Lmx1b and FoxC Combinatorially Regulate Podocin Expression in Podocytes

Bing He,* Lwaki Ebarasi,†‡ Zhe Zhao,§ Jing Guo,* Juha R.M. Ojala,* Kjell Hultenby,∥ Sarah De Val,§ Christer Betsholtz,†‡ and Karl Tryggvason*¶

*Department of Medical Biochemistry and Biophysics, Division of Matrix Biology, and †Department of Medical Biochemistry and Biophysics, Division of Vascular Biology, and ∥Department of Laboratory Medicine, Division of Clinical Research Centre, Karolinska Institute, Stockholm, Sweden; ‡Department of Immunology, Genetics and Pathology, Rudbeck Laboratory, Uppsala University, Uppsala, Sweden; §Ludwig Institute for Cancer Research, Oxford University, Oxford, United Kingdom; and ¶Cardiovascular & Metabolic Disorders Program, Duke-NUS, Singapore

ABSTRACT

Podocin is a key protein of the kidney podocyte slit diaphragm protein complex, an important part of the glomerular filtration barrier. Mutations in the human podocin gene NPHS2 cause familial or sporadic forms of renal disease owing to the disruption of filtration barrier integrity. The exclusive expression of NPHS2 in podocytes reflects its unique function and raises interesting questions about its transcriptional regulation. Here, we further define a 2.5-kb zebrafish nphs2 promoter fragment previously described and identify a 49-bp podocyte-specific transcriptional enhancer using Tol2-mediated G0 transgenesis in zebrafish. Within this enhancer, we identified a cis-acting element composed of two adjacent DNA-binding sites (FLAT-E and forkhead) bound by transcription factors Lmx1b and FoxC. In zebrafish, double knockdown of Lmx1b and FoxC orthologs using morpholino doses that caused no or minimal phenotypic changes upon individual knockdown completely disrupted podocyte development in 40% of injected embryos. Co-overexpression of the two genes potently induced endogenous nphs2 expression in zebrafish podocytes. We found that the NPHS2 promoter also contains a cis-acting Lmx1b-FoxC motif that binds LMX1B and FoxC2. Furthermore, a genome-wide search identified several genes that carry the Lmx1b-FoxC motif in their promoter regions. Among these candidates, motif-driven podocyte enhancer activity of CCNC and MEIS2 was functionally analyzed in vivo. Our results show that podocyte expression of some genes is combinatorially regulated by two transcription factors interacting synergistically with a common enhancer. This finding provides insights into transcriptional mechanisms required for normal and pathologic podocyte functions.


Normal glomerular filtration function depends on structural integrity of the filtration barrier. Glomerular podocytes play a key role in establishing and maintaining this unique filtration barrier structure. Mature podocytes are characterized by cell cycle arrest, foot process formation, and the presence of the slit diaphragm,1 which bridges the gaps between the interdigitating foot processes of neighboring podocytes and functions as a size-selective filtration barrier.2,3 For their differentiation, as well as for the maintenance of their complex architecture, podocytes require the expression of several specific genes in a correct spatial and temporal fashion. This notion is supported by the identification of many mutations in podocyte-expressed genes as the underlying cause of inherited renal diseases.4 Moreover, recent studies from genetically modified mice

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Correspondence: Dr. Bing He, Division of Matrix Biology, Department of Medical Biochemistry and Biophysics, Karolinska Institute, 171 77 Stockholm, Sweden. Email: Bing.He@ki.se. Dr. Karl Tryggvason, Division of Matrix Biology, Department of Medical Biochemistry and Biophysics, Karolinska Institute, 171 77 Stockholm, Sweden. Email: Karl.Tryggvason@ki.se

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and the identification of genes responsible for human podocyte diseases have revealed a complex transcriptional network in podocytes critical for podocyte specification, differentiation, and contributing to renal disease pathogenesis. However, transcriptional regulatory mechanisms by which the transcription factors govern expression of their target genes in podocytes remain incompletely understood.

*NPHS2* was identified by positional cloning because its mutations cause familial or sporadic forms of steroid-resistant nephrotic syndrome. Podocin is a key component of the slit diaphragm, where it interacts with nephrin, NEPH1, and CD2AP. In contrast to many other podocyte genes, *NPHS2* is exclusively and constitutively expressed in podocytes. This likely reflects its unique function, and in particular implies the presence of a podocyte-specific enhancer. A putative enhancer element in *NPHS2* has been localized within a 2.5-kb DNA fragment upstream of its transcriptional start site, and it drives reporter gene expression in transgenic mouse podocytes.

We recently identified a zebrafish podocyte-specific enhancer element, which also lies within the 2.5-kb 5′ flanking region. However, the precise DNA-binding motifs in these regions and their potential interaction with specific transcription factors remain unknown.

Previous studies have shown that Lmx1b is essential for mouse *Nphs2* expression. Lmx1b is a LIM-homeobox transcription factor that controls dorsal-ventral limb patterning during vertebrate development. Mutations in human *LMX1B* cause nail-patella syndrome, which is characterized by skeletal abnormality, nail hypoplasia, and nephropathy. In mice, genetic ablation of *Lmx1b* leads to loss of *Nphs2* expression as well as loss of expression of the glomerular basement membrane (GBM) collagens Col4a3 and Col4a4, suggesting that Lmx1b potentially acts as a common upstream regulator of these genes through binding to the FLAT element, which also lies within the 2.5-kb 5′ flanking region.

Further, we genome-wide detected 26 genes carrying the LMX1b-FoxC motifs in their promoter regions. Among them, motif-driven podocyte enhancer activity of Lmx1b-FoxC motifs in their promoter regions. Among them, motif-driven podocyte enhancer activity of *CCNC* and *MEIS2* was functionally analyzed in vivo. The findings provide insights into the transcriptional regulatory mechanisms required for normal podocyte functions, and for the development of certain kidney diseases.

**RESULTS**

Identification of a Podocyte-Specific Enhancer by Analysis of Reporter Gene Expression in Zebrafish

Using a 2.5-kb zebrafish *nphs2* promoter fragment (Supplemental Figure 1A), we previously generated a Tg(podocin:GFP) zebrafish line, in which green fluorescence protein (GFP) is exclusively expressed in podocytes. To fine-map this 5′ sequence by *in vivo* experiments, we first compared the GFP expression patterns driven by this promoter between injected G0 and Tg(podocin:GFP) G1 embryos. The two types of embryos exhibited similar podocyte-specific GFP expression pattern at 4 days postfertilization (dpf), despite the expected mosaic expression in G0 embryos (Supplemental Figure 1B). The robust expression was observed in 18% ± 4% of G0 embryos, verified by three independent injections. Thus, we found it plausible to use G0 transgenic zebrafish for a rapid fine-mapping of the promoter fragment.

Further analysis of the 2.5-kb promoter fragment revealed that deletions from −2.5 kb to an ApaI site situated at −1.0 kb preserved the podocyte specificity and frequency of GFP expression (Figure 1A). However, further deletion to a KpnI site situated at −185 bp increased the expression rate from 18% to 33% and also induced a low but significant frequency (5%–10%) of ectopic GFP expression in extrarenal tissues (Figure 1A). This expression pattern was preserved upon further deletion to −110 bp (Figure 1A), suggesting that a podocyte-specific enhancer element resides within 110 bp upstream of the transcription start site. Within the 110-bp sequence, we observed
two adjacent DNA-binding sites: a 5′ located FLAT-E element (TAATTA) and a 3′ located forkhead-binding site (ATAAACA) separated by seven nucleotides (Figure 1B). To test the cis-acting potential, we coupled the zebrafish motif element between 2110 and 250 bp to a mouse c-fos minimal promoter and analyzed GFP expression in G0 embryos (Figure 1C). This resulted in glomerular GFP expression in 25% of embryos, whereas no glomerular GFP expression was observed in 211 control embryos (Figure 1C), suggesting that the 49-bp element containing a FLAT-E/forkhead motif constitutes a podocyte-specific enhancer sufficient to direct GFP expression in podocytes independent of the native nphs2 minimal promoter.

**FLAT-E/forkhead Motif Defines the Enhancer Required for Podocyte-Specific Expression**

By analyzing the 5-kb Nphs2 promoter with a complete sequence in all vertebrates available, the motif with two adjacent binding sites is present in 90% of analyzed species (28 of 31 species) and the motif with 5-bp or 7-bp spacing between two sites accounts for 75% of 28 species (Supplemental Table 1), suggesting that the binding motif is highly conserved. To characterize the motif, we generated mutations targeting the FLAT-E and forkhead sites, respectively. A single mutation (A→C) in the FLAT-E element almost completely abolished expression (reduced to 3%; P<0.001) in comparison with the wild-type (32%). A point mutation at the forkhead site (A→G) likewise resulted in a significantly decreased expression frequency (11%; P<0.001) (Figure 2A). Further deletion of the entire forkhead site (A→G) likewise resulted in a significantly decreased expression frequency (11%; P<0.001) (Figure 2A). Further deletion of the entire forkhead site led to a complete loss of glomerular expression (Figure 2B), suggesting that the two sites are both required for podocyte expression.

It is known that Lmx1b binds to the FLAT elements.21 Moreover, Lmx1b and Foxc2 are critical for the Nphs2 expression.14,19,24 Therefore, we hypothesized that the two proteins bind to the FLAT-E/forkhead motif with a synergistic effect on Nphs2 transcription. In zebrafish, no Foxc2 homolog exists. Instead, two foxc1 paralogs (foxc1a, b) have been identified, of which only foxc1a is expressed in the pronephros.29 We next performed EMSA to test binding potentials of the two proteins to the zebrafish promoter element with two putative binding sites marked in red between 2185 and 262 bp was subcloned in the Tol2-cfos-GFP plasmid (zpmotif-cfos-GFP) and is schematically illustrated. The mouse c-fos minimal promoter (arrow) in the plasmid is indicated. The empty plasmid was used as a control. GFP expression rate described above is shown. Microscopic imaging laterally and dorsally shows GFP expression in G0 glomerulus (arrow). GFP is also visible in other tissues.
indicated in lowercase letters. GFP expression rate and number of G0 embryos in LMX1B with zebrafish unlabeled mutant probes with different amounts (Figure 4). Unlabeled wild-type probes with different amounts, but not by unlabeled mutant probes (lanes 6–8, lanes 13–15 in Lmx1b panel; lanes 5–7 in panels for foxc1a, Foxc1, and Foxc2).

Figure 2. Characterization of two putative DNA-binding sites bound by Lmx1b and FoxC proteins. (A) Mutagenesis analysis of two putative DNA-binding sites. Two point-mutation carrying plasmids and their wild-type (ZP-0.2k) are illustrated. The putative binding sites are marked in bold, and the mutation at the −87 or −73 position is indicated in lowercase letters. GFP expression rate and number of G0 embryos in parentheses are shown. (B) Deletion of the forkhead-binding site. The sequence between −185 and −77 bp, in which the forkhead-binding site was completely deleted, was subcloned in the Tol2-based cfos-GFP plasmid (see Figure 1C). GFP expression rate controlled by the 15-bp deletion plasmid and its wild-type (zpmotif-cfos-GFP) are displayed. (C) EMSA. Radiolabeled oligonucleotide probes (wt) encompassing the forkhead-binding sites and recombinant Lmx1b and FoxC proteins were used in EMSA. The mutations of the mutant probe (mu) are identical to that used in mutagenesis assay, shown in part A. An efficient binding of zebrafish Lmx1b.1 and human LMX1B to the FLAT-E site was observed (lanes 2, 9 in Lmx1b panel). Zebrafish foxc1a strongly bound to the forkhead-binding site (lane 1 in foxc1a panel). The binding of Foxc1 and Foxc2 was notably weaker than zebrafish foxc1a (lanes 1 in panels for Foxc1 and Foxc2). To test binding specificity, 12.5-fold, 25-fold, and 37.5-fold excess unlabeled wild-type probes or mutant probes, indicated with gradients from low to high, were used for competition assays. Labeled probes were efficiently competed by unlabeled wild-type probes with elevated amounts for five tested proteins (lanes 3–5, lanes 10–12 in Lmx1b panel; lanes 2–4 in panels for foxc1a, Foxc1, and Foxc2), but not by unlabeled mutant probes (lanes 6–8, lanes 13–15 in Lmx1b panel; lanes 5–7 in panels for foxc1a, Foxc1, and Foxc2).

unlabeled wild-type probes with different amounts, but not by the unlabeled mutant probes with different amounts (Figure 2C). High homology (84% identity and 89% similarity) of LMX1B with zebrafish lmx1b.130 is in agreement with sufficient binding of LMX1B to the zebrafish enhancer. Although repeated attempts with different methods and conditions were made, we could not detect a tertiary band that indicates co-binding of two proteins to the probe at the same time by a conventional EMSA in vitro.

Lmx1b and Foxc1a Are Both Required for Nphs2 Expression and Slit Diaphragm Formation in Zebrafish

To determine whether two proteins are required for nphs2 expression in vivo, we knocked down lmx1b.1, lmx1b.2, foxc1a and foxc1b by injection of morpholinos (MOs) in Tg(podocin:GFP) embryos. Pericardial edema, associated with pronephric kidney dysfunctions,31,32 and glomerular GFP loss in 4-dpf embryos were used to evaluate the kidney phenotypic consequences of the knockdowns (Figure 3A). Double knockdown of lmx1b.1 and lmx1b.2 led to kidney phenotype in 90% of morphants. Single knockdown of lmx1b.1 resulted in a phenotype that was almost as severe as the double knockdown, whereas single knockdown of lmx1b.2 resulted in a less penetrant, but qualitatively similar, phenotype. These results suggest nonredundant but overlapping roles of lmx1b.1 and lmx1b.2. Double knockdown of foxc1a and foxc1b also led to severe phenotype, but so did single foxc1a knockdown (89% and 85%, respectively). Foxc1b knockdown lacked these effects. To test a combinatorial requirement of the two proteins for nphs2 expression in vivo, we first determined subphenotypic doses of MOs for individual knockdown. Individual injection of 0.25 ng lmx1b.1-MO or 1 ng foxc1a-MO led to no or little phenotype (Figure 3B). Combinatorial knockdown of lmx1b.1 and foxc1a with the same doses of MOs resulted in severe kidney phenotype in 40% of morphants (Figure 3B).

Glomerular ultrastructure of lmx1b.1 and foxc1a morphants exhibit significant developmental defects in podocytes as well as capillary endothelium, which disrupt the glomerular filtration barrier integrity, resulting in the phenotype observed (Figure 4). At low magnification, defects in the lmx1b.1 morphant appear to be podocyte-restricted. However, the foxc1a morphant shows severe defects in developing podocytes and migration of glomerular capillary endothelial cells, although the podocyte specification already occurs (Figure 4, A, C, and E). At higher magnification, two morphants showed a common defect in glomeruli: aberrant or flattened podocyte foot processes and a complete loss of the slit diaphragm in comparison to wild-type (Figure 4, B, D, and F). This is compatible with the known role of podocin in podocyte foot process formation.31,33,34 Taking
Figure 3. Lmx1b and FoxC are both required for *nphs2* expression in zebrafish. (A) MO-mediated knockdown of *lmx1b* and *foxc1a* genes. Microscopic images of 4-dpf morphants of individual or double knockdown as well as controls are displayed. Pericardial edema is indicated (arrow) in bright-field imaging, and glomerular GFP expression is indicated (arrowhead) in dark-field imaging. The percentage of pericardial edema is shown. Number of morphants is indicated in parentheses. (B) Combinatorial knockdown of *lmx1b.1* and *foxc1a* genes. A low dose of *lmx1b.1*-MO (0.25 ng) or *foxc1a*-MO (1 ng) was individually injected in *Tg(podocin:GFP)* embryos. Glomerular GFP expression is indicated (arrowhead) in dark-field imaging. For double knockdown, 0.25 ng *lmx1b.1*-MO and 1 ng *foxc1a*-MO were coinjected in embryos. The percentage of pericardial edema in 4-dpf morphants is shown. Number of morphants is indicated in parentheses.

Together, *lmx1b* and *foxc1a* are both required for *nphs2* expression through a combinatorial action on the enhancer and the formation of a functional pronephric kidney.

**Lmx1b.1** and **Foxc1a** Synergistically Induce *nphs2* Expression in Zebrafish

To test whether coexpression of *lmx1b* and *foxc1a* could induce endogenous and/or ectopic *nphs2* expression in *in vivo*, we generated the transgene of *lmx1b.1* and *foxc1a* in zebrafish G9 embryos (see Concise Methods). To visualize potential ectopic GFP expression, the *Tg(podocin:GFP)* embryos were used for injection. At 1 dpf, quantitative PCR showed no significant difference of *nphs2* expression between any of the experimental situations, although *lmx1b.1* and *foxc1a* were strongly overexpressed (17- to 124-fold) individually or in combination (Figure 5). At 2 dpf, however, co-overexpression (12- to 27-fold) of the combined *lmx1b.1* and *foxc1a* potently induced *nphs2* expression, whereas overexpression (7- to 36-fold) of the single transcription factor lacked effect (Figure 5). The results provide additional evidence for that *lmx1b* and *foxc1a* combinatorially regulate *nphs2* expression in zebrafish podocytes. In addition, we did not observe notable ectopic expression, implying that a unique cellular environment in the podocytes also plays a role in *nphs2* expression.

**Lmx1b-FoxC Motif Is Present in Human NPHS2 and Many Other Genes**

By conservation analysis (Supplemental Table 1), we found that the binding motif is also present in the human *NPHS2* proximate promoter, suggesting a similar regulatory mechanism controlling its expression by mammalian orthologs Lmx1b and FoxC2. Thus, we evaluated its binding potentials by EMSA. LMX1B and FoxC2 potently and specifically bound to the FLAT-F site and the forkhead site, respectively (Figure 6A). The bindings were specific because the binding signals were completely competed out by unlabeled wild-type probes with three different titrations, but not by unlabeled mutant probes. We observed that the binding by Foxc1 was significantly weaker than that by Foxc2 (Figure 6A).

We then tested cis-acting potentials of the *NPHS2* motif using the transgenic zebrafish *in vivo*. For this experiment, the *NPHS2* element containing the putative motif was subcloned in the Tol2-based *cfos-GFP* plasmid (Figure 6B) and was injected in wild-type embryos. The *NPHS2* motif drove glomerular GFP expression in 5.5% (11 of 200) of embryos, compared with no glomerular expression in controls injected with an empty *cfos-GFP* plasmid (0 of 211). A low homology (48% identity) between FoxC2 and zebrafish foxc1a may lead to this low expression rate. As shown in Figure 6B, glomerular expression driven by the *NPHS2* motif element was validated through their location and appearance. Together, the motif is likely to be a cis-acting element controlling podocyte expression of *NPHS2*.

It is interesting to know whether the identified motifs could predict novel genes coexpressed with *NPHS2* in podocytes. On the basis of the motif consensus sequences (Figure 7A), we detected 26 candidates genome-wide that carry the motifs in their promoter regions in addition to *NPHS2* (Table 1). Among them, glomerular expression levels of *CCNC*, which encodes the cyclin C protein, and *MEIS2*, which encodes the Meis homeobox 2 protein, were downregulated almost 3-fold in *MEIS2* knockout mice, suggesting that their expression is regulated by Foxc2. Thus, we tested cis-acting potentials of these two genes. To verify podocyte expression for the two predicted podocyte genes, we reconstructed Tol2-based plasmids, where the CCNC and MEIS2 motifs were coupled with the mCherry
cDNA, and then injected them in the Tg(podocin:GFP) embryos. Colocalization of mCherry (red) with glomerular GFP (green) analyzed using confocal microscopy could provide direct evidence for podocyte expression. In addition, we also examined whether the proteins are present in mouse glomeruli. The CCNC element drove glomerular mCherry expression in 7% (7 of 100) of embryos. Similar to the positive control for glomerular colocalization (Figure 7B, upper panel), mosaic mCherry expression driven by the CCNC motif was co-localized with glomerular GFP expression (Figure 7B, middle panel). The MEIS2 motif also drove mCherry expression in 5.4% (6 of 110) of injected embryos, which was colocalized with glomerular GFP (Figure 7B, lower panel).

Furthermore, immunostaining of mouse kidney sections showed that cyclin C was present in glomeruli, and was mainly localized in cytoplasm (Figure 7C). Confocal images showed partial overlap (arrow) between cyclin C and nephrin, suggesting its expression in podocytes as well as other cells such as mesangial cells and endothelial cells (Figure 7C). MEIS2 shows strong nuclear expression in human glomeruli (http://www.proteinatlas.org). This antibody did not work in our immunofluorescence staining on mouse kidney sections. Glomerular expression of mouse Meis2 was, however, clearly detected by Western blotting (Figure 7D).

We further asked whether a low rate of glomerular reporter expression driven by the human motifs in zebrafish could reflect real podocyte enhancer activity. To answer this question, we knocked down zebrafish ccnc and tested whether the phenotype generated by MOs was related to podocyte defects. As shown in Figure 8A, injection of MOs, targeting two different regions of ccnc, in Tg(podocin:GFP) embryos resulted in severe pericardial edema and glomerular GFP loss (Figure 8, A and C). The phenotype was significantly rescued by co-injection of heterologous mouse mRNA (P<0.001) (Figure 8C). Glomerular ultrastructure of ccnc morphants displayed widespread effacement of podocyte foot processes and lack of the slit diaphragm (Figure 8D). Together, the observed phenotype caused by ccnc MOs suggests that the

Figure 4. Glomerular ultrastructures in lmx1b.1 and foxc1a morphants at 4 dpf. At low magnification, whole glomeruli (arrow) are exhibited (A, C, and E). Compared with wild-type (A), capillary lumens in the lmx1b.1 morphant are abnormally enlarged (C), and the foxc1a morphant shows that two glomeruli fail to merge at the midline and glomerular capillaries are missing (E). Detailed morphology of the glomerular filtration barrier is displayed at higher magnification (B, D, and F). In wild-type, fine foot processes, slit diaphragm (arrowhead) and fenestrated endothelium together with GBM are clearly visible (B). The lmx1b.1 morphant displays typical effacement (arrowhead), lack of endothelial fenestration, and a complete loss of the slit diaphragm (D). The foxc1a morphant shows aberrant podocyte foot processes on disorganized GBM and absence of endothelial cells. Normal cell-cell junctions by the slit diaphragm disappear and instead typical adherens junctions (arrowhead) occur between adjacent podocytes (F). cap, capillary; DA, dorsal aorta; E, endothelium; NC, notochord; P, podocyte. Magnification is shown by scale bars 10 μm and 500 nm.
DISCUSSION

Expression of NPHS2 is highly restricted to podocytes and is constantly active once it is activated during the podocyte development.7 Here, we show that podocyte-specific expression of NPHS2 or zebrafish nphs2 is combinatorially regulated by Lmx1b and FoxC through binding to two adjacent DNA-binding sites. We designate this cis-acting motif as the Lmx1b-FoxC enhancer, in which the forkhead site is bound by FoxC2 in mammals and foxc1a in zebrafish. This enhancer is also present in many other genes.

Combinatorial control represents an important gene regulatory mechanism in which multiple transcription factors come together to exert specific transcriptional control.35 This regulatory manner has been hypothesized for some tissue-restricted expression in multicellular organisms. A recent study revealed that transcriptional regulation of endothelium-specific gene expression follows this principle, and specifically that FoxC and Ets transcription factors cooperate by interacting with neighboring binding sites in a FOX:ETS enhancer present in several endothelial-specific genes.36 Accordingly, this principle is likely to apply to a portion of podocyte-specific gene expression. Thus, NPHS2 may represent an ideal gene for test because of its unique expression pattern. Using in vivo zebrafish models, we identified a 49-bp podocyte-specific enhancer that contains two adjacent DNA-binding sites, potentially bound by Lmx1b and FoxC. To claim combinatorial control, several basic points should be addressed: the DNA binding sites of the enhancers, coexpression of transcription factors required in a target cell, and cooperativity of protein-protein as well as protein-DNA. For the binding site, we found that the putative binding motif within the zebrafish nphs2 proximal promoter is highly conserved across species, implying a functional potential. Interestingly, 5- or 7-bp spacing between two sites represents a major form. The spacing in the motif may be critical in configuration of the DNA-protein complex. The DNA double-helical periodicity is 10.4 bp per turn,37 and therefore the interaction between two transcription factors may or may not be possible depending on what side of the double helix they bind. By in vitro EMSA and in vivo mutagenesis analyses, we demonstrate that the two adjacent DNA-binding sites are specifically bound by Lmx1b and FoxC and are both required for the activation of transcription, providing molecular basis for the combinatorial binding by two proteins. At the structure level, DNA binding of multiple proteins may lead to various alterations in protein structure, including the formation of additional secondary structural elements, reorientation of loops, rearrangements of hydrophobic cores, and changes of their quaternary structure.35 These alterations in the stereo-specific complex may highly depend on the cellular microenvironment, and detecting the co-binding of two proteins to DNA as a complex by a conventional EMSA in vitro is likely to be difficult.

Lmx1b and FoxC2 are both expressed in mammalian podocytes,14,15,24 In frog glomerulus, foxc2 is one of the earliest expressed transcription factors at stage 20, and lmx1b and nphs2 simultaneously appear at stage 27.38 In zebrafish, foxc1a expression appears in podocyte progenitors at 8-somite stage, while nphs2 expresses in mature podocytes at 1.5 dpf.39 In agreement with data from mouse knockout studies,14,15 our zebrafish data support that lmx1b.1 is essential for nphs2 expression because the ultrastructural alteration by lmx1b.1 knockdown is compatible with the observed pronephros dysfunction and with the known role of podocin in podocyte foot process formation.31,33,34 The role of foxc1a in developing
glomeruli seems to be more complex. Our data show that fox1a knockdown is unlikely to affect the podocyte specification; instead it significantly disrupts podocyte differentiation and recruitment of endothelial cells, suggesting that fox1a plays a role in cross-talking between two types of cells for functional glomerulus formation in addition to regulating the podocyte development and function. Through a genome-wide search for the motifs in the promoter regions throughout all human genes, we identified 26 novel podocyte-expressed genes, but, to our knowledge, none of them have been previously demonstrated to play a functional role in podocytes. Therefore, we are cautious in characterizing the predicted data. To validate podocyte expression of these predicted genes, we used two evaluating assays: (1) in vivo reporter expression in zebrafish and (2) endogenous expression in mouse glomeruli, preferably using double immunofluorescence staining. Two selected genes—CCNC and MEIS2—for follow-up analysis met the criteria. Podocyte expression of CCNC was further supported by the zebrafish ccnc knockdown, demonstrating that CCNC is required for podocyte development and normal podocyte functions. Together, the Lmx1b-FoxC motif may be an efficient predictor to identify novel podocyte-expressed genes, although they are not necessarily podocyte-specific. It is interesting how a member of the cyclin protein family that is
involved in regulating cell cycle progression plays an important role in terminally differentiated podocytes. Cyclins function through activating their partners, cyclin-dependent kinases. The role of cyclin C in the cell cycle is unclear.43 Thus far, Cdk8 and Cdk3 are the only known kinases associated with cyclin C.44,45

Podocytes rapidly lose their characteristic and specific protein expression pattern when cultured in vitro.1 To evaluate cis-regulatory elements controlling podocyte gene expression, we therefore reasoned that in vivo models are likely required. In this study, we validate that G0 embryos are suitable for rapid evaluation of potential cis-acting elements in vivo. Interestingly, the human Lmx1b-FoxC motif can be activated in zebrafish podocytes, albeit at a relatively low expression rate. Thus, this system may be useful in identifying other cell type-specific enhancers.

In summary, the complex architecture and unique function of podocytes apparently require the cooperation of common transcription factors to achieve specific podocyte gene expression. We show that through binding to a unique DNA motif, Lmx1b and FoxC combinatorially regulate gene expression in podocytes. Our findings also indicate novel podocyte genes coexpressed with Nphs2. Our results provide insights into the transcriptional regulatory mechanisms required for normal podocyte functions and likely also important for renal diseases.

CONCISE METHODS

Zebrafish
The wild-type AB zebrafish and the Tg(podocin: GFP) line13 were maintained at the Karolinska Institute zebrafish core facility.

Transgenic Zebrafish
To generate transgenic zebrafish, we used the Tol2 transposon-mediated transgenesis in two distinct bands (arrows), one with approximately 52-kD (predicted molecular mass, 52 kD) and one with about 110 kD, in mouse whole glomerular lysates (Glom). In nuclear extract of HEK293 cells, only a single strong band with about 110 kD was detected.
zebrafish as described.46 The Tol2 transposon-based plasmids contain two Tol2 transposable sequences flanking the insertion fragment sites, and they were comicroinjected, together with the Tol2 transposase mRNA, into one- to two-cell zebrafish embryos. The translated transposase protein from injected mRNA in fertilized eggs catalyzes excision of the Tol2 transposable elements from the plasmid, leading to stable integration of the excised DNA fragments in the genome. Generally, the insertion fragment consists of a promoter and a downstream reporter gene such as GFP or any cDNA sequences to be expressed in zebrafish. In this study, we analyzed glomerular expression of GFP or mCherry in injected G0 embryos at 4 dpf as a rapid in vivo expression system. We showed that despite its mosaic pattern, this transient expression assay accurately reflects expression of germline zebrafish (see Results and Supplemental Figure 1). The Ethics Committee of Karolinska Institute has approved the experiments using the transgenic manipulation in zebrafish.

### Table 1. Candidate podocyte genes identified by a genome-wide search of the Lmx1b-FoxC motif in human and mouse genomes

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**Morpholino-Mediated Knockdown**

MOs were used to knock down zebrafish genes lmx1b.1, lmx1b.2, foxc1a, foxc1b, and ccnc. The sequences of these MOs used are shown in Supplemental Table 2. An unrelated standard control MO (control-MO), provided by the manufacturer, was used as a negative control. In addition, a mismatch lmx1b.1 MO was also used as a gene-specific control. About 4–6 ng of MOs were normally injected into the yolk of one- or two-cell Tg(podocin:GFP) embryos. For knockdown of zebrafish ccnc gene, a translation-blocking MO (ATG-ccnc-MO) and a splice-blocking MO (E3I3-ccnc-MO) were used. The splice-blocking efficacy by the E3I3-ccnc-MO was evaluated by using RT-PCR. As shown in Figure 8B, an in-frame deletion of exon 3 caused by this MO at 4 dpf was detected as a decrease in amplicon size (448 bp) compared with wild-type (533 bp). For the mRNA rescue, mouse ccnc mRNA (400 pg), in vitro transcribed from a mouse full-length ccnc cDNA plasmid (Origene) using the mMESSAGE mMACHINE kit (Ambion), was co-injected with MOs. Embryos injected with 4–6 ng control-MO and with 6 ng mismatch lmx1b.1-MO did not produce any discernable pericardial edema, similar to wild-type embryos.
digestions at naturally occurring restriction sites (Supplemental Figure 1A), followed by religations. PCR was also used to generate the plasmid carrying the shortest element (ZP-0.1k) between −110 and 63 bp. For the heterologous promoter analysis and the forkhead site deletion analysis, the elements between −185 and −62 and between −185 and −77 were generated by PCR and then subcloned upstream of the mouse c-fos minimal promoter in the Tol2-based pGW-cfos-GFP plasmid, provided by Dr. Andrew S. McCallion (Johns Hopkins University School of Medicine, Baltimore, MD). This plasmid, with some modification, such as replacement of GFP by mCherry, was also used to evaluate cis-acting potentials of putative enhancer motifs in CCNC and MEIS2 in vivo. In addition, a mCherry plasmid coupled with zebrafish nphs2 promoter (nphs2-mCherry) was used as a positive control for glomerular localization. To induce zebrafish endogenous nphs2 expression, full-length cDNA of zebrafish foxc1a and lmx1b.1 was amplified from cDNA of 4-dpf wild-type embryos and was constructed in the pT2KXIG plasmid, in which GFP sequence was replaced by the lmx1b.1-IRES-foxc1a cDNA fragment. This construct contains a Xenopus EF1α enhancer/promoter that strongly drives ubiquitous expression in zebrafish. A pcDNA plasmid encoding human LMX1B was provided by Dr. Brendan Lee (Baylor College of Medicine, Houston, TX). In addition, two pcDNA plasmids expressing mouse Foxc1 and Foxc2 were obtained elsewhere. The point mutations were generated using QuickChange II Site-Direct.

Figure 8. Morpholino-mediated knockdown of zebrafish ccnc gene. (A) Individual injection of two ccnc MOs, ATG-ccnc-MO and E3I3-ccnc-MO, in zebrafish embryos resulted in similar kidney phenotype (pericardial edema and glomerular GFP loss) at 4 dpf, compared with the control-MO. Pericardial edema (arrowhead) in bright-field imaging and glomerular GFP signal (arrow) in dark-field imaging are indicated, respectively. (B) RT-PCR demonstrated efficacy of ccnc knockdown generated by E3I3-ccnc-MO. At 4 dpf, compared with control mRNA showing a single 533-bp band, ccnc morphants appear as two bands (533 bp and 448 bp), indicating deletion of the 85-bp exon 3 generated by this MO. (C) The phenotype caused by ccnc MOs was significantly rescued by coinjection of mouse Ccnc mRNA (400 pg) (P < 0.001). The phenotype penetrance generated by MOs and mRNA rescue is illustrated by bar graphs. Phenotypic number of morphants and total number of morphants are indicated. (D) Glomerular ultrastructural analysis of ccnc morphants at 4 dpf. In the wild-type glomerulus, fine foot processes of podocytes (arrowhead), fenestrated endothelial cells and GBM are clearly visible (upper panel). At higher magnification (lower panel), the slit diaphragm (arrowhead) is visible. In contrast, podocytes in the morphants generated by injection of the ATG-ccnc-MO exhibit irregular, abnormally flattened podocyte foot processes (arrow in upper panel) and lack of the slit diaphragm (lower panel), suggesting diffuse podocyte effacement. Normal glomerular endothelium in ccnc morphants becomes invisible at higher magnification (lower panel). Compared with wild-type, GBM shows no notable difference (upper panel), capillary lumen; E, endothelium; P, podocyte; RBC, red blood cells. Magnification is shown by scale bars 2 µm and 500 nm.
Mutagenesis kit (Stratagen) according to the manufacturer’s instructions. The primer sequences used for cloning and mutagenesis are shown in Supplemental Table 3.

Quantitative PCR
Total RNA was isolated from a mixture of five whole embryos using the RNeasy Mini Kit (Qiagen). The first-strand cDNA synthesis was carried out using the iScript cDNA synthesis kit (Bio-Rad). Quantitative PCR was performed on the ABI PRISM 7300 Sequence Detection System using the TaqMan or SYBR Green method (Applied Biosystems). Triplicates for each sample were carried out. The relative quantification of gene expression was analyzed using the comparative threshold method. Data were presented as mean ± SEM 2^ΔΔCt.

Immunofluorescence Staining and Western Blotting
Kidneys from C57BL/6 mice were snap-frozen and embedded in optimal cutting temperature media. Cryosections (8 μm) were post-fixed with cold acetone for 10 minutes, followed by blocking in 5% normal goat or donkey serum. For immunofluorescence staining, the primary antibodies to cyclin C (1:100, ab2950; Abcam), MEIS2 (1:100, HPA003256; Sigma-Aldrich) and nephrin (1:200, guinea pig; Acris Antibodies GmbH) were incubated at 37°C for 1 hour, followed by 45-minute incubation with corresponding Alexa Fluor (Invitrogen) secondary antibodies. Western blotting was performed with standard procedures. Whole glomerular lysates isolated from adult C57BL/6 mice using Dynabead perfusion and nuclear extract of HEK293 cells were used. The primary antibody to MEIS2 (1:800, HPA003256; Sigma-Aldrich) was used. The local ethical committee (the North Stockholm district court) approved studies in mice.

EMSA
EMSA’s were performed as previously described. Recombinant proteins were in vitro synthesized using the TNT Quick Couple Transcription/Translation System (Promega) according to the manufacturer’s protocols. mRNA used for these protein synthesis was transcribed from plasmids expressing LMX1B, Lmx1b.1, Foxc1a, Foxc1, and Foxc2 using SP6 or T7 polymerase. Specificity of EMSA was evaluated by competition assay, where unlabeled wild-type or mutant probes in three titrations of 12.5-fold (0.25 μg), 25-fold (0.5 μg), and 37.5-fold (0.75 μg) excess were used as competitor to labeled wild-type probes. Oligonucleotide sequences used for EMSA are provided in Supplemental Table 4.

Confocal Imaging
For in vivo imaging of transgenic zebrafish glomerulus, confocal microscopy (Leica TCS SP8) was performed (magnification ×20). Larvae at 5 dpf were anesthetized by 0.1% tricaine and embedded in 1% low-melt agarose gel. Confocal imaging of mouse kidney immunostaining was performed using Zeiss LSM 700 (magnification ×63).

Transmission Electron Microscopy
Transmission electron microscopy was performed as previously described. Briefly, larvae were fixed in the fixation solution buffer (2% glutaraldehyde, 0.5% paraformaldehyde, 0.1 M cacodylate, 0.1 M sucrose, 3 mM CaCl2) and washed in 0.1 M cacodylate buffer (pH, 7.4) before staining in 2% OsO4 in cacodylate buffer for 1 hour at room temperature. Samples were dehydrated and en bloc staining was performed in 2% uranyl acetate in absolute ethanol for 1 hour at room temperature; samples were then taken through an Epon 812/acetone series and embedded at 60°C in pure Epon 812. Thin sections of 70 nm thickness were made on a Leica EM UC6 Ultratome and mounted on Formvar-coated copper slot grids. Poststaining was done with 2% aqueous acetate (pH, 3.5) and Venable and Coggleshall lead citrate. Grids were analyzed on an FEI TECNAI electron microscope.

Genome-Wide Identification of the Conserved Lmx1b-FoxC Motifs
The 5′ flanking DNA sequences within 10 kb in length upstream from the transcription start sites of all human and mouse genes were downloaded from the Ensembl databases (http://www.ensembl.org/downloads.html) and were scanned using the Lmx1b-FoxC motifs. The position weight matrix for the motif scanning, based on the two motif sequences from zebrafish and human with 5- or 7-bp spacing, was shown in Figure 7A. Resulting hits from the two genomes were further analyzed. Final candidates were determined if the hit was (1) present in two genomes and (2) protein-coding locus or noncoding locus with transcript evidence. Pseudogenes were excluded.

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DISCLOSURES
None.

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