

Transcriptional induction of slit diaphragm genes by *Lmx1b* is required in podocyte differentiation

Jeffrey H. Miner,^{1,2} Roy Morello,³ Kaya L. Andrews,¹ Cong Li,¹ Corinne Antignac,⁴ Andrey S. Shaw,⁵ and Brendan Lee³

¹Department of Medicine, Renal Division, and

²Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri, USA

³Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas, USA

⁴Institut National de la Santé et de la Recherche Médicale, Unité 423, Necker Hospital, Paris, France

⁵Center for Immunology and Department of Pathology, Washington University School of Medicine, St. Louis, Missouri, USA

Address correspondence to: Jeffrey H. Miner, Renal Division, Box 8126, Washington University School of Medicine, 660 S. Euclid Avenue, St. Louis, Missouri 63110, USA.
Phone: (314) 362-8235; Fax: (314) 362-8237; E-mail: minerj@pcg.wustl.edu.

Jeffrey H. Miner and Roy Morello contributed equally to this work.

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LMX1B encodes a LIM-homeodomain transcription factor. Mutations in *LMX1B* cause nail-patella syndrome (NPS), an autosomal dominant disease with skeletal abnormalities, nail hypoplasia, and nephropathy. Expression of glomerular basement membrane (GBM) collagens is reduced in *Lmx1b*^{-/-} mice, suggesting one basis for NPS nephropathy. Here, we show that *Lmx1b*^{-/-} podocytes have reduced numbers of foot processes, are dysplastic, and lack typical slit diaphragms, indicating an arrest in development. Using antibodies to podocyte proteins important for podocyte function, we found that *Lmx1b*^{-/-} podocytes express near-normal levels of nephrin, synaptopodin, ZO-1, α 3 integrin, and GBM laminins. However, mRNA and protein levels for CD2AP and podocin were greatly reduced, suggesting a cooperative role for these molecules in foot process and slit diaphragm formation. We identified several *LMX1B* binding sites in the putative regulatory regions of both *CD2AP* and *NPHS2* (podocin) and demonstrated that *LMX1B* binds to these sequences in vitro and can activate transcription through them in cotransfection assays. Thus, *LMX1B* regulates the expression of multiple podocyte genes critical for podocyte differentiation and function. Our results indicate that reduced levels of proteins associated with foot processes and the glomerular slit diaphragm likely contribute, along with reduced levels of GBM collagens, to the nephropathy associated with NPS.

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Introduction

Podocytes are specialized cells of the renal glomerulus with both epithelial and mesenchymal characteristics. They lie atop the glomerular basement membrane (GBM) in Bowman's space and enwrap the glomerular capillaries with long, interdigitated foot processes. Podocytes are receiving increased attention and recognition as critical components of the kidney's ultrafiltration barrier (1, 2). They not only synthesize and secrete GBM components but also assemble the glomerular slit diaphragms, which are thought to be the kidney's ultimate size-selective filtration barrier (3, 4).

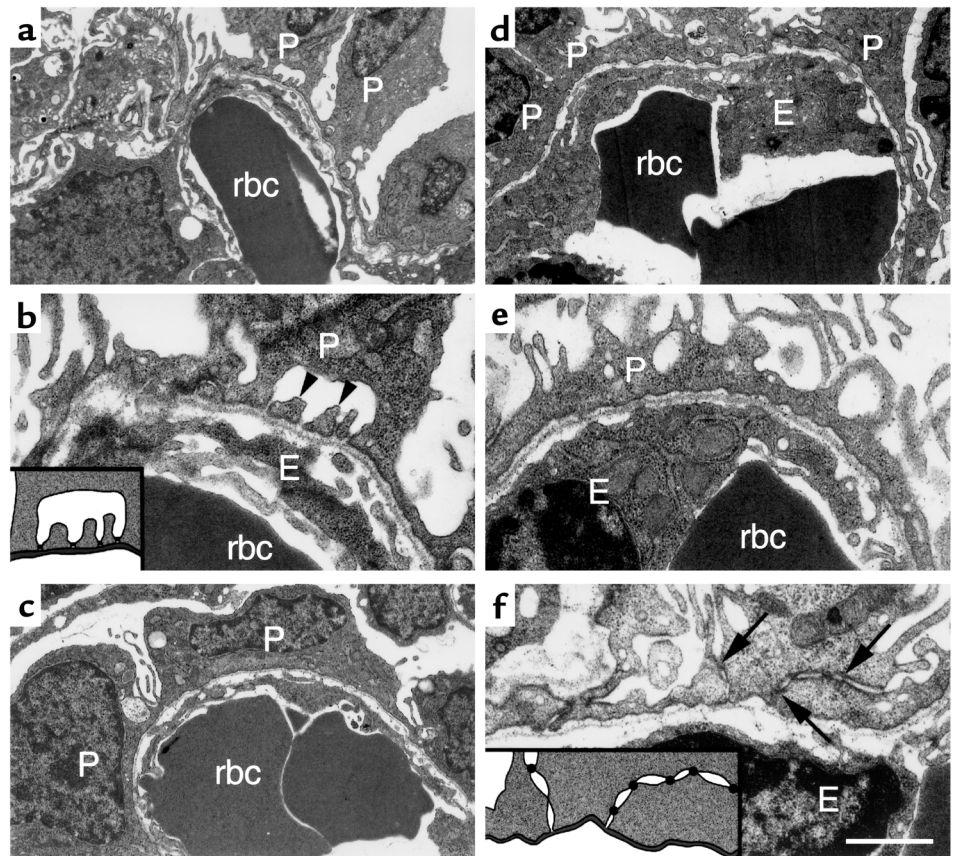
Several genes have recently been shown, through either positional cloning or gene knockout approaches, to be involved in podocyte function. Most of these genes encode structural proteins that are important in formation, function, and/or maintenance of foot process architecture and slit diaphragm integrity (2, 5, 6). However, two encode transcription factors presumed to regulate expression of genes in podocytes. The first, *Pod1*, encodes a basic-helix-loop-helix protein expressed in podocytes and in other cell types in the kidney and

elsewhere (7). The phenotype of the *Pod1*^{-/-} kidney is complex, but it is clear that podocytes are developmentally arrested (8). This suggests that *Pod1* normally activates expression of genes important for podocyte maturation, but specific downstream genes have not been identified. The second transcription factor, *Lmx1b*, encodes a LIM-homeodomain protein (9). Mutations in *LMX1B* cause nail-patella syndrome (NPS) (10), an autosomal dominant disorder characterized by skeletal abnormalities, nail hypoplasia, and nephropathy. Consistent with this, *Lmx1b*^{-/-} mice exhibit kidney defects as well as patterning defects in appendicular skeletal structures and associated soft tissues, and they die shortly after birth (11). Because *Lmx1b* is expressed in the kidney primarily in podocytes, these data suggest that *Lmx1b* regulates expression of genes required for proper podocyte function.

Two genes that we previously showed to be regulated by *Lmx1b* in podocytes, *Col4a3* and *Col4a4*, encode two of the three specialized type IV collagen chains found in the GBM. Mutations in human *COL4A3* and *COL4A4* cause Alport syndrome, a hereditary nephritis leading to

Figure 1

Ultrastructural analysis of podocytes from control and *Lmx1b*^{-/-} newborn mice. Low- and high-magnification micrographs from control (a and b) and mutant (d and e) show reduced foot process formation in the mutant. Those processes that do form have aberrant cell-cell junctions (f, arrows). Normal and aberrant processes and junctions have been schematized for clarity (insets in b and f). Mutant podocytes resemble those observed in immature control glomeruli (c), suggesting that mutant podocytes are developmentally arrested. P, podocyte; rbc, red blood cell; E, endothelial cell; arrowheads, foot processes. Bar, 2.3 μ m for a, c, and d; 0.7 μ m for b, e, and f.



end-stage renal failure (12), so their importance for renal function is well recognized. Levels of *Col4a3* and *Col4a4* RNA and protein are reduced in *Lmx1b*^{-/-} glomeruli, and LMX1B binds to a site in their common regulatory region (13). Thus, reduced expression of *COL4A3* and *COL4A4* in NPS patients is a likely consequence of *LMX1B* haploinsufficiency. This reduction would contribute to the nephropathy which, like Alport syndrome, is characterized by the presence of distinct GBM abnormalities.

Here, we have characterized the *Lmx1b*^{-/-} podocytes in detail. We found significant morphological defects as well as defects in podocyte gene expression, suggesting that *Lmx1b*^{-/-} podocytes do not differentiate properly. Our results indicate that reduced levels of proteins associated with foot processes and the glomerular slit diaphragm likely contribute, along with reduced levels of GBM collagens, to the nephropathy associated with *LMX1B* haploinsufficiency in NPS.

Methods

Electron microscopy. Kidneys from newborn control and *Lmx1b*^{-/-} mice were fixed in 2% paraformaldehyde/2% glutaraldehyde in 0.15 M sodium cacodylate. They were then rinsed in cacodylate buffer, stained with osmium tetroxide and uranyl acetate, dehydrated, and embedded in Poly/Bed 812. Ultrathin sections were stained with uranyl acetate and lead citrate and viewed with an electron microscope. Reagents were obtained from Polysciences Inc. (Warrington, Pennsylvania, USA).

Immunohistochemistry. Kidneys were frozen in OCT compound and sectioned at 7 μ m. Sections were fixed in 2% paraformaldehyde in PBS for 10 minutes and rinsed in PBS. Primary antibodies were diluted in 1% BSA in PBS and applied for 1 hour. After a PBS rinse, fluorophore-conjugated secondary antibodies were applied for 1 hour. Sections were rinsed in PBS, mounted in 0.1 \times PBS/90% glycerol/1 mg/ml *p*-phenylenediamine, and viewed with a fluorescence microscope. Images were captured with a Spot 2 cooled color digital camera (Diagnostic Instruments Inc., Sterling Heights, Michigan, USA).

Antibodies. Antibodies obtained from generous colleagues were: rabbit anti-human nephrin (14, 15) from Karl Tryggvason (Karolinska Institute, Stockholm, Sweden); rabbit anti-chick integrin α 3 (16) from C. Michael Dipersio (Albany Medical College, Albany, New York, USA); mouse anti-human synaptopodin (17) from Peter Mundel (Albert Einstein College of Medicine, Bronx, New York, USA); and rabbit anti-human α -actinin-4 (18) from Alan Beggs (Children's Hospital, Boston, Massachusetts, USA). Rat anti-mouse laminin γ 1 (MAB1914) was purchased from Chemicon International (Temecula, California, USA). Rat anti-mouse ZO-1 (19) was purchased from the Developmental Studies Hybridoma Bank (Iowa City, Iowa, USA). Rabbit anti-mouse laminin α 5 (20), rabbit anti-mouse CD2AP (21), and rabbit anti-human podocin (22) were generated as described.

Secondary antibodies were conjugated to FITC (ICN Biomedicals Inc., Costa Mesa, California, USA) or Cy3 (Chemicon International).

In situ hybridizations. For *Cd2ap* in situ, kidneys were frozen fresh in OCT and sectioned at 12 μ m. Digoxigenin-UTP-labeled sense and antisense *Cd2ap* riboprobes were hybridized to the sections and detected as described (23). The segment of mouse *Cd2ap* cDNA used to make the probes extended from nucleotides 430 to 926 (GenBank accession no. AF149092). For podocin in situ, kidneys were fixed in 4% paraformaldehyde in PBS and embedded in paraffin. Sectioning and in situ hybridization were carried out as described (24). The mouse podocin cDNA probe was homologous to nucleotides 250–632 of human podocin (GenBank accession no. AJ279254). The probe was generated by RT-PCR amplification of newborn mouse kidney RNA using primers designed from a mouse expressed sequence tag (GenBank accession no. AW106985). Primers were: sense, 5'-CCGCCACCGTAGTGGACGTG-3'; antisense, 5'-CTCCAT-TATAACATATCTTTGG-3'. Antisense and sense probes were labeled with α -³⁵S-UTP (ICN Biomedicals Inc.).

Electrophoretic mobility shift assay and transfections. Expression plasmids contained cDNAs encoding the full-length human *LMX1B* cDNA, its wild-type homeodomain, or the N246K mutant homeodomain in pcDNA3.1 (10). In vitro transcription/translation reactions were performed with unlabeled or ³⁵S-labeled methionine using the TNT Quick Coupled Transcription/Translation System (Promega Corp., Madison, Wisconsin, USA). Labeled products were analyzed by PAGE to estimate efficiency of translation. Putative LMX1B binding sites (FLAT sequences) found in 5' genomic sequences of *CD2AP* and *NPHS2* (GenBank accession nos. AL355353 and AL160286, respectively) were generated by synthesis and annealing of two complementary oligonucleotides, as follows. *CD2AP* -2855: 5'-ATACGTACATTAATTATGGCAATA-3' and 5'-TCAGTTATTGCCATAATTAATGTA-3'; *CD2AP* -1817: 5'-CTCATACTGTTAATTAACCTTTGTGTT-3' and 5'-GATACGAACACAAAGTTAATTAACAGTA-3'; *CD2AP* -1170: 5'-GCTTGCTGAAATAATTATACTTCTT-3' and 5'-TACGAAGAAGTATAATTATTCAGC-3'; *NPHS2* -825: 5'-GCATAAGCATTAAATAAGACCCTAAATAATAACAG-3' and 5'-GGCTCTGTTATTATTTAGGGTCTTTATTAATGCTT-3'. Each different ³²P end-labeled probe (2 fmol) and each in vitro transcribed/translated LMX1B or homeodomain polypeptide (3 μ l) were used in electrophoretic mobility shift assay (EMSA) as previously described (25).

Transient transfection assays in NIH 3T3 cells were performed in triplicate using Lipofectamine-Plus (Invitrogen Corp., Carlsbad, California, USA). Reporter plasmids contained four head-to-tail copies of either the *NPHS2* -825 FLAT site and flanking sequences (CATAAGCATTAAATAAGACCCTAA) or a mutated version (CATAAGCATTGCTAAAGACCCTAA) in front of the *Col2a1* minimal promoter (26) and a luciferase reporter. These plasmids were cotransfected with pcDNA3.1-LMX1B or empty pcDNA3.1 (Invitrogen Corp.), with pSV2 β gal (Promega Corp.) included as a control for transfection

efficiency. Luciferase and β -galactosidase activities were assayed as described previously (13) 16 hours after an initial 4-hour incubation with the transfection mixture.

Results

Aberrant foot process and slit diaphragm formation in *Lmx1b*^{-/-} podocytes. Examination of glomeruli in *Lmx1b*^{-/-} kidneys revealed an aberrant morphology at birth, including a dilated Bowman's space and atypical cellular organization (data not shown). These abnormalities were similar to those visible in the initial report of the *Lmx1b*^{-/-} phenotype (11). *Lmx1b* has been shown to positively regulate expression of *Col4a3* and *Col4a4* (13), genes that encode GBM collagens. However, reduced expression of only these genes cannot fully explain the glomerular abnormalities, because mice with inactivating mutations in *Col4a3* and *Col4a4* have unremarkable glomeruli at young ages (27–29). Here, we further investigated the nature of the glomerular defect in *Lmx1b* mutants, paying particular attention to podocytes, the specialized glomerular cell that expresses *Lmx1b* (13).

We performed an ultrastructural analysis of podocyte morphology in *Lmx1b*^{+/-} and *Lmx1b*^{-/-} glomeruli (Figure 1). Electron microscopy was used to evaluate the refined structure of podocytes that is so critical for proper

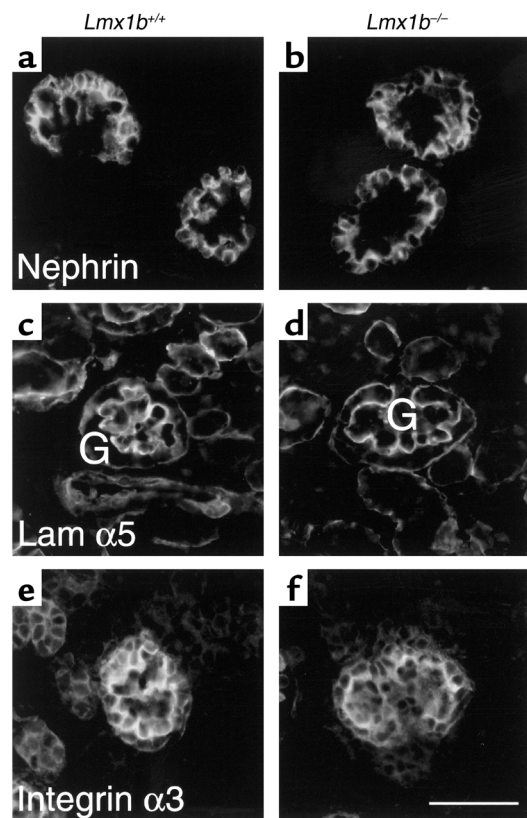


Figure 2

Immunohistochemical analysis of podocyte gene expression in *Lmx1b*^{+/-} and *Lmx1b*^{-/-} glomeruli from newborn mice. No differences in levels of nephrin (a and b), laminin (Lam) α 5 (c and d), or integrin α 3 (e and f) were detected, suggesting that *Lmx1b* does not normally regulate their expression. G, glomerulus. Bar, 50 μ m.

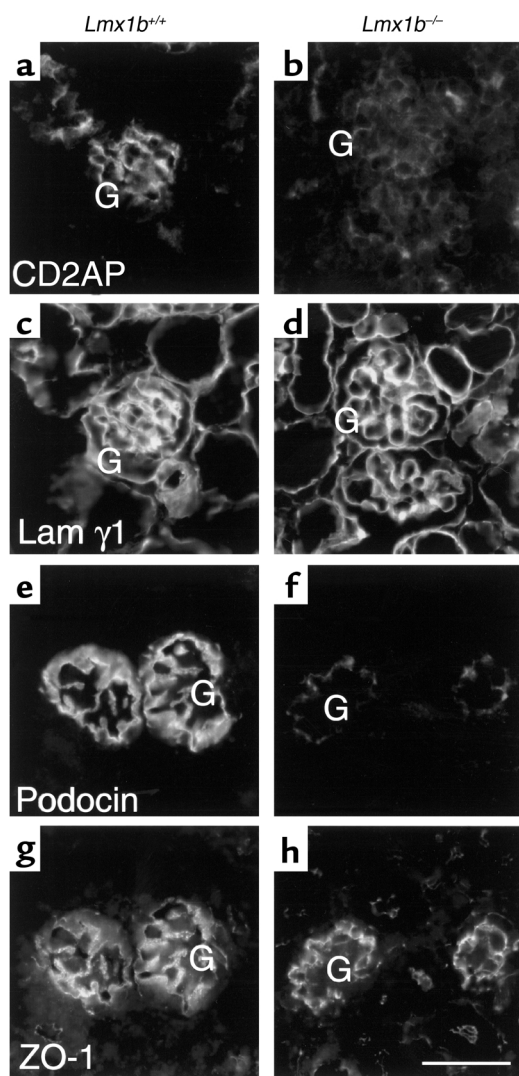


Figure 3
Immunohistochemical analysis of podocyte gene expression in *Lmx1b*^{+/+} and *Lmx1b*^{-/-} glomeruli from newborn mice. Levels of CD2AP (a and b) and podocin (e and f) were greatly reduced in the mutant, suggesting that *Lmx1b* regulates their expression. Sections in a and b were doubly labeled with an antibody to laminin γ 1 chain (c and d, respectively), and sections in e and f were doubly labeled with an antibody to ZO-1 (g and h, respectively). Laminin γ 1 was not reduced, and ZO-1 was only slightly reduced. G, glomerulus. Bar, 50 μ m.

glomerular filtration (1). In comparison to control, *Lmx1b*^{-/-} podocytes exhibited striking defects. Foremost was the lack of normal foot process formation. Though mutant podocytes were properly juxtaposed to the GBM, they only rarely elaborated processes (Figure 1, a, b, d, and e). Most mutant podocytes appeared quite similar to control podocytes present in immature glomeruli (Figure 1c), suggesting that there is an arrest in podocyte development at the stage when foot processes normally form. (The mutant podocytes examined were from the most mature glomeruli, so our interpretation was not confounded by comparing immature mutant podocytes to mature control podocytes.)

Another defect was observed in the morphology of those processes that did form: many did not have the flattened, side-by-side appearance typical of normal foot processes (Figure 1b, arrowheads). Instead, they had a somewhat stacked arrangement, with one process appearing to invaginate slightly beneath an adjacent one (Figure 1f, arrows). In addition, there was a lack of typical slit diaphragms between the processes. Slit diaphragms are specialized cell-cell junctions with characteristics of both adherens junctions (30) and tight junctions (31). They extend across the gap between foot processes and are thought to serve as the kidney's ultimate filtration barrier. Slit diaphragms were visible in controls as electron-dense material lying between the foot processes parallel to the GBM (Figure 1b). In *Lmx1b*^{-/-} mice, two classes of cell-cell junctions were observed: those between the rare processes, and those between adjacent podocyte cell bodies. For both classes, electron-dense material was not always observed in the spaces between the cells, and when it was, it was usually not parallel to the GBM (Figure 1f and data not shown). Thus, there were clearly defined morphological defects in *Lmx1b*^{-/-} podocytes, suggesting perturbation of podocyte development. In addition, the nature of the defects suggests that renal function should be impaired. Consistent with this prediction, little if any urine was found in the bladders of *Lmx1b*^{-/-} mice, and there were protein casts in a subset of tubules in the mutant kidney (data not shown). This indicates that what little urine was produced by *Lmx1b*^{-/-} mice contained significant levels of protein, one hallmark of filtration barrier defects.

Aberrant podocyte gene expression in Lmx1b mutants. Based on these results, we hypothesized that *Lmx1b*, in addition to regulating collagen IV genes, also regulates podocyte genes encoding proteins involved in slit diaphragm and foot process formation. Over the last few years, several proteins from diverse families have been shown or hypothesized to be critically important for proper podocyte and/or slit diaphragm development, maturation, and function (2, 5, 6). It is unknown whether the genes encoding these proteins are regulated by *Lmx1b*. Those that are should exhibit reduced expression in *Lmx1b*^{-/-} podocytes. We used immunohistochemistry to assay for expression of podocyte proteins thought to be important for filtration. The data shown were derived from the deepest glomeruli to ensure that only the most mature podocytes were assayed. Levels of several proteins were not significantly changed. These included nephrin, a component of the slit diaphragm (32–35); laminin α 5 and γ 1 chains, components of the GBM (20, 36); integrin α 3, a podocyte receptor for laminin in the GBM (37); and α -actinin-4, a cytoskeletal protein mutated in one form of familial focal segmental glomerulosclerosis (18) (Figures 2 and 3). Thus, the genes encoding these proteins are not likely to be directly regulated by *Lmx1b* on a transcriptional level. Slight reductions in levels of ZO-1, a cytoplasmic protein found associated with tight junctions and slit diaphragms (31), and synaptopodin, an actin-asso-

ciated protein found in foot processes (38), were consistently observed (Figure 3, g and h; and data not shown). However, because the reductions were slight, we suspect that they are more likely attributable to the paucity of cell-cell junctions and foot processes (respectively) than to decreased gene expression.

In contrast to these results, levels of CD2AP, an adapter protein that binds to the cytoplasmic tail of nephrin at the slit diaphragm (39), and podocin, a transmembrane protein which has been localized to the slit diaphragm (22, 40), were drastically reduced in mutant podocytes (Figure 3). Reduction was apparent not only in the deepest, most mature glomeruli, but also in the very primitive S-shape and capillary loop stage glomeruli (data not shown). Importantly, CD2AP and podocin are known to have crucial roles in podocyte function. Mice lacking CD2AP and humans with homozygous or compound heterozygous mutations in *NPHS2*, the gene encoding podocin, exhibit nephrotic syndrome accompanied by foot process effacement (41, 42). We therefore conclude that the podocyte abnormalities observed in *Lmx1b*^{-/-} kidneys are, at least in part, caused by reduced levels of CD2AP and podocin.

We next hypothesized that *Lmx1b* directly regulates transcription of the *Cd2ap* and *Nphs2* genes. To begin to test this hypothesis, we performed *in situ* hybridization on sections of control and *Lmx1b*^{-/-} kidneys with probes for *Cd2ap* and podocin mRNA. This was done to distinguish potential posttranscriptional effects on gene expression; rather than regulating *Cd2ap* and *Nphs2* directly, *Lmx1b* might regulate expression of other genes whose products stabilize CD2AP and podocin. However, the *in situ* analysis revealed significant reductions in the levels of both *Cd2ap* and podocin mRNAs (Figure 4). The reduction was most evident for *Cd2ap* in early capillary loop structures, the stage at which CD2AP protein is first detectable in normal kidney (14). Importantly, the reduction was specific to podocytes; *Cd2ap* RNA and protein levels were not significantly reduced in mutant collecting ducts (Figure 4a and data not shown), consistent with the fact that *Lmx1b* is not normally expressed in collecting ducts (11, 13). Podocin RNA was essentially undetectable in *Lmx1b* mutant kidney but was easily detected in the control (Figure 4b). Semiquantitative RT-PCR demonstrated that total podocin mRNA levels were reduced by 75% (data not shown). Together, these data provide strong evidence that *Lmx1b* is involved in regulating transcription of *Cd2ap* and *Nphs2*. Consistent with this notion, *Lmx1b* is expressed in primitive structures in the nephrogenic zone (11, 13), at stages before the onset of *Cd2ap* and *Nphs2* expression (14, 42).

LMX1B binds to putative regulatory elements in CD2AP and NPHS2 promoters. If *Lmx1b* directly regulates transcription of *Cd2ap* and *Nphs2*, then their regulatory regions should contain LMX1B binding sites. Unfortunately, the regulatory regions of these genes have not yet been fully characterized. Nevertheless, we were able to retrieve genomic DNA sequences of human *CD2AP* and *NPHS2* from GenBank. Candidate LMX1B binding sequences

(FLAT elements) (25) were identified in both genes in the sequences flanking the first exons (Figure 5a), where transcriptional regulatory elements are expected to be located. In *CD2AP*, five FLAT sites flanked the predicted first exon, at -2855, -1817, -1170, +874, and +1053 nucleotides relative to the ATG translational start codon. In *NPHS2*, three FLAT sites flanked the predicted first exon, at -1251, -825, and +1275 relative to the ATG.

To determine whether LMX1B is capable of binding to these elements, we performed EMSA. Oligonucleotides representing both strands of the three 5' *CD2AP* sites and the -825 *NPHS2* site were synthesized and annealed to make double-stranded FLAT sites. Oligonucleotides were end-labeled with ³²P and incubated with either full-length

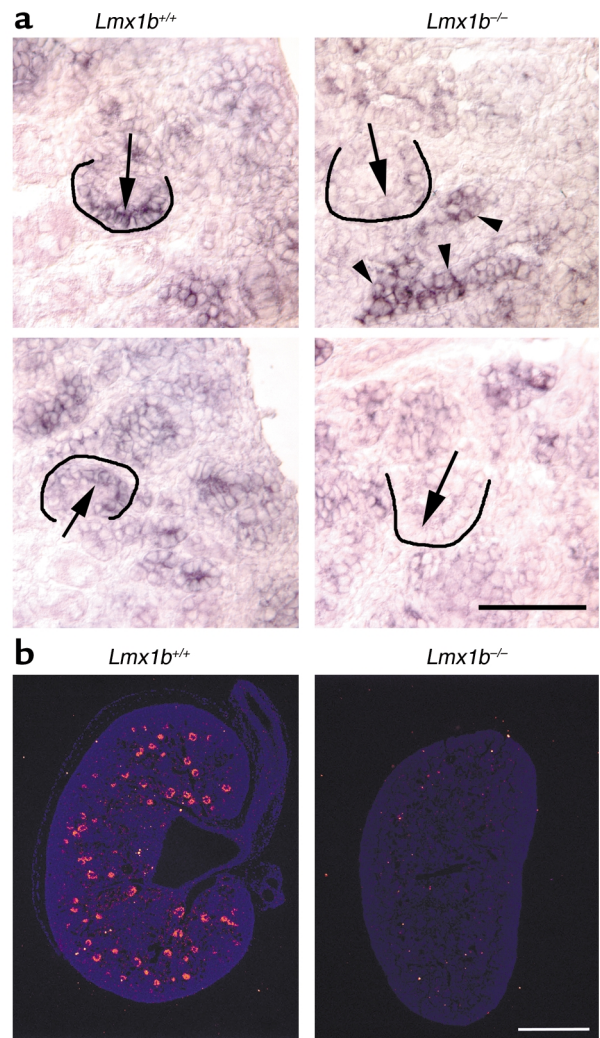


Figure 4 *In situ* hybridization analysis of CD2AP and podocin mRNA in *Lmx1b*^{+/+} and *Lmx1b*^{-/-} glomeruli from newborn mice. (a) In podocytes (arrows) of capillary loop stage glomeruli (outlined in black), CD2AP transcripts were detected in control (*Lmx1b*^{+/+}), but levels were greatly reduced in mutant (*Lmx1b*^{-/-}). Transcripts were easily detected in the branched mutant collecting duct (arrowheads). (b) Podocin transcripts were detected in control but not mutant podocytes. These data suggest that *Lmx1b* regulates podocyte transcription of *Cd2ap* and *Nphs2* (podocin). Bar in a, 50 μm; in b, 500 μm.

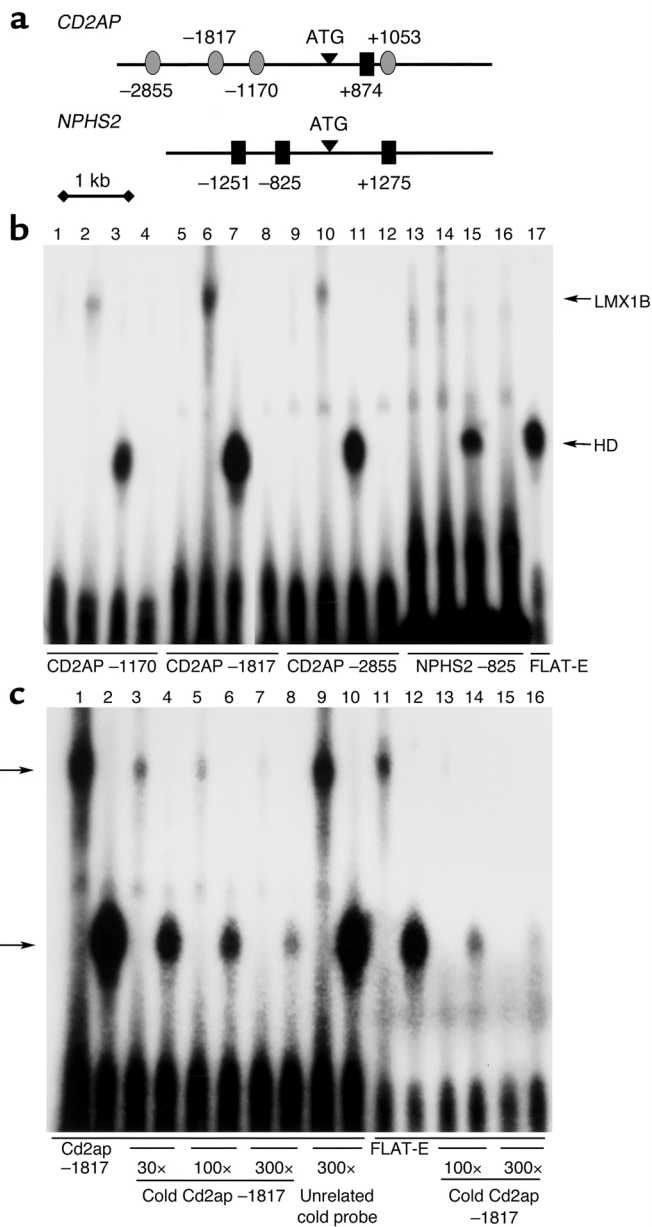


Figure 5

LMX1B interacts specifically with putative regulatory elements in *CD2AP* and *NPHS2*. **(a)** Schematic diagram of *CD2AP* and *NPHS2* 5' genomic structures. The putative LMx1B binding sites (FLAT sites) are numbered relative to the ATG translation start codon. Ovals represent FLAT-E sites (consensus TAATTA) and rectangles represent FLAT-F sites (consensus TTAAKAM). **(b)** EMSA of full-length LMX1B (lanes 2, 6, 10, 14), LMX1B homeodomain (HD) (lanes 3, 7, 11, 15, 17), mutant LMX1B HD (N246K) (lanes 4, 8, 12, 16), and empty vector (lanes 1, 5, 9, 13) using the following probes: *CD2AP* -1170, *CD2AP* -1817, *CD2AP* -2855, *NPHS2* -825, and the *COL4A3-COL4A4* enhancer element (FLAT-E) as positive control. Arrows indicate the shifted bands containing full-length LMX1B and LMX1B HD protein/DNA complexes. **(c)** Binding of full-length LMX1B (lanes 1, 3, 5, 7, 9, 11, 13, 15) and LMX1B HD (lanes 2, 4, 6, 8, 10, 12, 14, 16) to the *CD2AP* -1817 probe (lanes 1-10) was successfully competed with 30-, 100-, and 300-fold excess of cold *CD2AP* -1817 but not with excess unrelated cold probe. Binding to FLAT-E (lanes 11-16) was successfully competed with 100- and 300-fold excess of cold *CD2AP* -1817 probe. Arrows indicate the full-length LMX1B and LMX1B HD DNA/polypeptide complexes.

LMX1B or its DNA-binding homeodomain. The mixtures were then separated by native PAGE and subjected to autoradiography (Figure 5b). All four sites were bound by the full-length protein and were bound even more efficiently by the homeodomain polypeptide. No significant mobility shifts were observed if a mutant homeodomain (N246K, described in an NPS patient) or a negative control was used. This suggests that binding of LMX1B to the FLAT sites was specific.

To further demonstrate specificity of LMX1B binding, additional assays with the *CD2AP* -1817 site were performed (Figure 5c). An excess of the unlabeled (cold) -1817 probe was incubated along with the ³²P-labeled probe. The cold probe was able to compete for binding to both full-length LMX1B and the homeodomain fragment, as shown by the reduction in intensity of the shifted bands, but 300-fold excess

of an unrelated cold site did not compete effectively. Finally, an excess of the *CD2AP* -1817 site was able to compete for LMX1B binding to a FLAT-E site from the *COL4A3-COL4A4* regulatory region (13). Taken together, these data show that LMX1B can efficiently bind to potential regulatory elements from both *CD2AP* and *NPHS2* in a dose-related and specific fashion, consistent with the hypothesized regulation of these genes by *Lmx1b*.

LMX1B upregulates a reporter construct containing *NPHS2* -825 FLAT sites. LMX1B is considered a relatively weak transactivator that requires coactivators for optimal activity (43). The coactivators required for Lmx1b-mediated expression of *Cd2ap* and *Nphs2* in podocytes are unknown. Nevertheless, we attempted to demonstrate LMX1B-mediated transactivation of the *NPHS2* -825 FLAT site in heterologous cells.

Four copies of this site and its flanking sequences were cloned upstream of a minimal promoter and a luciferase reporter. A similar reporter with a mutated version of the site was also constructed. These were cotransfected into NIH 3T3 cells with either LMX1B expression plasmid or empty vector, together with a β -galactosidase expression plasmid to control for transfection efficiency. LMX1B upregulated expression from the wild-type FLAT site, but not from the mutant site (Figure 6). Together with our immunohistochemical, in situ, and gel shift data, these results strongly suggest that LMX1B is involved in the transcriptional regulation of *NPHS2* and *CD2AP* in podocytes, providing an additional basis for the nephropathy observed in NPS.

Discussion

The fact that heterozygous mutations in *LMX1B* cause a glomerulopathy suggests that *LMX1B* regulates genes critical for podocyte function. Homozygous *Lmx1b*^{-/-} mice are an excellent model system for defining which genes expressed in podocytes are regulated by *Lmx1b*, and therefore which genes might be under-expressed – perhaps only subtly as a consequence of haploinsufficiency – in NPS. We previously reported reduced levels of Col4a3 and Col4a4 protein and RNA in *Lmx1b*^{-/-} glomeruli (13); here, we found greatly reduced levels of both CD2AP and podocin protein and RNA (Figures 3 and 4). As these four proteins are known to be required for normal glomerular function, the glomerulopathy in NPS could stem from a reduction in any or all of them. In addition, there may be other genes whose expression is regulated by *Lmx1b* and whose products are important for podocyte differentiation and function.

Our electron microscopic analysis of *Lmx1b*^{-/-} podocytes (Figure 1) showed that *Lmx1b* is required for normal podocyte differentiation. Mutant podocytes appeared to be developmentally arrested: they did not elaborate normal foot processes, and cell-cell junctions did not have typical slit diaphragms. Both CD2AP and podocin have been localized to the slit diaphragm (22, 39, 40) and are necessary for normal glomerular function (41, 42). We therefore suggest that reduced levels of CD2AP and podocin contribute to the developmental arrest and conclude that they normally act cooperatively to promote foot process and slit diaphragm formation during glomerulogenesis. Indeed, podocin has recently been shown to interact directly with CD2AP (40).

The ultrastructure of the cell-cell junctions that form between the mutant podocytes bears some resemblance to that reported for homozygous *Nphs1* (nephrin) mutant podocytes (44). Nephrin is a major component of the slit diaphragm (32–35), and mutations in *NPHS1* cause congenital nephrotic syndrome of the Finnish type (45, 46). The fact that we found significant levels of nephrin protein in *Lmx1b* mutant podocytes (Figure 2) suggests that nephrin is not

sufficient for normal slit diaphragm formation. As CD2AP and podocin interact with each other (40), and CD2AP interacts with nephrin (39, 41), CD2AP and podocin may cooperate to organize or stabilize nephrin. Interestingly, podocytes in mice lacking nephrin also have reduced numbers of foot processes and exhibit some effacement (44), but the severity of these defects is less than we observed in the *Lmx1b*^{-/-} podocytes. Thus, proteins other than nephrin must coordinate foot process formation, and CD2AP and podocin are likely to be involved.

The demonstration of LMX1B binding sites in putative regulatory regions of the *CD2AP* and *NPHS2* genes (Figure 5) and the ability of LMX1B to transactivate the *NPHS2* –825 site (Figure 6) strengthen our conclusion that *Lmx1b* positively regulates their expression. We previously showed that CD2AP and nephrin begin to accumulate in differentiating podocytes at approximately the same stage and predicted that they would be coregulated (14). However, we did not find a significant reduction in nephrin mRNA (13) or protein (Figure 2) in the absence of *Lmx1b*. This is consistent with the fact that a 1.25-kb segment of the human nephrin promoter drives expression of a reporter in podocytes (47), but it lacks consensus LMX1B binding sites. It will be interesting to determine whether Pod1, a basic-helix-loop-helix protein required for podocyte development (7, 8), and WT1, a zinc finger transcription factor expressed in podocytes (48) and mutated in Denys-Drash syndrome and isolated diffuse mesangial sclerosis (49, 50), are involved in regulating expression of any of the genes, such as *Nphs1*, that do not appear to be regulated by *Lmx1b* (Figure 2 and data not shown). These transcription factors could each control expression of nonoverlapping sets of podocyte genes necessary for proper glomerulogenesis and podocyte function. Haploinsufficiency of LMX1B (as occurs in NPS) or *POD1* could lead

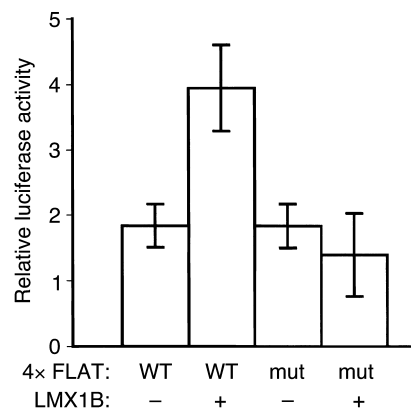


Figure 6

LMX1B transactivates expression of a reporter driven by *NPHS2* –825 FLAT sites. Luciferase reporter constructs containing four copies of the wild-type (WT) or mutant (mut) FLAT site and a minimal promoter were cotransfected with an empty vector (-) or with an LMX1B expression plasmid (+) into NIH 3T3 cells. LMX1B upregulated expression of luciferase directed by the wild-type but not by the mutant FLAT sites.

to either subtly or significantly reduced expression of a number of podocyte genes. Over time or with the appropriate genetic or environmental cofactors, this could in turn lead to nephropathy.

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- Smoyer, W.E., and Mundel, P. 1998. Regulation of podocyte structure during the development of nephrotic syndrome. *J. Mol. Med.* **76**:172–183.
- Somlo, S., and Mundel, P. 2000. Getting a foothold in nephrotic syndrome. *Nat. Genet.* **24**:333–335.
- Tryggvason, K., Ruotsalainen, V., and Wartiovaara, J. 1999. Discovery of the congenital nephrotic syndrome gene discloses the structure of the mysterious molecular sieve of the kidney. *Int. J. Dev. Biol.* **43**:445–451.
- Tryggvason, K. 1999. Unraveling the mechanisms of glomerular ultrafiltration: nephrin, a key component of the slit diaphragm. *J. Am. Soc. Nephrol.* **10**:2440–2445.
- Kerjaschki, D. 2001. Caught flat-footed: podocyte damage and the molecular bases of focal glomerulosclerosis. *J. Clin. Invest.* **108**:1583–1587. DOI:10.1172/JCI200114629.
- Miner, J.H. 2002. Focusing on the glomerular slit diaphragm: podocin enters the picture. *Am. J. Pathol.* **160**:3–5.
- Quaggin, S.E., Vanden Heuvel, G.B., and Igarashi, P. 1998. Pod-1, a mesoderm-specific basic-helix-loop-helix protein expressed in mesenchymal and glomerular epithelial cells in the developing kidney. *Mech. Dev.* **71**:37–48.
- Quaggin, S.E., et al. 1999. The basic-helix-loop-helix protein pod1 is critically important for kidney and lung organogenesis. *Development.* **126**:5771–5783.
- Hobert, O., and Westphal, H. 2000. Functions of LIM-homeobox genes. *Trends Genet.* **16**:75–83.
- Dreyer, S.D., et al. 1998. Mutations in LMX1B cause abnormal skeletal patterning and renal dysplasia in nail patella syndrome. *Nat. Genet.* **19**:47–50.
- Chen, H., et al. 1998. Limb and kidney defects in Lmx1b mutant mice suggest an involvement of LMX1B in human nail patella syndrome. *Nat. Genet.* **19**:51–55.
- Kashtan, C.E. 1997. Alport syndrome. *Kidney Int.* **51**(Suppl. 58):S-69–S-71.
- Morello, R., et al. 2001. Regulation of glomerular basement membrane collagen expression by LMX1B contributes to renal disease in nail patella syndrome. *Nat. Genet.* **27**:205–208.
- Li, C., Ruotsalainen, V., Tryggvason, K., Shaw, A.S., and Miner, J.H. 2000. CD2AP is expressed with nephrin in developing podocytes and is found widely in mature kidney and elsewhere. *Am. J. Physiol. Renal Physiol.* **279**:F785–F792.
- Patrakka, J., et al. 2001. Expression of nephrin in pediatric kidney diseases. *J. Am. Soc. Nephrol.* **12**:289–296.
- DiPersio, C.M., Shah, S., and Hynes, R.O. 1995. Alpha 3A beta 1 integrin localizes to focal contacts in response to diverse extracellular matrix proteins. *J. Cell Sci.* **108**:2321–2336.
- Mundel, P., Gilbert, P., and Kriz, W. 1991. Podocytes in glomerulus of rat kidney express a characteristic 44 KD protein. *J. Histochem. Cytochem.* **39**:1047–1056.
- Kaplan, J.M., et al. 2000. Mutations in ACTN4, encoding alpha-actinin-4, cause familial focal segmental glomerulosclerosis. *Nat. Genet.* **24**:251–256.
- Stevenson, B.R., Siliciano, J.D., Mooseker, M.S., and Goodenough, D.A. 1986. Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. *J. Cell Biol.* **103**:755–766.
- Miner, J.H., et al. 1997. The laminin α chains: expression, developmental transitions, and chromosomal locations of α 1-5, identification of heterotrimeric laminins 8-11, and cloning of a novel α 3 isoform. *J. Cell Biol.* **137**:685–701.
- Dustin, M.L., et al. 1998. A novel adaptor protein orchestrates receptor patterning and cytoskeletal polarity in T-cell contacts. *Cell.* **94**:667–677.
- Roselli, S., et al. 2002. Podocin localizes in the kidney to the slit diaphragm area. *Am. J. Pathol.* **160**:131–139.
- Schaeren-Wiemers, N., and Gerfin-Moser, A. 1993. A single protocol to detect transcripts of various types and expression levels in neural tissue and cultured cells: in situ hybridization using digoxigenin-labelled cRNA probes. *Histochemistry.* **100**:431–440.
- Albrecht, U. 1998. Studying gene expression on tissue sections using in situ hybridization. In *Human genome methods*. K.W. Adolph, editor. CRC Press. Boca Raton, Florida, USA. 93–119.
- German, M.S., Wang, J., Chadwick, R.B., and Rutter, W.J. 1992. Synergistic activation of the insulin gene by a LIM-homeo domain protein and a basic helix-loop-helix protein: building a functional insulin minihancer complex. *Genes Dev.* **6**:2165–2176.
- Lefebvre, V., et al. 1996. An 18-base-pair sequence in the mouse pro-alpha1(II) collagen gene is sufficient for expression in cartilage and binds nuclear proteins that are selectively expressed in chondrocytes. *Mol. Cell Biol.* **16**:4512–4523.
- Miner, J.H., and Sanes, J.R. 1996. Molecular and functional defects in kidneys of mice lacking collagen α 3(IV): implications for Alport syndrome. *J. Cell Biol.* **135**:1403–1413.
- Cosgrove, D., et al. 1996. Collagen COL4A3 knockout: a mouse model for autosomal Alport syndrome. *Genes Dev.* **10**:2981–2992.
- Lu, W., et al. 1999. Insertional mutation of the collagen genes Col4a3 and Col4a4 in a mouse model of Alport syndrome. *Genomics.* **61**:113–124.
- Reiser, J., Kriz, W., Kretzler, M., and Mundel, P. 2000. The glomerular slit diaphragm is a modified adherens junction. *J. Am. Soc. Nephrol.* **11**:1–8.
- Schnabel, E., Anderson, J.M., and Farquhar, M.G. 1990. The tight junction protein ZO-1 is concentrated along slit diaphragms of the glomerular epithelium. *J. Cell Biol.* **111**:1255–1263.
- Ruotsalainen, V., et al. 1999. Nephrin is specifically located at the slit diaphragm of glomerular podocytes. *Proc. Natl. Acad. Sci. USA.* **96**:7962–7967.
- Kawachi, H., et al. 2000. Cloning of rat nephrin: expression in developing glomeruli and in proteinuric states. *Kidney Int.* **57**:1949–1961.
- Holthofer, H., et al. 1999. Nephrin localizes at the podocyte filtration slit area and is characteristically spliced in the human kidney. *Am. J. Pathol.* **155**:1681–1687.
- Holzman, L.B., et al. 1999. Nephrin localizes to the slit pore of the glomerular epithelial cell. *Kidney Int.* **56**:1481–1491.
- Sorokin, L.M., Pausch, F., Durbejj, M., and Ekblom, P. 1997. Differential expression of five laminin α (1-5) chains in developing and adult mouse kidney. *Dev. Dyn.* **210**:446–462.
- Kreidberg, J.A., et al. 1996. Alpha 3 beta 1 integrin has a crucial role in kidney and lung organogenesis. *Development.* **122**:3537–3547.
- Mundel, P., et al. 1997. Synaptopodin: an actin-associated protein in telencephalic dendrites and renal podocytes. *J. Cell Biol.* **139**:193–204.
- Shih, N.-Y., et al. 2001. CD2AP localizes to the slit diaphragm and binds to nephrin via a novel C-terminal domain. *Am. J. Pathol.* **159**:2303–2308.
- Schwarz, K., et al. 2001. Podocin, a raft-associated component of the glomerular slit diaphragm, interacts with CD2AP and nephrin. *J. Clin. Invest.* **108**:1621–1629. DOI:10.1172/JCI200112849.
- Shih, N.-Y., et al. 1999. Congenital nephrotic syndrome in mice lacking CD2-associated protein. *Science.* **286**:312–315.
- Boute, N., et al. 2000. NPHS2, encoding the glomerular protein podocin, is mutated in autosomal recessive steroid-resistant nephrotic syndrome. *Nat. Genet.* **24**:349–354.
- Dreyer, S.D., et al. 2000. LMX1B transactivation and expression in nail-patella syndrome. *Hum. Mol. Genet.* **9**:1067–1074.
- Putaala, H., Soininen, R., Kilpelainen, P., Wartiovaara, J., and Tryggvason, K. 2001. The murine nephrin gene is specifically expressed in kidney, brain and pancreas: inactivation of the gene leads to massive proteinuria and neonatal death. *Hum. Mol. Genet.* **10**:1–8.
- Kestila, M., et al. 1998. Positionally cloned gene for a novel glomerular protein—nephrin—is mutated in congenital nephrotic syndrome. *Mol. Cell.* **1**:575–582.
- Patrakka, J., et al. 2000. Congenital nephrotic syndrome (NPHS1): features resulting from different mutations in Finnish patients. *Kidney Int.* **58**:972–980.
- Wong, M.A., Cui, S., and Quaggin, S.E. 2000. Identification and characterization of a glomerular-specific promoter from the human nephrin gene. *Am. J. Physiol. Renal Physiol.* **279**:F1027–F1032.
- Mundlos, S., et al. 1993. Nuclear localization of the protein encoded by the Wilms' tumor gene WT1 in embryonic and adult tissues. *Development.* **119**:1329–1341.
- Pelletier, J., et al. 1991. Germline mutations in the Wilms' tumor suppressor gene are associated with abnormal urogenital development in Denys-Drash syndrome. *Cell.* **67**:437–447.
- Jeanpierre, C., et al. 1998. Identification of constitutional WT1 mutations, in patients with isolated diffuse mesangial sclerosis, and analysis of genotype/phenotype correlations by use of a computerized mutation database. *Am. J. Hum. Genet.* **62**:824–833.