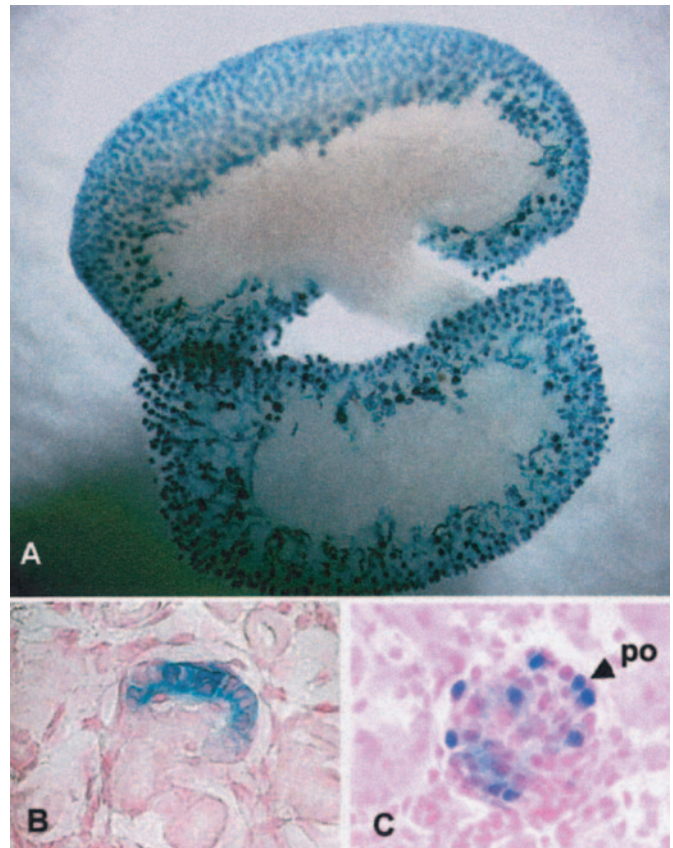


Figure 4. LacZ staining was specific to podocytes within transgenic kidneys. (A) Whole-mount kidney demonstrates lacZ expression within glomeruli (blue “balls”) of a transgenic newborn mouse kidney (original magnification, $\times 25$). (B) Cryosection of a newborn kidney demonstrates lacZ staining in a late S-shape stage glomerulus in the nephrogenic zone (original magnification, $\times 250$). The blue cells are podocyte precursors. (C) Cryosection of a mature glomerulus from a newborn transgenic kidney demonstrates lacZ staining specifically within podocytes (po) (original magnification, $\times 200$). Notably, only a subset of podocytes are staining in this mosaic transgenic line.

canonical WT1 binding site (lanes 6 to 7) but was unaffected by a probe that did not contain WT1 binding sites (lanes 4 to 5). Formation of the Neph186-WT1 complex was specifically reduced by a WT1 antibody (lane 9) but unaffected by equal amounts of control IgG (lane 8).

WT1 Activates a Nephrin-Luciferase Reporter Transgene

To determine whether WT1 directly activates nephrin expression, four copies of the 186-bp nephrin enhancer were inserted into a promoter-less luciferase reporter (pGL₂-nephrin). The nephrin luciferase reporter was transfected into 293T cells along with expression vectors for wild-type and mutant WT1-A isoform (WTA) (Figure 6). WTA activated the promoter up to 30-fold compared with empty vector control. As expected, WTA-112, a tumor associated missense mutant that is deficient for transcriptional activation, did not activate the nephrin enhancer (18).

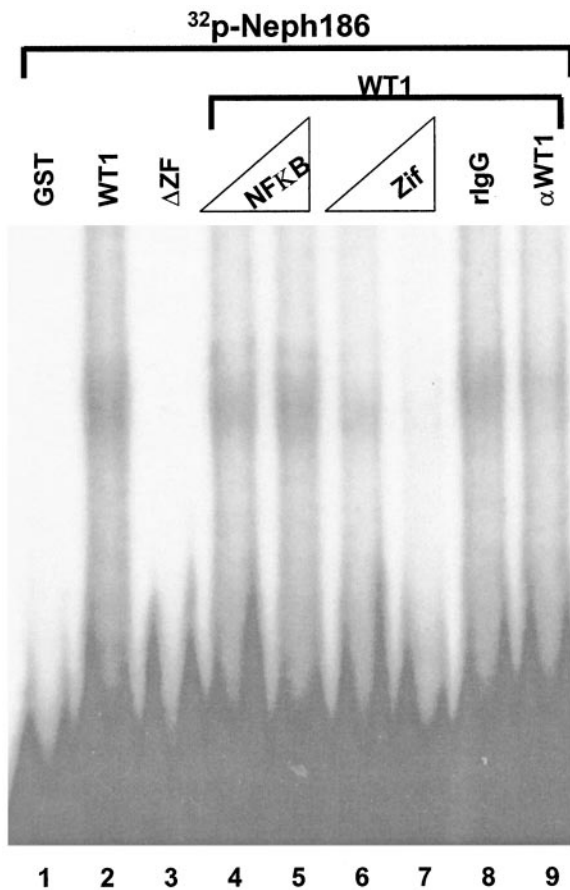


Figure 5. Gel shift binding assay. WT1 binds specifically to the nephrin promoter fragment that directs podocyte-specific expression in transgenic mice. A DNA probe corresponding to a 186-bp fragment of the human nephrin promoter was incubated with GST (lane 1), GSTWT1 (lanes 2 and 4 to 9) or GST Δ ZF (lane 3). For competition experiments WT1 was preincubated with unlabeled competitor probes NF κ B (lanes 4 to 5) and Zif3 (lanes 6 to 7) and rabbit IgG (lane 8) or a WT1 antibody (lane 9).

Discussion

Glomerular injury is a leading cause of end-stage kidney failure worldwide. Unfortunately, few successful therapies exist to treat damaged glomeruli. Increased understanding of glomerular-specific gene regulation should provide insight into the molecular basis of renal disease. We demonstrate here that 186 bp of the human nephrin promoter is capable of directing gene expression specifically to the developing and mature podocyte in transgenic mice. We chose to examine this fragment because it is highly conserved between the human and mouse nephrin promoters, with over 80% identity at the nucleotide level. Although mutations have been reported in the nephrin promoter region in three patients with congenital Finnish nephropathy (19,20), they do not occur within this 186-bp region. After the identification of the nephrin gene, another podocyte-restricted gene named podocin was identified as the gene responsible for some cases of autosomal recessive focal segmental glomerulosclerosis (21). Moeller, *et al.* (22) have previously identified a 2.5-kb fragment of the podocin gene

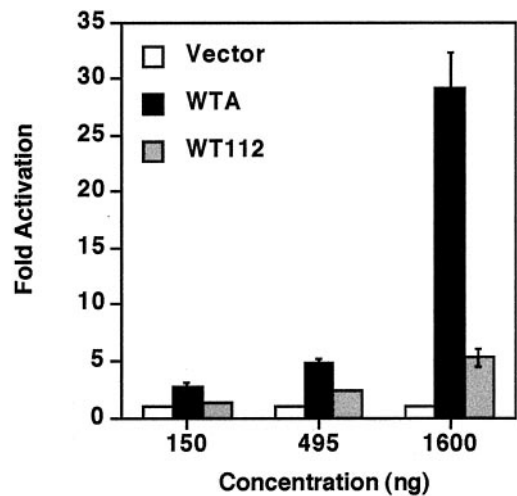


Figure 6. Transcriptional activation of the human nephrin “enhancer” by WT1. The pGL₂-nephr-luc reporter plasmid (150 ng) was transiently cotransfected into 293T cells along with increasing concentrations of empty vector, vector expressing WTA or WTA-112, and a Renilla internal control (5 ng). At 48 h after transfection, a dual luciferase assay was performed. Luciferase data are expressed as fold activation (\pm SD) relative to empty expression vector and is representative of four experiments each performed in triplicate.

that is also capable of podocyte-specific expression. By using the human genome database and the pairwise BLAST program at NCBI, we also compared the 186-bp fragment to the podocin promoter but were unable to identify a homologous region and suggests that regulation of these two genes may differ. Accordingly, Miner, *et al.* (23) have shown that podocin expression disappears in *lmx1b* (a lim-homeodomain containing transcription factor) null mice but nephrin levels remain unaffected.

Only 50% of the founder mice expressed the β -galactosidase transgene, and this did not correlate with transgene copy number. The most likely explanation for lack of expression in some founder mice is the site of integration of the transgene within the genome that is not controlled in these experiments. Of note, lacZ expression was detected as early as late S-shape stage glomeruli when podocytes are not fully differentiated. In contrast, both the 1.125-kb human and the 4.125-kb mouse promoter are incapable of driving expression in podocyte precursors (S-shape stage). Why this discrepancy in temporal expression exists is not clear, but endogenous nephrin is expressed in S-shape stage podocytes. Tryggvason and coworkers have reported that kidney regulatory elements reside within the fragment between -2.1 to -4 kb of the mouse nephrin gene, which does not contain the 186-bp fragment reported here (12,22). In a separate paper, the team of Moeller, *et al.* (22), by using 1.25 kb of the proximal murine nephrin promoter, were able to show podocyte-specific expression. It is likely that endogenous regulation of nephrin expression requires more than one regulatory element.

To date, a number of transcription factors have been identified that are expressed within the developing and mature podocyte (for review, see (24,25)). Null mutations in most of

these genes have been generated by using homologous recombination and gene targeting in embryonic stem cells in mice. However, no transcription factor has been shown to be essential for nephrin expression, although its expression is reduced in DNA-binding mutants of the Kreisler (*Krml1/MafB*) gene (26) and retinoic acid signaling leads to activation of the murine *NPHS1* promoter in cultured podocytes (27). The Wilms tumor suppressor protein is a zinc-finger containing transcription factor that is required for early nephrogenesis; null mutant mice die during midgestation with multiple defects and fail to form kidneys or gonads, precluding the analysis of nephrin expression in glomeruli (28). In addition to its expression in the metanephric mesenchyme, *WT1* becomes restricted to differentiating and mature podocytes later in nephrogenesis. Rescue experiments that used a human *WT1*-containing YAC in *WT1* null embryos results in mesangial sclerosis or crescentic glomerulonephritis depending on the gene dose (29,30), suggesting that *WT1* is required for normal podocyte differentiation and function. Furthermore, patients with point mutations in the *WT1* gene develop Denys-Drash syndrome, which is characterized by glomerular defects and nephrotic syndrome, genital anomalies, and Wilms tumor. Also, mice carrying a common Denys-Drash syndrome mutation develop glomerulosclerosis (31). Finally, *WT1* mutations have been identified in patients with nephrotic syndrome and sporadic glomerulosclerosis. Although *WT1* transcriptional targets were identified *in vitro* and *in vivo* (32,33), downstream targets for *WT1* in the podocyte have remained elusive. However, *NPHS1* expression was dramatically decreased in mice with reduced levels of the *WT1* protein (29), suggesting that nephrin is downstream of *WT1* and in *Drosophila*, the *WT1* and nephrin orthologues—*Klumpfuss* (34,35) and *IrreC* (36)—were shown to interact genetically. By using the TRANSFAC database and visual inspection of the 83-bp core fragment of homology in the human and murine nephrin promoters, we identified a putative *WT1* binding element. Gel shift assays demonstrated that a recombinant *WT1* (-KTS) (A isoform) (37), does bind specifically to the nephrin enhancer because it can be competed by excess cold oligonucleotide and can be reduced with antisera to *WT1*. Furthermore, *WT1* is able to transactivate a nephrin-enhancer luciferase reporter gene *in vitro*.

In summary, we report the first glomerular specific enhancer and demonstrate that *WT1* can bind and activate this enhancer *in vitro*. Identification of this enhancer should facilitate the identification of other cis elements required for glomerular-specific expression through standard approaches such as footprinting and consensus sequence analysis. This element may also be used as the “bait” in yeast-one-hybrid assays or in DNA-Sepharose column purification experiments to pull out additional transacting factors (38) required for nephrin expression. Finally, future studies will determine whether *NPHS1* is a direct target for *WT1* *in vivo*.

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