

**Kidney filtration barrier components nephrin and Neph3:
transcriptional regulation and role in cell adhesion**

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ACADEMIC DISSERTATION

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“The first step in the journey is to lose your way.”
- Galway Kinnell

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals (I-IV):

- I Heikkilä E, Ristola M, Havana M, Jones N, Holthöfer H and Lehtonen S. (2011) *Trans*-interaction of nephrin and Neph1/Neph3 induces cell adhesion that associates with decreased tyrosine phosphorylation of nephrin. *Biochem J* 435 (3): 619-28.
- II Ristola M, Arpiainen S, Saleem MA, Mathieson PW, Welsh GI, Lehtonen S and Holthöfer H. (2009) Regulation of Neph3 gene in podocytes – key roles of transcription factors NF- κ B and Sp1. *BMC Mol Biol* 10: 83.
- III Ristola M, Arpiainen S, Saleem MA, Holthöfer H and Lehtonen S. (2012) Transcription of nephrin-Neph3 gene pair is synergistically activated by WT1 and NF- κ B and silenced by DNA methylation. *Nephrol Dial Transplant* 27 (5): 1737-45.
- IV Ristola M, Arpiainen S, Shimokawa T, Ra C, Saleem MA, Holthöfer H and Lehtonen S. Regulation of nephrin by the Ets factor, GA-binding protein. Submitted.

Publication I was included in the thesis of Eija Heikkilä (Molecular basis of the kidney filtration barrier: role of the nephrin protein complex, University of Helsinki 2010). All of the publications are reproduced with the permission of their copyright holders. In addition, some unpublished material is presented.

ABBREVIATIONS

ATRA	all- <i>trans</i> retinoic acid
bp	base pair(s)
CASK	calmodulin-associated serine/threonine kinase
CD2AP	CD2-associated protein
ChIP	chromatin immunoprecipitation
CNF	congenital nephrotic syndrome of the Finnish type
DBD	DNA binding domain
DMEM	Dulbecco's Modified Eagle's Medium
DNMT	DNA methyltransferase
EGFP	enhanced green fluorescent protein
GABP	GA-binding protein
GBM	glomerular basement membrane
Grb2	growth factor receptor-bound protein 2
H	histone
Ig	immunoglobulin
IKK	I κ B kinase
IL	interleukin
kb	kilo base pair(s)
MM	metanephric mesenchyme
mRNA	messenger ribonucleic acid
Nck1/2	non-catalytic region of tyrosine kinase adaptor protein 1/2
NF- κ B	nuclear factor- κ B
NPS	nail-patella syndrome
PA	puromycin aminonucleoside
PCR	polymerase chain reaction
PIC	preinitiation complex
PDZ	PSD-95/disc large/ZO-1
PHN	passive Heymann nephrosis
PI3K	phosphatidylinositol 3-kinase
Pol II	RNA polymerase II
PPAR	peroxisome proliferator-activated receptor
PPRE	PPAR response element
PTIP	Pax transactivation domain-interacting protein
qRT-PCR	quantitative reverse transcription-PCR
RAR(E)	retinoic acid receptor (element)
RXR	retinoid X receptor
SD	slit diaphragm
Sp1	specificity protein 1
TAD	transactivation domain
TF	transcription factor
TNF	tumour necrosis factor
TSS	transcription start site
UB	ureteric bud
VDR(E)	vitamin D receptor (element)
WT1	Wilms' tumour 1
ZO-1	zonula occludens 1

ABSTRACT

Glomerular epithelial cells, podocytes, and the specialized cell junctions termed slit diaphragms (SDs) between interdigitating foot processes of the podocytes form the essential components of the filtration barrier in the kidney glomerulus. Nephrin is a transmembrane immunoglobulin superfamily member and a crucial structural and signalling component of the SD. Mutations in the nephrin gene cause congenital nephrotic syndrome of the Finnish type (CNF), leading to disruption of the SD and leakage of plasma proteins into the urine. The Neph-protein family comprises Neph1, Neph2 and Neph3; these are nephrin homologues and also important components of the SD. Interactions between nephrin and Neph1-3 are suggested to play a role in cell adhesion and thus serve as a structural framework of the SD. This thesis investigated the interactions and cell adhesion activities of nephrin and Neph-family members and specifically focused on examining whether Neph3 has similar functions as nephrin, Neph1 and Neph2. Neph3 was shown to belong to the nephrin protein complex and to form homodimers, indicating that Neph3 shares similar binding properties with nephrin, Neph1 and Neph2. Both Neph1 and Neph3 were demonstrated to participate in the formation of cell-cell contacts by their homophilic interactions. Cell adhesion was also promoted by heterophilic *trans*-interactions between nephrin and Neph1 or Neph3. These adhesive activities may play an important role in the formation and function of the SD. In nephrin-deficient mice, where SDs are replaced by tight junction-like structures, Neph3 was shown to be up-regulated. This suggests that Neph3 may be involved in molecular mechanisms that are associated with morphological changes in injured podocytes.

Nephrin and Neph3 genes, *NPHS1* and *KIRREL2*, respectively, are located on human chromosome 19q13.12 in a head-to-head orientation separated by an approximately 5-kb intergenic region. A similar bidirectional arrangement of nephrin and Neph3 genes is present in mouse and rat chromosomes. The bidirectional and conserved arrangement of nephrin and Neph3 genes together with their similar protein structure and location suggest that nephrin and Neph3 genes may share key features in their regulation. This work focused on investigating transcriptional regulatory mechanisms and factors important for nephrin and Neph3 genes. Transcription factors WT1 and NF- κ B were shown to function cooperatively in both nephrin and Neph3 gene regulation. Further, DNA methylation participated in silencing both nephrin and Neph3 gene expression. These similar mechanisms in regulating transcription of nephrin and Neph3 genes may produce their similar spatial and temporal expression as well as similar function. In addition, transcription factors GABP and Sp1 were found to regulate nephrin and Neph3 genes, respectively.

The results of this thesis widen the current understanding of the role of nephrin and Neph-family members in the formation of the SD by showing that Neph3 belongs to the nephrin protein complex and that interactions of nephrin, Neph1 and Neph3 promote cell adhesion. Further, nephrin and Neph3 genes were demonstrated to share similar transcriptional regulatory mechanisms. This may improve understanding of the transcriptional regulation of podocytes in general.

1 REVIEW OF THE LITERATURE

1.1 Kidney – anatomy and function

Kidneys are paired bean-shaped organs located in the posterior abdominal cavity on each side of the vertebral column that are vital in maintaining normal body functions. They filter plasma to secrete metabolic waste products from the bloodstream into the urine and participate in the regulation of acid-base, electrolyte and fluid balance of the body. Kidneys also secrete hormones that regulate blood pressure and red blood cell formation. The functional unit of the kidney is a nephron, and each human kidney contains approximately one million nephrons. The nephron consists of the glomerulus, which is a capillary tuft surrounded by Bowman's capsule, and the tubular system comprising the proximal tubule, the loop of Henle and the distal tubule. Glomeruli form about 180 l of primary urine daily that is modified in the tubular system by effective reabsorption of valuable substances, such as water, electrolytes, glucose and amino acids, into the circulation (Tisher and Madsen, 1991).

1.2 Glomerular filtration barrier

The ultrafiltration of plasma in the glomerulus (Figure 1A) occurs through the glomerular filtration barrier (Figure 1B) between the capillary lumen and the urinary space. The barrier consists of three layers and filtration occurs in a size-, charge- and shape-selective manner. The fenestrated endothelium on the capillary loop side represents the initial barrier and lines the middle layer, the glomerular basement membrane (GBM). Highly differentiated glomerular epithelial cells, podocytes, in the urinary space form the final filter by their cell-cell contacts called slit diaphragms (SDs). In addition to these three layers, the intraglomerular mesangium located among the glomerular capillaries contributes to the regulation of ultrafiltration (reviewed by Haraldsson et al., 2008).

1.2.1 Podocytes and the slit diaphragm (SD)

Podocytes are polarized, highly differentiated epithelial cells with a unique architecture comprising a cell body and long, extending primary processes that divide further into thinner, numerous secondary foot processes. The podocyte cell body lies in the urinary space facing Bowman's capsule, while the foot processes line the outside of the glomerular capillaries. The basal side of the foot processes is anchored to the GBM via adhesion molecules, including $\alpha_3\beta_1$ -integrin and α - and β -dystroglycan (Figure 2) (Korhonen et al., 1990; Adler, 1992; Raats et al., 2000; Regele et al., 2000). The apical side of the podocyte foot processes faces the urinary space and possesses the heavily sialylated glycoprotein podocalyxin as one the main molecular components, giving a negative charge to the apical membrane (Figure 2) (Kerjaschki et al., 1984). The foot processes attached to the GBM are arranged in a highly organized manner so that the foot processes from adjacent podocytes

interdigitate and are separated by about a 40-nm-wide slit. A specialized cell junction termed slit diaphragm (SD), located between the basal and apical side of the podocyte foot processes, connects the interdigitating foot processes to each other (Figure 2) (Rodewald and Karnovsky, 1974). Based on electron microscopic examination, the SD has been described as a zipper-like structure with pores whose dimensions are almost the same as the size of albumin (70 kDa) (Rodewald and Karnovsky, 1974).

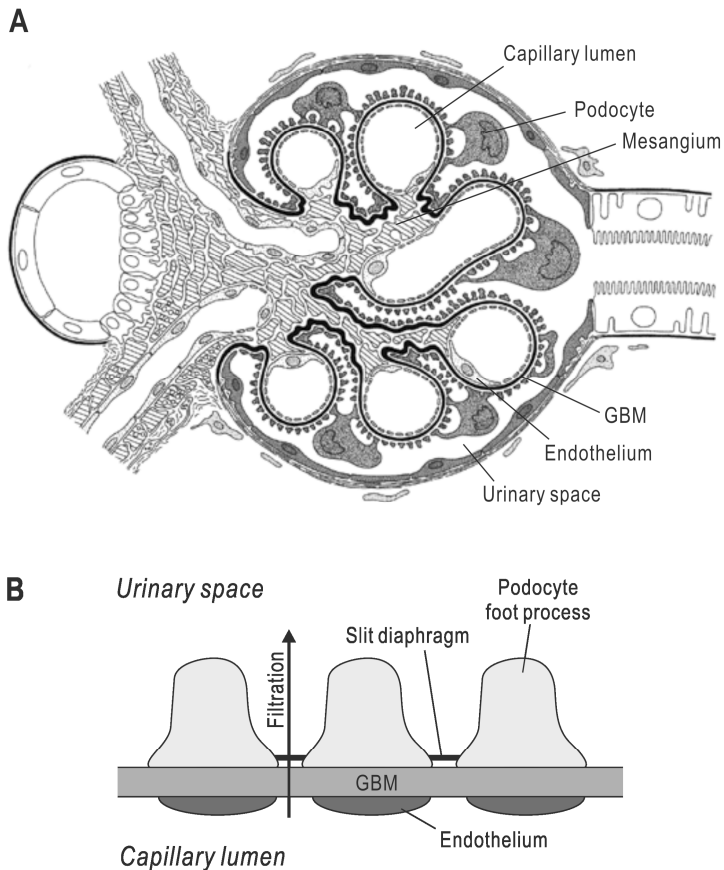


Figure 1. (A) Schematic structure of the glomerulus. Modified from Kriz et al. (1998). (B) Schematic representation of the glomerular filtration barrier. GBM, glomerular basement membrane.

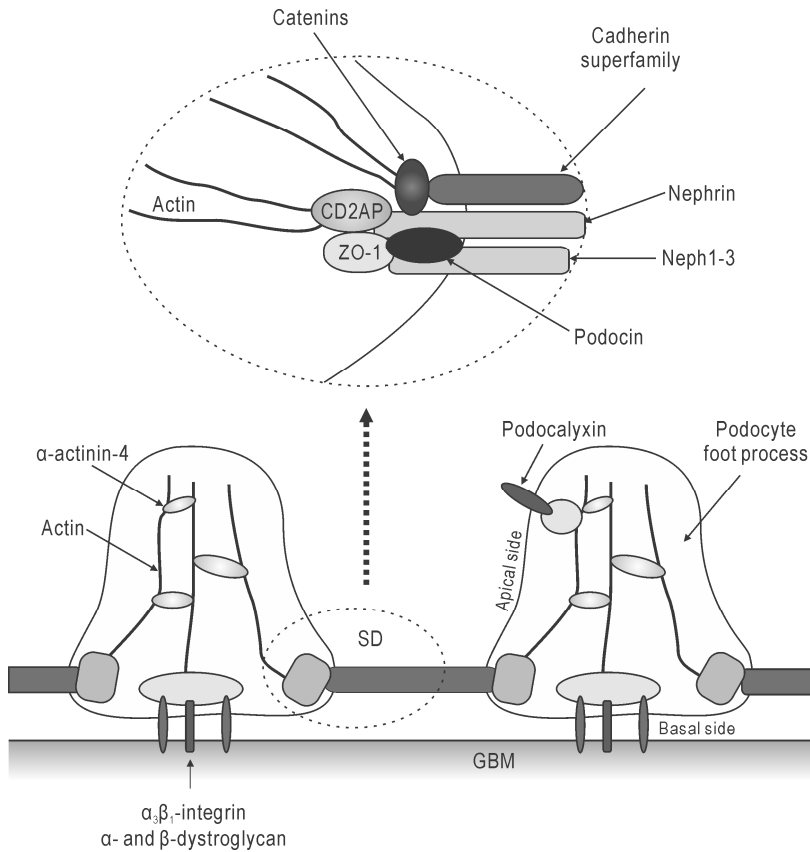


Figure 2. Simplified presentation of the molecular composition of the podocyte foot processes and the SD, indicating key proteins and protein families. Modified from Cohen et al. (2006). SD, slit diaphragm; GBM, glomerular basement membrane.

1.2.1.1 Composition and functions of the SD

The SD is a protein complex representing adhesion and signalling functions (Figure 2). The complex includes proteins that are common for most cell junctions, but also proteins that are not found in other junctions. Transmembrane proteins nephrin and Neph-protein family (Neph1-3) are suggested to form the framework of the SD (Section 1.3) (Ruotsalainen et al., 1999; Barletta et al., 2003; Gerke et al., 2003; Khoshnoodi et al., 2003; Liu et al., 2003; Wartiovaara et al., 2004; Gerke et al., 2005). In addition, the SD contains several scaffolding proteins often associated with tight junctions such as ZO-1 (Zonula occludens-1) (Schnabel et al., 1990), MAGI-1 (membrane-associated guanylate kinase inverted-1) (Hirabayashi et al., 2005), MAGI-2 (Lehtonen et al., 2005) and CASK (calmodulin-associated serine/threonine kinase) (Lehtonen et al., 2004). Proteins present in adherens junctions, including catenins (Reiser et al., 2000; Lehtonen et al., 2004) and cadherin

superfamily proteins P-cadherin (Reiser et al., 2000), VE-cadherin (Cohen et al., 2006) and FAT1 (Inoue et al., 2001) as well as tight junction proteins JAM-A (junctional adhesion molecule A), occludin and cingulin (Fukasawa et al., 2009), are also present in the SD. Based on its composition, the SD is considered a specialized adherens junction which harbours characteristics of both adherens and tight junctions (Schnabel et al., 1990; Reiser et al., 2000). Adherens junctions mediate cell-cell adhesion, whereas tight junctions help to maintain the polarity of cells and provide a cellular barrier for the passage of water, molecules and ions. Similarly, the SD functions as a bridge between neighbouring podocytes, polarizes podocytes, and also has a structural function as a molecular sieve.

The SD also represents a signalling platform that contributes to, for instance, the regulation of cytoskeletal organization, cell polarity and cell survival of podocytes (reviewed by Benzing, 2004). These signalling pathways are influenced by the action of certain SD proteins. Phosphorylation of nephrin and Neph1 is known to activate signalling pathways in podocytes (see Sections 1.3.1.2 and 1.3.2). Another SD protein, podocin, contributes to the intracellular signalling cascade by interacting with nephrin (Boute et al., 2000; Huber et al., 2003c; Li et al., 2004). An adaptor protein CD2AP (CD2-associated protein) in the podocyte cytoplasm is linked to activation of PI3K (phosphoinositide 3-kinase) and stimulation of a serine/threonine kinase AKT-dependent signalling (Li et al., 2000; Huber et al., 2003a). CD2AP, as well as ZO-1, functions also in linking the SD to the actin cytoskeleton (Fanning et al., 2002; Lehtonen et al., 2002; Huber et al., 2003b), and adaptor proteins Nck1 (non-catalytic region of tyrosine kinase adaptor protein 1) and Nck2 have an important function affecting the regulation of actin assembly in podocytes (Jones et al., 2006; Verma et al., 2006; Blasutig et al., 2008). Furthermore, cell polarity complex composed of Par3, Par6 and aPKC (atypical protein kinase C) has been located at the cytoplasmic side of the SD and shown to play a role in podocyte polarization and correct positioning of the SD (Hartleben et al., 2008; Hirose et al., 2009; Huber et al., 2009).

1.2.1.2 Podocyte cytoskeleton

The unique morphology of podocytes is influenced by a complex cytoskeleton. Microtubules and intermediate filaments dominate in the cell body and primary processes. Foot process architecture, in turn, is supported by a network of actin filaments cross-linked by α -actinin-4 (Figure 2) (reviewed by Pavenstadt et al., 2003). α -actinin-4 gene is mutated in a kidney disease called familial focal segmental glomerulosclerosis characterized by podocyte foot process effacement and proteinuria, emphasizing the role of the actin cytoskeleton in maintaining functional foot process architecture (Kaplan et al., 2000). The foot process cytoskeleton is linked by several adapter proteins to all three podocyte plasma membrane domains, the basal, lateral (SD) and apical domains, and is therefore responsible for, for instance, keeping podocytes attached to the GBM. The cytoskeleton also serves as a platform for transmitting signals from the extracellular environment and neighbouring podocytes, and provides mechanical stability and a high dynamic capacity to respond to physical stress (reviewed by Pavenstadt et al., 2003).

1.2.1.3 Nephrogenesis and development of podocytes and the SD

Mammalian kidney development contains three kidney structures: pronephros, mesonephros and metanephros. The development of metanephros, the permanent kidney, is a process that involves reciprocal interactions between the ureteric bud (UB) and the metanephric mesenchyme (MM). These interactions induce a series of events starting with the condensation of MM cells at the tips of the UB. The aggregated cells then begin to undergo mesenchyme-to-epithelial conversion and form renal vesicles. Renal vesicle stage is followed by comma-shaped and S-shaped stages. The S-shaped body fuses with the UB-derived collecting duct to form a capillary loop stage glomerulus that differentiates into a mature glomerulus (reviewed by Horster et al., 1997).

Podocyte precursors appear first at the S-shape body stage and are recognized by the expression of podocyte-specific markers such as transcription factor WT1 (Wilms' tumour 1) (Mundlos et al., 1993) and transmembrane proteins *Glepp1* (Sharif et al., 1998) and *nephrin* (Ruotsalainen et al., 2000). At this stage, the developing podocytes are columnar epithelial cells that are connected by apical junctional complexes composed of typical tight and adherens junction proteins (Schnabel et al., 1990). As podocytes differentiate, junctions migrate down the cell membrane towards the basal side of podocytes during the capillary loop stage, and the formation and interdigitation of the podocyte foot processes begins. During interdigitation podocyte cell bodies become independent of each other, but podocytes remain connected by tight junctions between the interdigitated foot processes (Figure 3). Tight junctions are replaced by the SDs in mature glomeruli (Reeves et al., 1978).

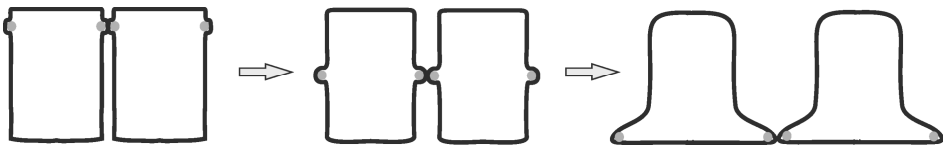


Figure 3. Schematic illustration of podocyte development from an S-shaped body stage to a mature glomerulus with respect to the junction translocation and foot process formation.

1.2.2 Glomerular basement membrane

The glomerular basement membrane (GBM) separates endothelial cells and podocytes. GBM is a tight, highly cross-linked meshwork of laminin, type IV collagen and nidogen. Heparan sulphate proteoglycans (HSPGs) bind to collagen and laminin, providing anionic charge to the GBM due to their negatively charged glycosaminoglycan side-chains. This has long been considered to be an important contributor to the charge-selectivity of the filtration barrier (reviewed by Haraldsson et al., 2008).

1.2.3 Endothelial cells

Glomerular endothelial cells line the interior surface of the glomerular capillaries and the luminal side of the GBM. The glomerular endothelium has numerous round or ovoid transcellular holes, fenestrations (70-100 nm in diameter in humans), that allow the passage of fluid across the cell layer. The luminal side of the endothelial cells and the fenestrae are covered by an endothelial cell surface layer consisting of a glycocalyx and an endothelial cell coat. The components of the endothelial cell surface layer, glycoproteins, glycosaminoglycans and proteoglycans, give a negative charge to the endothelial cells and are hypothesized to contribute to the charge-selective properties of the glomerular filtration barrier (reviewed by Haraldsson et al., 2008).

1.2.4 Mesangium

The mesangial cells and their surrounding matrix material constitute the mesangium, which is located between the capillary loops of the glomerular tuft. The main functions of the mesangium are to provide structural support for the glomerular capillaries and to participate in the regulation of glomerular filtration. Mesangial cells resemble smooth muscle cells having contractile properties. Cell contraction is stimulated by a variety of vasoactive agents. Mesangial cells also have phagocytic activity and thus may contribute to the clearance of debris from the filtration barrier (reviewed by Stockand and Sansom, 1998).

1.2.5 Glomerular filtration

High blood pressure within the glomerulus forces ultrafiltration of plasma through the filtration barrier. The selective function of the filtration barrier almost completely restricts the passage of albumin and larger plasma proteins as well as blood cells into the urine, while some proteins of smaller molecular size pass the filtration barrier freely. This is not, however, a problem since the majority of these proteins are returned back to the circulation by active reabsorption in the tubular system. Dysfunction of the glomerular filtration barrier leads to leakage of proteins that are normally kept in the circulation across the filtration barrier. This overloads the tubular reabsorption capacity and proteins pass into the collecting duct and subsequently into urine. This condition is called proteinuria and can be caused by several factors such as autoimmune diseases, local inflammation, toxic effects, metabolic products and genetic mutations (reviewed by Nielsen and Christensen, 2010).

It is generally accepted that filtration across the glomerular filtration barrier restricts the passage of solutes depending on their size, charge and shape. There have long been speculations about which of the three layers of the glomerular filtration barrier – endothelial cells, glomerular basement membrane or podocyte slit diaphragm – restricts the passage of proteins the most. However, it is now becoming increasingly apparent that the filtration barrier should be seen as a single unit where each layer makes a significant contribution to

the selective permeability. Each layer provides resistance to the movement of water and solutes across the barrier in a manner predicted by biophysical models. In addition, the structure and composition of each layer of the barrier bear a consequence for adjacent layers of the barrier through biophysical interactions affecting the manner and rate at which solutes and solvent enter and/or exit adjacent layers of the barrier (reviewed by Haraldsson et al., 2008).

1.3 Nephrin and Neph-protein family

Nephrin and the members of the Neph-protein family, Neph1, Neph2 and Neph3, are homologous molecules and crucial components of the SD. They are assumed to play key roles in cell-cell adhesion through their interactions, thus serving as a structural framework of the SD. Nephrin and Neph-family members also impact the signalling events in podocytes.

1.3.1 Nephrin

The nephrin gene *NPHS1* was identified in 1998 (Kestilä et al., 1998) and later the protein was localized to the SD (Holthöfer et al., 1999; Holzman et al., 1999; Ruotsalainen et al., 1999). The human nephrin gene *NPHS1* has 29 exons spanning a 26-kb area on chromosome 19q13.12 (Kestilä et al., 1998). *NPHS1* is mutated in the congenital nephrotic syndrome of the Finnish type (CNF), a serious renal disease described already in 1956 (Hallman et al., 1956; Kestilä et al., 1998). In mice, inactivation of the nephrin gene leads to morphological alterations, resembling findings in CNF patients (Putala et al., 2001; Rantanen et al., 2002), further demonstrating the importance of nephrin in the SD structure and function.

1.3.1.1 Congenital nephrotic syndrome of the Finnish type (CNF)

Congenital nephrotic syndrome of the Finnish type (CNF), an autosomal recessively inherited disease, occurs worldwide but is highly concentrated in the Finnish population. The incidence of CNF in Finland is approximately one in 8000 live births (Hallman et al., 1956; Norio, 1966). CNF is characterized by heavy proteinuria already *in utero*, oedema, premature birth and large placenta (Hallman et al., 1973; Huttunen et al., 1976). Macroscopically, the kidneys of CNF patients are 2-3 times larger than the kidneys of normal children, and microscopic findings include increased glomerular size and number (Tryggvason and Kouvalainen, 1975; Tryggvason, 1978; Autio-Harminen and Rapola, 1981). The most characteristic microscopic finding is effacement and fusion of podocyte foot processes. The number of the slits between podocytes is decreased, the width of the slits varies greatly and the typical zipper-like structure of the SD is lacking (Patrakka et al., 2000; Ruotsalainen et al., 2000; Lahdenkari et al., 2004). Historically, CNF patients have had a poor prognosis, with most of them dying during the neonatal period. During the last

25 years the only life-saving treatment has been dialysis and intravenous nutrition, followed by kidney transplantation in early childhood (reviewed by Holmberg et al., 1995).

CNF is caused by mutations in *NPHS1*, and, to date, over 100 mutations in *NPHS1* have been reported worldwide (www.biobase-international.com), most of them leading to deficiency of nephrin protein and severe disease. There are two main mutations among the Finnish population, Fin_{major} and Fin_{minor} , accounting for over 90% of the cases. Both mutations lead to a truncated nephrin protein; Fin_{major} is a two base pair frameshift deletion in exon 2, resulting in an early stop codon, and Fin_{minor} is a nonsense mutation in exon 26 (Kestilä et al., 1998). In contrast to the Finnish CNF patients, most non-Finnish patients have individual mutations. These include deletions and insertions and nonsense, missense and splicing mutations that are scattered along the whole gene (Lenkkeri et al., 1999; Beltcheva et al., 2001). In addition to mutations in the coding region of *NPHS1*, mutations in the regulatory region of the nephrin gene have been identified. Deletions of multiple nucleotides in a GA repeat region in the nephrin proximal promoter have been observed in several patients (Lenkkeri et al., 1999). Further, G to C substitution in the nephrin proximal promoter has been described in two independent studies (Gigante et al., 2002; Koziell et al., 2002).

1.3.1.2 Structure and functions of nephrin protein

Nephrin is a transmembrane protein and belongs to the immunoglobulin (Ig) superfamily. It has a short C-terminal intracellular domain, a single-span transmembrane region and an extracellular domain with eight Ig-like motifs and one fibronectin type III domain (Figure 4) (Kestilä et al., 1998). The Ig-like motifs are type C2, which is characteristic for proteins participating in cell-cell or cell-matrix interactions (reviewed by Chothia and Jones, 1997). Based on the structure of nephrin and its localization to the podocyte SD, nephrin molecules have been proposed to form a zipper-like structure, where extracellular domains of nephrin extend from neighbouring podocyte foot processes and interact with each other via the Ig-like domains in the centre of the SD (Ruotsalainen et al., 1999; Gerke et al., 2003; Khoshnoodi et al., 2003; Wartiovaara et al., 2004).

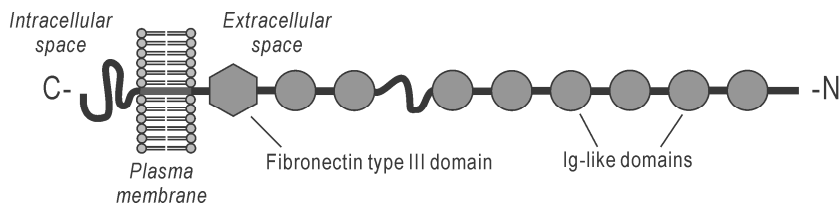


Figure 4. Domain structure of nephrin. Ig, immunoglobulin.

Besides being a structural component of the SD, nephrin also has signalling functions. This is enabled by the association of nephrin with specific microdomains of the cell membrane called lipid rafts (Simons et al., 2001) and the presence of tyrosine residues in the nephrin intracellular domain serving as a target for phosphorylation (Verma et al., 2003). Fyn, a member of the Src family kinases, has been shown to directly bind to the intracellular domain of nephrin and mediate nephrin phosphorylation (Lahdenperä et al., 2003; Verma et al., 2003). Fyn and nephrin phosphorylation seem to be important for the maintenance of the SD, as Fyn-deficient mice have foot process effacement and proteinuria (Yu et al., 2001; Verma et al., 2003). Tyrosine-phosphorylated nephrin creates docking sites for many proteins, and these interactions lead to a stimulation of various signalling events. For example, binding of p85, a regulatory subunit of PI3K, to the phosphorylated nephrin activates AKT and further inhibits podocyte apoptosis by regulating apoptosis-associated molecules (Huber et al., 2003a). Nephrin interaction with another SD protein podocin, leading to augmented nephrin-mediated signalling, is also enhanced by nephrin phosphorylation (Huber et al., 2001; Li et al., 2004). Nephrin phosphorylation is further linked to the pathways that lead to an elevation in cytosolic calcium levels (Harita et al., 2009) and raft-mediated endocytosis of nephrin (Qin et al., 2009). Association of Nck adaptor proteins with phosphorylated nephrin and cytoskeletal regulator proteins leads to actin cytoskeleton regulation (Jones et al., 2006; Verma et al., 2006). This seems to be important for foot process formation since deletion of Nck1/Nck2 in developing and adult podocytes in mice results in podocyte foot process effacement (Jones et al., 2006; Jones et al., 2009). Nephrin is suggested to participate in organizing the podocyte actin cytoskeleton also by interacting with actin-associating and actin-modifying proteins like CD2AP, CASK, IQGAP1 and α -actinin-4 (Lehtonen et al., 2002; Palmén et al., 2002; Lehtonen et al., 2004; Lehtonen et al., 2005).

1.3.1.3 *Nephrin expression and its splicing variants*

In the human kidney, nephrin is expressed only in the podocytes where it localizes at the SD (Kestilä et al., 1998; Ruotsalainen et al., 1999). Extensive studies of human and porcine foetal, newborn and infant tissues did not show nephrin expression outside the kidney (Kuusniemi et al., 2004), but other studies have reported nephrin expression in adult human pancreas and lymphoid tissue (Palmén et al., 2001; Zanone et al., 2005; Åström et al., 2006). In mice and rats, nephrin is expressed, in addition to podocytes (Kawachi et al., 2000; Putaala et al., 2000), also in the central nervous system (Putaala et al., 2000; Li et al., 2011), and in mice also in the pancreas, spleen, thymus and testis (Putaala et al., 2000; Liu et al., 2001). In addition to the full-length sequence, different splice variants of nephrin have been indentified; one in humans and four in rats. The human splice variant lacks the whole transmembrane domain encoded by exon 24, and the rat splice variants lack entirely or partly exons 24-27 (Ahola et al., 1999; Holthöfer et al., 1999). These splicing variants appear to have distinct tissue specificity. Mouse nephrin has an alternative transcription start site (TSS) located 1808 bp upstream from the previously detected first exon, and its expression is detected only in the central nervous system (Beltcheva et al., 2003). Further, in the mouse, the endogenous antisense mRNA of nephrin is expressed in the brain, lymphoid tissue and embryonic stem cells (Ihalmo et al., 2004).

1.3.2 Neph-protein family

The first Neph-family member Neph1 was identified in 2001 (Donoviel et al., 2001) and led to the establishment of the Neph-protein family when two other closely related proteins, Neph2 and Neph3, were identified two years later (Ihalmo et al., 2003; Sellin et al., 2003). Members of the Neph-protein family, similarly to nephrin, belong to the Ig superfamily and are transmembrane proteins composed of a short intracellular domain, a single-span transmembrane region and an extracellular domain with five Ig-like motifs (Figure 5) (Ihalmo et al., 2003; Sellin et al., 2003). Further, they all localize to the podocyte SD (Barletta et al., 2003; Liu et al., 2003; Gerke et al., 2005; Ihalmo et al., 2007). The phenotype of mice lacking Neph1 resembles that of nephrin-deficient mice, and these mice exhibit podocyte foot process effacement, develop proteinuria and die during the early postnatal period (Donoviel et al., 2001). Mice lacking Neph2- or Neph3 have not been created thus far. However, disruption of the functions of Neph2 and Neph3 has been achieved in zebrafish, whose pronephros is composed of the same cell types as found in the higher vertebrate kidney, including podocytes and SDs (Drummond et al., 1998). Disruption of Neph2 expression in zebrafish results in podocyte foot process effacement, lack of SDs and leaking of the glomerular filtration barrier (Neumann-Haefelin et al., 2010). A similar phenotype was noted when Neph1 expression was disrupted (Neumann-Haefelin et al., 2010). Disruption of Neph3 expression in zebrafish led to a distorted body curvature, transient pericardial oedema and altered pronephros morphology, suggesting that Neph3 is involved in the regulation of glomerular development (Wang et al., 2012).

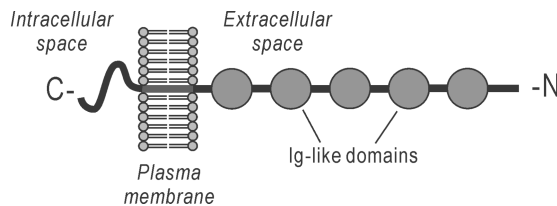


Figure 5. Domain structure of Neph1-3. Ig, immunoglobulin.

The members of the Neph-protein family are suggested to share many functional similarities with nephrin. Neph1 localizes to lipid rafts (Barletta et al., 2003), and therefore, similarly to nephrin, it may function as a signalling protein. All three Neph-family members have potential tyrosine phosphorylation sites, and highly conserved Grb2 (growth factor receptor-bound protein 2) and PDZ (PSD-95/disc large/ZO-1) -binding motifs in their intracellular domains (Sellin et al., 2003). Neph1 is tyrosine-phosphorylated by Fyn, enabling the interaction of Grb2 with Neph1 and leading to actin polymerization (Sellin et al., 2003; Garg et al., 2007). Phosphorylation of the Grb2 binding motif in Neph1 also regulates the interaction between Neph1 and podocin (Sellin et al., 2003). Moreover, the association of Neph1 with ZO-1 is mediated by the PDZ-binding motif and increases tyrosine phosphorylation of Neph1, further inducing signal transduction (Huber et al., 2003b).

Neph1 and Neph2 form homodimers and the extracellular domains of Neph1 and Neph2 have been shown to interact with nephrin. These interactions are mediated by the Ig-like motifs present in their extracellular domains (Barletta et al., 2003; Gerke et al., 2003; Liu et al., 2003; Gerke et al., 2005). Disruption of the nephrin-Neph1 interaction *in vivo* by injecting combinations of nephrin and Neph1 antibodies into the rat has proven the importance of the nephrin-Neph1 interaction. Injection results in proteinuria, but the podocyte foot processes are preserved. However, the expression of the intracellular binding partner of nephrin and Neph1, ZO-1, is dramatically reduced in antibody-injected animals (Liu et al., 2003). The interactions between Neph-family members and nephrin are proposed to play a role in the cell-cell adhesion in the SD and also to have an impact on the intracellular signal transduction machinery.

Interestingly, *KIRREL2*, the gene encoding Neph3, locates in close proximity to the nephrin gene *NPHS1* on human chromosome locus 19q13.12. The genes are arranged in a head-to-head orientation and separated by an approximately 5-kb intergenic region (Ihalmo et al., 2003). In mouse 7qB1 and rat 1q21 chromosomes, nephrin and Neph3 genes also have a bidirectional arrangement, but the genes are separated by about a 3-kb region. This arrangement implies that nephrin and Neph3 genes may share similar mechanisms in transcriptional regulation. Neph3 mRNA expression is down-regulated in human proteinuric diseases, and its expression level shows a positive correlation with nephrin mRNA in patients with diabetic nephropathy, suggesting common regulatory mechanisms controlling nephrin and Neph3 gene expression (Ihalmo et al., 2007). In the kidney, besides the podocyte SD, Neph3 expression has been detected in proximal and distal tubuli (Ihalmo et al., 2003). Outside the kidney, Neph3 is expressed in the human brain, pancreas and lymph nodes and also in the mouse central nervous system (Sun et al., 2003; Minaki et al., 2005; Rinta-Valkama et al., 2006). Different splicing variants with distinct tissue specificity have been suggested for Neph3. These represent forms of Neph3 that lack exon 2 or exon 4 or consist only of the sequence coding for the N-terminal extracellular part fused to the C-terminal intracellular part (Ihalmo et al., 2003, Sun et al., 2003).

1.3.3 Orthologues of nephrin and Neph-family members in *Drosophila* and *Caenorhabditis elegans*

Nephrin and Neph-family members are evolutionarily conserved, and a cell-cell recognition module composed of these proteins is also highly conserved across species (Neumann-Haefelin et al., 2010). In *Drosophila*, two nephrin orthologues, Hibris and Sticks-and-stones (Sns), and two orthologues of Neph-family members, irreC/Roughest (Rst) and Kin of irre (Kirre/Dumbfounded) have been identified (Bour et al., 2000; Ruiz-Gomez et al., 2000; Dworak et al., 2001; Strunkelberg et al., 2001). In *C. elegans*, the nephrin orthologue is SYG-2 and the orthologue of Neph-family members is SYG-1 (Shen and Bargmann, 2003; Shen et al., 2004). These proteins share similar functions in processes such as development of muscle and eye in *Drosophila* (Dworak et al., 2001; Bao and Cagan, 2005) and synapse development in *C. elegans* (Shen et al., 2004). The proteins are expressed in different cell types and promote junction formation and/or localization through their interactions.

During muscle development in *Drosophila*, heterophilic interaction between Hibris and Kin of irre mediates adhesion and fusion between myoblasts (Dworak et al., 2001). In pupal eye development, heterophilic interaction of Hibris and Rst promotes cell adhesion, which is required for proper patterning of the eye unit (Bao and Cagan, 2005; Carthew, 2007). In *C. elegans*, SYG-1 and SYG-2 are involved in synapse formation via their heterophilic interaction in *trans* (Shen and Bargmann, 2003; Shen et al., 2004; Wanner et al., 2011). Studies in *C. elegans* also show that all mammalian Neph-family members are able to drive synapse formation, suggesting a functional overlap between these three homologues (Neumann-Haefelin et al., 2010). Further, a highly conserved intracellular PDZ-binding motif of SYG-1, similarly to a PDZ-binding motif of mammalian Neph1-3, is suggested to be essential for its signalling functions and subsequent morphogenetic events (Neumann-Haefelin et al., 2010; Wanner et al., 2011).

Drosophila and *C. elegans* lack a glomerular filtration structure comparable with SD. However, studies investigating functions of nephrin and Neph-family members in these model organisms, even though in different settings, may contribute to the functional understanding of nephrin and Neph-family members in the SD.

1.4 Transcriptional regulation in podocytes

1.4.1 General aspects of transcriptional regulation

Transcriptional regulation is one of the steps in the regulation of gene expression. Gene expression covers the entire process from transcription to the synthesis of a protein that carries out the function specified by the gene. The regulation of gene expression can take place at any point in this pathway and the purpose of the regulation is to assure spatial and temporal expression of proteins enabling cells to respond to changing conditions and to undergo normal cell growth, development and differentiation. Specifically, regulation can occur at the levels of transcription and chromatin structure, which are responsible for producing precursor mRNA (pre-mRNA). Regulation at the post-transcriptional level includes conversion of pre-mRNA into mature mRNA by 5' and 3' processing and splicing, control of mRNA transport from nucleus into cytoplasm and stabilization or degradation of mRNA. Translational mechanisms control the production of protein from mRNA by ribosomes, and protein stabilizations and modifications such as glycosylation, phosphorylation, acetylation, methylation and disulphide bond formation constitute the post-translational levels of gene expression regulation (reviewed by Orphanides and Reinberg, 2002).

Transcription can be divided into four discrete stages: initiation, promoter clearance, elongation and termination. Although biologically significant regulation may occur at any stage in the process, most of the control is achieved by regulating the transcription initiation. Transcription initiation is thought to occur via binding of specific transcription factors to the regulatory elements in promoter regions of a given gene. This recruits the

general transcription factors and the RNA polymerase II (Pol II) to the core promoter, and Pol II can initiate the synthesis of mRNA. Afterwards, Pol II progresses to elongation to produce the nascent transcript by RNA Pol II elongation factors.

1.4.1.1 Transcription initiation complex

General transcription factors and RNA polymerase II

RNA polymerase II (Pol II) is responsible for all protein-encoding gene synthesis in eukaryotes. Pol II is capable of unwinding DNA, synthesizing RNA and rewinding DNA, but alone it is unable to recognize promoter DNA and initiate transcription. Instead, the general transcription factors, known as TFIIB, TFIID, TFIIE, TFIIH and TFII, mediate the binding of Pol II to DNA. The assembly of this transcription preinitiation complex (PIC) to the core promoter is initiated by association of specific general transcription factors with specific DNA elements, such as the TATA box or other sequences that specify the transcription start site (TSS), and occurs in a stepwise manner, orchestrated by protein-DNA and protein-protein interactions (Figure 6) (reviewed by Kornberg, 2007). Mediator, a large evolutionarily conserved complex consisting of approximately 25-30 subunits, also binds Pol II and general transcription factors (Figure 6), and is required for transcription of almost all Pol II promoters. Mediator is believed to act during assembly of the PIC. Mediator also provides important interaction sites for transcription factors (Figure 6), therefore transducing regulatory information from regulatory elements to basal transcription machinery on the core promoter (reviewed by Borggreffe and Yue, 2011).

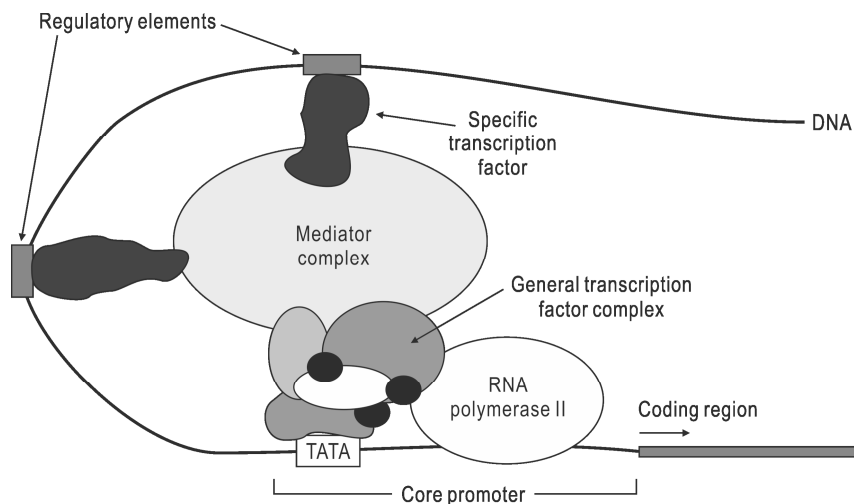


Figure 6. Schematic presentation of transcription initiation complex comprising RNA polymerase II, general and specific transcription factors and mediator complex. Modified from Kornberg (2007).

Specific transcription factors

Specific nucleotide sequences, regulatory elements, in the promoter DNA are bound by specific transcription factors that either enhance or reduce the level of transcription, confirming that the gene is transcribed at the right time and at the proper level in a particular cell type. DNA binding domain (DBD) of the transcription factors is responsible for mediating the interaction with DNA. There are various structures of DBDs, including helix-turn-helix, homeodomain, zinc finger and beta-scaffold, and these can be used to classify transcription factors (Pabo and Sauer, 1992; Stegmaier et al., 2004). The regulatory elements to which transcription factors bind are short sequences, usually about 10 bp in length. These binding sequences are occupied by only a few amino acids of transcription factors. Regulatory elements are generally located within several hundred base pairs upstream from the TSS of the gene, but they can also be found tens of thousands of base pairs away either upstream or downstream of the coding DNA region (reviewed by Farnham, 2009). Depending on where transcription factors bind in the promoter, they can contact several proteins within the basal transcription machinery directly or they can interact with the basal transcription machinery through the mediator, thus enhancing DNA looping (Figure 6).

Transcription factors co-operate with each other, and many transcription factors bind to DNA as homo- or heterodimers, increasing specificity and affinity for a cognate binding site. Most transcription factors are highly conserved among mammals, and they usually bind similar regulatory elements in different species. The conservation of regulatory elements between orthologous gene promoters may indicate their significant role in the transcriptional regulation of a certain gene (reviewed by Farnham, 2009). The activity of transcription factors can be regulated not only by transcription factor synthesis, but also in response to various extra- and intracellular signals. Growth factors, cytokines, presence or absence of metabolites, temperature, light, stress, oxidative stress, mechanical forces and xenobiotics, among others, can activate or inhibit the binding of transcription factor to the DNA or affect other characteristics of transcription factors to modify their activation state. The signalling can be mediated by a number of mechanisms, including protein modifications such as phosphorylation, presence of ligands and availability of transcription factors in the nucleus (Alberts, 2008). Transcription factors not only recruit the basal transcription machinery to the promoter but are also believed to stimulate transcription through interaction with chromatin-modifying activities (reviewed by Narlikar et al., 2002).

1.4.1.2 Controlling the assembly and regulation of chromatin

DNA of eukaryotes is packed into a nucleoprotein complex known as chromatin, which is composed of repeating units, nucleosomes (Figure 7). Each nucleosome contains 146 bp of DNA wrapped around histone octamers formed of two molecules of each of the core histones H2A, H2B, H3 and H4. Between the nucleosomes, the DNA is occupied by a linker histone, H1, which additionally compacts the chromatin. Chromatin plays a structural role, but it also plays a critical role in transcriptional regulation. Tightly packed chromatin restricts the access of transcription factors and Pol II to the DNA (Figure 7). However, chromosome structure is dynamic and at least three processes, ATP-dependent chromatin remodelling, histone modifications and DNA methylation, control the assembly and regulation of chromatin. Ultimately, all of these processes appear to work in concert and

further, transcription factors are believed to participate in these processes via interactions with chromatin and DNA-modifying enzymes (reviewed by Paranjape et al., 1994).

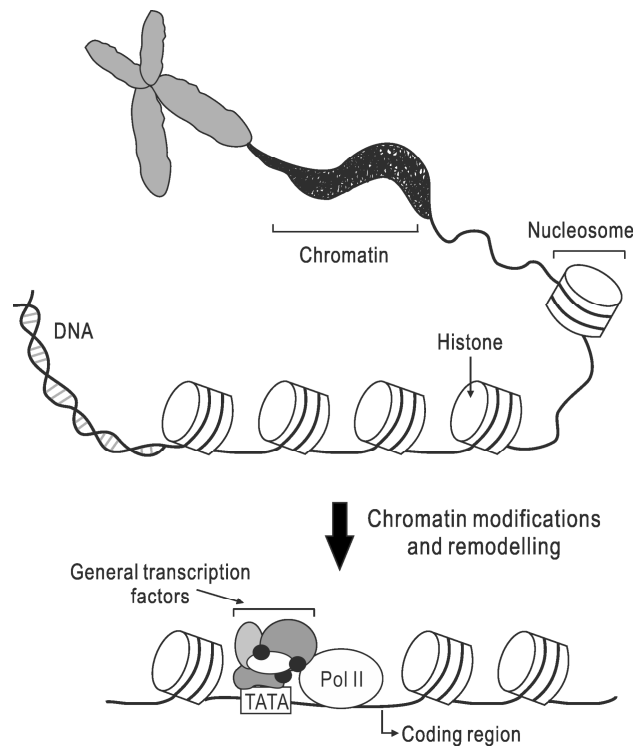


Figure 7. Schematic representation of chromatin structure and activation of transcription via chromatin modifications and remodelling. Modified from Kornberg (2007). Pol II, RNA polymerase II.

ATP-dependent chromatin remodelling

Chromatin remodelling is the enzyme-assisted change in nucleosome organization and seems to be crucial for both the assembly of chromatin structures and their dissolution, thus mediating transcription by either facilitating or preventing access of transcriptional regulators to promoters (Figure 7). Chromatin remodelling is performed by chromatin remodelling complexes, which are ATP-dependent large multiprotein complexes that change DNA-histone interactions. In mammals, there are at least four classes of chromatin remodelling complexes: SWI2/SNF2, Mi-2, ISWI/SNF2 and INO80 (reviewed by Wu et al., 2009 and Ho and Crabtree, 2010).

Histone modifications

Histone proteins are subject to a number of covalent post-translational modifications that occur at particular amino acid positions, primarily on the histone tails protruding from the nucleosome. Histone modifications are epigenetic modifications, heritable changes in DNA that occur without a change in the nucleotide sequence. Histone modifications can control chromatin packaging by altering the interactions of histones with DNA or affecting the contact between different histones in adjacent nucleosomes, thus enabling or blocking the passage of transcription machinery to the DNA. Modifications may also recruit various non-histone proteins and other regulatory proteins to specific chromosomal regions and eventually create a defined structure that is able to enhance gene expression. To date, at least eight different classes of modifications have been characterized, including acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP ribosylation, deimination and proline isomerization. These modifications may act alone or in concert, being highly interactive, influencing and regulating each other by several mechanisms. Most of the histone modifications are dynamic and reversible, and various histone-modifying enzymes control these processes (reviewed by Bannister and Kouzarides, 2011).

DNA methylation

Similarly to histone modifications, DNA methylation is an epigenetic modification. It is associated with gene transcription and chromatin structure. DNA methylation usually occurs in the context of CpG dinucleotides targeting the cytosine located next to a guanine and describes the addition of a methyl group to the 5' carbon of cytosine. The methylation targets are not equally distributed in the genome, but are found in long GC-rich sequences called CpG islands. In mammalian genomes, CpG islands are typically a few hundred to a few thousand nucleotides long and situated at significant regions of the genome, like in the promoters and start regions of many genes. If CpG islands are present in promoters, their methylation is typically associated with transcriptional silencing. Most CpG islands are unmethylated in normal cells, but become methylated in various cell types during development and differentiation and in certain pathological situations, resulting in relevant gene silencing (Siegfried et al., 1999; Strathdee et al., 2004).

DNA methylation is catalysed in mammals by three DNA methyltransferases (DNMTs), namely DNMT1, DNMT3A and DNMT3B, which transfer a methyl group from the universal methyl donor, S-adenosyl-L-methionine (SAM). DNMT1 is the most abundant methyltransferase in mammalian cells and is generally described as maintenance DNA methyltransferase, whereas DNMT3A and DNMT3B are considered *de novo* methyltransferases. However, DNMT1 has well-established *de novo* activity, and all DNMTs have maintenance activity. DNMTs are associated with histone-modifying enzymes, including histone deacetylases, histone methyltransferases and ATP-dependent chromatin remodelling enzymes (reviewed by Hermann et al., 2004).

DNA methylation can modulate the transcription of genes by preventing the transcriptional regulatory proteins from binding to the appropriate sequence. However, demethylation is not always a prerequisite for the binding of transcription factor, as sometimes demethylation follows the binding of transcription factor. For example, the binding of transcription factors Sp1 (specificity protein 1) and NF- κ B (nuclear factor- κ B) to their regulatory elements plays a role in protecting DNA from methylation (Brandeis et al., 1994;

Macleod et al., 1994; Kirillov et al., 1996). Transcriptional silencing by DNA methylation is also influenced by a protein family called methyl-CpG binding proteins, including MeCP2 (methyl-CpG binding protein 2), MBD1 (methyl-CpG binding domain protein) and MBD2. These proteins specifically bind to methylated DNA and are associated with histone deacetylase and methylation activities, finally forming compact, inactive silent chromatin (reviewed by Bogdanovic and Veenstra, 2009).

1.4.1.3 Co-regulators

Co-regulators are proteins that either promote or repress transcription by regulating the recruitment of transcription machinery or acting through chromatin modifications. Co-regulators are characterized as co-activators or co-repressors. They do not bind to DNA, but usually are components of multisubunit co-regulator complexes through protein-protein interactions. Many co-regulators play significant roles in mediating the actions of various transcription factors. Depending on the cell type, target gene, DNA binding site and the actions of various signalling pathways, transcription factors can use distinct combinations of co-regulators. Many co-regulators are capable of modifying histone tails via diverse enzymatic activities such as acetylating/deacetylating and methylating/demethylating activities. Further, some co-factors have ATP-dependent remodelling activities (reviewed by Rosenfeld et al., 2006).

1.4.2 Transcription factors in podocytes

To date, a number of transcription factors specifically expressed in developing and mature podocytes have been identified. Further, genetic studies in mice have provided insight into the role of these transcription factors during kidney development and differentiation of podocytes and development of glomerular disease. The following sections present in more detail the transcription factors of specific interest in this thesis.

1.4.2.1 WT1

WT1, a member of the zinc finger family of transcription factors, was first identified as a tumour suppressor gene of Wilms' tumour, a paediatric malignancy of the kidneys (Call et al., 1990; Gessler et al., 1990). WT1 also plays a crucial role in the development of several organ systems, including the kidney, where WT1 is suggested to play a role at multiple stages. At the beginning of kidney development, WT1 is expressed in the metanephric mesenchyme and from the S-shaped stage onward the WT1 expression is detected in differentiating podocytes (Pritchard-Jones et al., 1990; Pelletier et al., 1991; Armstrong et al., 1993; Kreidberg et al., 1993; Mundlos et al., 1993; Davies et al., 2004). WT1 expression continues in mature podocytes and it has been shown to be crucial for maintaining adult glomerular structure and function (Mundlos et al., 1993; Chau et al., 2011). WT1 regulates nephrin and podocalyxin in podocytes (Palmer et al., 2001; Guo et al., 2004; Wagner et al., 2004).

Binding of WT1 to DNA is mediated by its zinc finger region. WT1 generally recognizes GC-rich sequences and rarely other binding sequences (Rauscher et al., 1990; Wang et al., 1993; Hamilton et al., 1995; Nakagama et al., 1995). WT1 has at least 24 different isoforms resulting from alternative splicing, RNA editing and an alternative translation start site. Isoforms seem to have different and sometimes overlapping functions and their expressions have tissue-, developmental stage- and species-specific differences (Kent et al., 1995; Renshaw et al., 1997). Four major isoforms are produced by alternative splicing events occurring in two different sites, in exon 5 and exon 9 (Haber et al., 1991). Exon 5 encodes 17 amino acids and alternative splicing results in the presence or absence of this amino acid sequence in between the transactivation domain (TAD) and the zinc finger region. An alternative splicing event occurring in a splice donor site at the end of exon 9 gives rise to isoforms differing by three amino acids, lysine-threonine-serine (KTS), between the third and fourth zinc finger (Haber et al., 1991). The KTS sequence changes the structure of the zinc finger domain, altering its DNA binding capabilities, and the isoform that lacks the KTS tripeptide (KTS-) is suggested to have a stronger effect on transcriptional regulation (Drummond et al., 1994; Laity et al., 2000).

In addition to Wilms' tumour at least three other human diseases are associated with mutations in WT1. Denys-Drash syndrome (DDS) results from mutations in the zinc finger region of WT1, indicating that DDS occurs as a result of altered or severely impaired DNA binding activity of WT1 (Pelletier et al., 1991; Little et al., 1993; Bardeesy et al., 1994). DDS is characterized by diffuse mesangial sclerosis within the glomeruli and patients develop partial gonadal dysgenesis, nephropathy and frequently also Wilms' tumour (Drash et al., 1970). WAGR syndrome is a combination of Wilms' tumour, aniridia, genitourinary abnormalities and mental retardation and results from chromosomal deletions of variable size at locus 11p13 (Crolla and van Heyningen, 2002). Mutation in the splice donor site in intron 9 of the WT1 gene results in a higher ratio of the KTS- splice isoform over KTS+ and leads to Frasier syndrome. Patients with Frasier syndrome typically have focal segmental glomerular sclerosis and pseudohermaphroditism (Barboux et al., 1997; Klamt et al., 1998).

A large number of different types of *Wt1*-mutant or -deficient mice have been studied, and they show diverse phenotypes and contradictory results with respect to the role of WT1 in kidney development. This may be due to the complex nature of WT1 and the lack of sufficient knowledge of the role of various WT1 isoforms in different biological processes and also a lack of information about co-operation of WT1 with other regulatory proteins and downstream targets of WT1. Mice homozygous null for WT1 die *in utero* at midgestation on E13.5 because of cardiac abnormality, and mice fail to develop kidneys (Kreidberg et al., 1993). This early lethal phenotype has been rescued using a human-derived WT1 YAC construct and these mice survive until birth. The mice have kidneys, but differentiated podocytes are not observed. Studies on these rescued mice demonstrate that WT1 is required continuously during nephrogenesis, in particular, in specification and maturation of the podocyte lineage (Moore et al., 1999). Ageing mice heterozygous for WT1 develop glomerulosclerosis and show proteinuria and podocyte foot process effacement. Further, nephrin and CD2AP are down-regulated in these mice (Menke et al., 2003). Transgenic mice expressing a mutant form of WT1 in podocytes have abnormally developed glomeruli and defects in capillary development, but the expression levels of nephrin, podocin, CD2AP, synaptopodin and podocalyxin are unaffected. This suggests that during the development of glomeruli, WT1 is involved in capillary formation, regulating

the expression of growth factors that affect capillary development (Natoli et al., 2002a). In terms of the role of different WT1 isoforms in kidney development, mice lacking exon 5 develop normally (Natoli et al., 2002b). Heterozygous transgenic mice expressing only KTS+ or KTS- isoforms show abnormal glomerular development, but KTS- null mice have a more severe phenotype, suggesting that there may be an earlier requirement for the KTS- isoform or the KTS- isoform may have a broader role during glomerular development. The expression of nephrin was decreased in both knock-out mice (Hammes et al., 2001). Altogether, these mouse models demonstrate the critical role of WT1 during kidney development. Very recently, WT1 was deleted ubiquitously in adult mice. The development of the kidney phenotype in these mice was extremely rapid and mice exhibited severe proteinuria, loss of podocyte foot processes and had hardly any nephrin and synaptopodin expression in the podocytes. This provides evidence that WT1 is crucial also for adult glomerular structure and function (Chau et al., 2011).

1.4.2.2 NF- κ B

NF- κ B (nuclear factor- κ B), a member of a beta-scaffold transcription factor family, was initially identified as a DNA-binding factor for the enhancer of the Ig-light chain activated in B-cells (Sen and Baltimore, 1986). Nowadays, NF- κ B is considered a stimulus-responsive pleiotropic regulator of gene control. It is present in all cell types, playing a significant role in promoting the expression of a number of genes involved in immune and inflammatory responses and in cell proliferation, differentiation and apoptosis. The NF- κ B family consists of five members, p50/p105, p52/p100, p65 (RelA), c-Rel and RelB, which share an N-terminal highly conserved Rel homology domain (RHD) responsible for DNA binding and dimerization (reviewed by Gilmore, 2006 and Hayden and Ghosh, 2008). The transcription activation domain (TAD) is present only in the C-terminal domains of the so-called Rel proteins of this family, p65, c-Rel and RelB. p105 and p100 represent the precursors for p50 and p52, respectively, and contain in C-terminal segment multiple copies of ankyrin repeats, which act to inhibit these proteins. In a proteolytic process occurring in the cytoplasm, the C-terminal segments of p105 and p100 are degraded, and these proteins become the shorter, active DNA-binding proteins p50 and p52 (Blank et al., 1991; Fan and Maniatis, 1991; Palombella et al., 1994). As p50/p105 and p52/p100 lack TAD, they are generally not activators of transcription unless they are associated with Rel proteins or other proteins capable of co-activator recruitment.

NF- κ B functions as a homo- or heterodimer when it is able to bind to 10 base pair response element with a great amount of variability (5' -GGGRNNYYCC -3'; R = A or G, N = any nucleotide, Y = C or T). All NF- κ B family members can form homodimers or heterodimers, except RelB, which only forms heterodimers. p50 and p65 are the most abundant subunits, and their homo- and heterodimers are the major NF- κ B dimers in many cells, including podocytes (Baldwin, 1996; Karin and Ben-Neriah, 2000; Mudge et al., 2001; Martinka and Bruggeman, 2006; Hussain et al., 2009). The combinatorial diversity of NF- κ B proteins gives rise to the complex nature of NF- κ B. Different dimers can have distinct DNA-binding site specificities or specific needs for certain co-regulators, or there can be different dimers acting in specific physiological conditions.

NF- κ B is an inducible transcription factor, and its activity is controlled by interaction with inhibitory proteins of the I κ B family. In its inactive state, NF- κ B is bound to I κ B in the cytoplasm, but in response to a large variety of stimuli, such as stress, cytokines and viruses, I κ B becomes degraded and NF- κ B is translocated to the nucleus. The I κ B family consists of I κ B α , I κ B β , I κ B γ , I κ B ϵ , Bcl-3 and the precursors p105 and p100. They all contain multiple ankyrin repeats by which they interact with the Rel homology region, masking the nuclear localization signal of the NF- κ B dimer. All I κ B family members have different affinities for individual NF- κ B dimers and I κ B α is the best-characterized member of the group (reviewed by Hayden and Ghosh, 2008). I κ B α knock-out mice are born normally but usually die a few days after birth due to extensive skin inflammation consistent with persistent NF- κ B activation (Klement et al., 1996).

NF- κ B is activated via two main pathways: the classical/canonical pathway and the alternative/non-canonical pathway. The classical pathway is usually rapid and mostly targets p50/p65 and p50/c-Rel dimers and is triggered by pro-inflammatory cytokines, such as TNF- α (tumour necrosis factor- α) and IL-1 (interleukin-1), as well as microbial products. Upon receptor stimulation, I κ B kinase (IKK) complex, consisting of catalytic kinase subunits IKK α and/or IKK β and a scaffold, sensing protein called NF- κ B essential modulator (NEMO), is activated, leading to phosphorylation of I κ B at two serine residues. This results in ubiquitination and degradation of I κ B by the proteasomes and NF- κ B is then free to enter the nucleus. The alternative NF- κ B pathway involves slow activation, and it targets the p100/RelB complex. This pathway is activated by a more limited number of stimuli and is mainly involved during B- and T-cell organ development. In this pathway, activation of NF- κ B-inducing kinase (NIK) leads to phosphorylation and activation of IKK α homodimer complex, resulting in the phosphorylation of p100 at two serine residues, subsequently leading to a proteasomal degradation of the C-terminal part of p100 and liberation of the p52/RelB complex (reviewed by Gilmore, 2006).

The role of NF- κ B in podocytes has been previously investigated in mouse models. No renal phenotype has been reported in mouse models deficient in any NF- κ B genes (Gerondakis et al., 1999), but constitutive activation of NF- κ B in mice, resulting from removal of a natural NF- κ B pathway inhibitor Par4, causes proteinuria and podocyte foot process effacement. Nephritin expression is down-regulated in the podocytes of these mice (Hussain et al., 2009). NF- κ B activation in podocytes has been reported in the pathogenesis of human kidney diseases, such as diabetic nephropathy (Mezzano et al., 2004), lupus nephritis and non-proliferative glomerulopathy (Zheng et al., 2006) and in experimental kidney disease models, including a passive Heyman nephritis model in rats (Mudge et al., 2001), a 5/6 renal ablation model (Fujihara et al., 2007) and HIV-associated nephropathy (Martinka and Bruggeman, 2006). Increased NF- κ B accumulation in the nuclei of podocytes has also been linked to ageing glomeruli (Wiggins et al., 2010). Moreover, NF- κ B has been suggested to be important for activating the expression of WT1, but results have been controversial (Dehbi et al., 1998; Chen and Williams, 2000).

1.4.2.3 Sp1

Sp1 (specificity protein 1) is a member of the Sp family of transcription factors. This family has a particular combination of three conserved Cys₂His₂ zinc fingers representing a DNA-

binding domain. Sp1 is ubiquitously expressed in mammalian cells and initially was considered a constitutive activator of housekeeping genes. However, it is now clear that Sp1 is involved also in regulating expression of many tissue-specific and inducible genes. Sp1 binds to GC-rich motifs and many promoters of Sp1-regulated genes lack TATA box (reviewed by Suske, 1999).

A role for Sp1 in nephrogenesis has been proposed since during nephrogenesis Sp1 is expressed in developing podocytes. The expression overlaps with that of WT1, and Sp1 is able to regulate WT1 (Cohen et al., 1997). Sp1 is expressed in podocytes in adult rat kidney (Cohen et al., 1997), and it also plays a role in regulating transcription of podocyte molecules CD2AP and podocalyxin (Butta et al., 2006; Lu et al., 2008; Su et al., 2009).

1.4.2.4 Other transcription factors

Pax2 is a member of the paired box (Pax) family of transcription factors and plays an essential role in kidney development since *Pax2*-null mice lack kidneys, ureters and genital tracts (Torres et al., 1995). During kidney development Pax2 expression becomes down-regulated in podocyte precursors simultaneously with a marked increase in WT1, suggesting that Pax2 could be a target gene for WT1 (Ryan et al., 1995; Donovan et al., 1999). Pax2 down-regulation is speculated to be required for proper podocyte maturation because the synthesis of Pax2 in transgenic mice results in podocyte damage (Dressler et al., 1993) and reactivation of Pax2 in terminally differentiated podocytes has been linked to formation of a more dedifferentiated phenotype (Wagner et al., 2006). Pax2 is shown to interact with an adaptor protein Pax transactivation domain interacting protein (PTIP), promoting assembly of the histone H3K4 methyltransferase complex. This suggests that Pax2 could provide the locus and tissue specificity for histone methylation, thus establishing a kidney-specific fate (Patel et al., 2007). Very recently, PTIP was conditionally deleted in podocytes in mice. Renal development and function were not impaired in young mice (aged 3 months), but in older mice (aged 6 and 12 months) deletion led to proteinuria and podocyte foot process effacement. Nephron expression was partially depleted in these mice (Lefevre et al., 2010).

LMX1B is a LIM-homeodomain transcription factor, mutations of which are responsible for nail-patella syndrome (NPS). Up to 40% of NPS patients exhibit kidney abnormalities, including proteinuria and disappearance of podocyte foot processes (Chen et al., 1998; Dreyer et al., 1998; Vollrath et al., 1998; Sweeney et al., 2003). Conventional knock-out of *Lmx1b* in mice leads to lack of normal podocyte foot processes and SDs, and mice die within 24 h of birth (Chen et al., 1998; Miner et al., 2002; Rohr et al., 2002). Podocyte-specific knock-out of *Lmx1b* in mice leads to survival for up to 2 weeks after birth, and the podocytes develop foot processes initially but subsequently lose them (Suleiman et al., 2007). These results suggest that LMX1B is important for the initial differentiation of podocytes, but also for maintenance of the podocyte phenotype. A physical interaction between LMX1B and Pax2 has been demonstrated, suggesting that they could act together at some embryonal stage (Marini et al., 2005). Podocyte molecules podocin and CD2AP are suggested to be regulated by LMX1B, even though the results have been controversial (Miner et al., 2002; Rohr et al., 2002; Heidet et al., 2003; Suleiman et al., 2007; Harendza et al., 2009).

Kreisler (also called MafB or Maf-1) is a member of the Maf family of transcription factors that is expressed in developing and mature podocytes (Imaki et al., 2000). The inactivation of Kreisler in mice results in proteinuria and loss of podocyte foot processes, demonstrating that Kreisler is essential for podocyte differentiation. Podocyte molecules nephrin, podocin and CD2AP are down-regulated in mice lacking Kreisler, suggesting that they could represent direct or indirect targets of Kreisler (Sadl et al., 2002; Moriguchi et al., 2006).

Pod1 (also known as epicardin and capsulin) is a transcription factor of the basic helix-loop-helix family showing expression in podocyte precursors during kidney development as well as in podocytes of adult kidneys (Quaggin et al., 1998). *Pod1* knock-out mice die at birth as a result of heart and lung defects, but they also possess major renal defects. Podocytes are formed, but they remain columnar-shaped and form only a few foot processes, suggesting that Pod1 plays an important role in kidney morphogenesis and differentiation of podocytes (Quaggin et al., 1999).

Foxc2 is a member of the forkhead box (FOX) transcription factor family. Foxc2 is expressed in developing and mature podocytes (Takemoto et al., 2006) and is suggested to have a role in podocyte differentiation since podocytes in mice lacking Foxc2 fail to form foot processes (Takemoto et al., 2006). Recently, using *Xenopus* pronephrons and zebrafish as models for podocyte development, the interplay between Foxc2 and WT1 and Notch signalling was demonstrated to be important for podocyte development (White et al., 2010; O'Brien et al., 2011).

1.4.3 Transcriptional regulation of nephrin and Neph3 genes

Several studies have focused on identifying transcription factors and agents and underlying mechanisms responsible for regulating the nephrin gene in podocytes of man and rodents, but the transcriptional regulation of the Neph3 gene in podocytes has thus far not been elucidated. Outside the podocytes, the transcriptional regulation of nephrin and Neph3 genes has been studied only in the developing central nervous system (Nishida et al., 2010).

1.4.3.1 Tissue-specific regulatory elements in nephrin promoter

Soon after discovery of nephrin, several studies have concentrated on identifying and characterizing regulatory sequences responsible for its tissue-specific expression. Wong et al. (2000) observed that the 1.25-kb fragment of the human nephrin proximal promoter directs the lacZ gene to glomeruli and specifically to podocytes. Later, Guo et al. (2004) demonstrated that a highly conserved 186-bp fragment within this region (locating -667 bp upstream of the TSS) drives the expression in podocytes. Using a mouse nephrin sequence, it was first shown that 8.3-kb and 5.4-kb promoter fragments drive podocyte-specific expression in the kidney, but the expression of these fragments was also detected in the pancreas and in structures of the developing and adult brain (Moeller et al., 2000; Putaala et al., 2001). Later, a 1.25-kb fragment of the mouse nephrin promoter was shown to drive the expression solely in podocytes (Moeller et al., 2002). Beltcheva and co-workers (2003)

have also used different fragments of the mouse nephrin promoter to identify the sequence essential for podocyte-specific expression. They demonstrated that 6.242-kb and 4.013-kb fragments are sufficient for driving expression in the podocytes, central nervous system and pancreas. However, in their experiments, in disagreement with other studies, 2.148-kb fragment did not yield expression in podocytes, or in any other tissues. Taken together, nephrin seems to have distinct promoter regions for tissue-specific expression.

1.4.3.2 Transcription factors and agents regulating nephrin gene in podocytes

Transcription factors in nephrin gene regulation

Two studies show that transcription factor WT1 is an important regulator of the nephrin gene (Guo et al., 2004; Wagner et al., 2004). Guo et al. (2004) at the same time as they found a 186-bp human nephrin promoter fragment capable of driving podocyte-specific expression identified within this 186-bp fragment an 83-bp area with 83% sequence homology between humans and mice and harbouring a putative binding site for WT1 at position -654 to -638 bp upstream from the human nephrin TSS. They further demonstrated that WT1 can bind *in vitro* to this region in the human sequence and activate the human nephrin promoter. Wagner et al. (2004), by contrast, showed a putative WT1 binding site at position -1043 to -1022 bp upstream from the mouse nephrin TSS. This element is not located in the homological region that Guo et al. (2004) identified, but approximately 600 bp upstream from it. However, Wagner et al. (2004) showed that this putative WT1 binding site is bound by WT1, and WT1 transactivates the mouse nephrin promoter via this element. The importance of this element was also demonstrated *in vivo*, showing that it is required for direct podocyte-specific expression in transgenic mice. Further, nephrin mRNA expression has been shown to be down-regulated in kidneys lacking either KTS+ or KTS- isoform of WT1 and the expression was lower in mice deficient for KTS- than for KTS+ (Wagner et al., 2004).

Beltcheva et al. (2010) analysed in more detail the mouse nephrin promoter region which they had previously found to be important for podocyte-specific expression (Beltcheva et al., 2003). Within the region between -4 and -2.1 kb upstream from the major TSS, a 237-bp area highly homologous with the human sequence was identified. This region is located in the mouse sequence between nucleotides -2299 and -2063, and a putative binding site for Sp1 was located within it at position -2133/-2103. Sp1 was shown to bind this element *in vitro*, suggesting a role for Sp1 in nephrin gene regulation.

Snail, a transcription factor implicated in regulating cell adhesion and the epithelial to mesenchymal transition, is linked to the regulation of the nephrin gene in injured podocytes. The expression of nephrin mRNA and protein was down-regulated and Snail protein expression up-regulated in the podocytes of rats with puromycin aminonucleoside (PA)-induced nephrosis, an experimental model showing podocyte injury. *In vitro* assays indicate that Snail represses rat nephrin promoter activity and binds possibly to E-box sites in the coding region of the rat nephrin gene between nucleotides +404 and +972 (Matsui et al., 2007).

Agents in nephrin gene regulation

To identify regulators of nephrin gene expression, Yamauchi et al. (2006) performed extensive studies in conditionally immortalized mouse podocytes expressing SEAP (secreted alkaline phosphatase) reporter gene under the control of the mouse nephrin promoter (reporter mouse podocytes). They found that both nephrin promoter activity and mRNA expression were increased by all-*trans* retinoic acid (ATRA), 1,25-dihydroxyvitamin D₃ and dexamethasone and also demonstrated that any combination of these agents further induced nephrin promoter activity. A couple of years later, studies from the same group using the same reporter mouse podocytes as a model system suggested that ATRA induces nephrin promoter activity through retinoic acid receptor (RAR) and 1,25-dihydroxyvitamin D₃ through co-operation of vitamin D receptor (VDR) and RAR. Elements for RAR and VDR in the mouse nephrin promoter were not specified in this study (Okamura et al., 2009). Very recently, another group shed light on the mechanisms underlying 1,25-dihydroxyvitamin D₃ up-regulation of nephrin mRNA and protein expression in cultured mouse podocytes. Deb et al. (2011) showed that VDR-RXR (retinoic X receptor) heterodimer binds to the VDR element (VDRE) located at position -327/-312 in the mouse nephrin promoter and further demonstrated that 1,25-dihydroxyvitamin D₃ treatment strongly induces nephrin promoter activity containing this VDRE and leads to increased binding of VDR to this element. *In vivo* validation with mice confirmed that in podocytes 1,25-dihydroxyvitamin D₃ stimulates nephrin mRNA and protein expression by acting on a VDRE (Deb et al., 2011). Two studies of ATRA and nephrin mRNA and protein expression have concentrated more on the therapeutic effects of ATRA in various animal models of kidney diseases. In rats with PA-induced nephrosis, the expression of nephrin mRNA was decreased, but treatment with ATRA maintained nephrin expression as well as prevented proteinuria. Human nephrin promoter was found to harbour at least three putative binding sites for RAR (RARE), at positions -1260/-1246, -1041/-1021 and -774/-757, and the activity of the promoter regions containing these elements was shown to be augmented by ATRA in HeLa cells (Suzuki et al., 2003). Another study revealed that nephrin protein expression is increased by ATRA in cultured mouse and rat podocytes. ATRA also reduces proliferation and induces differentiation of cultured podocytes (Vaughan et al., 2005). *In vivo* studies in the antibody-mediated mouse model of podocyte injury demonstrated that ATRA prevents the decrease in nephrin protein expression (Vaughan et al., 2005).

Inflammatory cytokines have also been shown to be involved in nephrin regulation. Using human embryonic A293 kidney cells, TNF- α and IL-1 β were found to up-regulate nephrin mRNA and protein expression, and this up-regulation apparently involved a rottlerin-sensitive protein kinase. In the same study, nephrin mRNA and protein expression was up-regulated by IL-1 β also in a primary culture of human podocytes (Huwiler et al., 2003). In contrast to research in human cell lines, studies on mouse podocytes show that nephrin promoter activity and mRNA expression are decreased by TNF- α and IL-1 β (Yamauchi et al., 2006). Using reporter mouse podocytes, it has further been shown that macrophages suppress nephrin promoter activity possibly via production of TNF- α and IL-1 β . Involvement of the PI3K/AKT pathway is speculated in the suppression of the nephrin gene by these cytokines (Takano et al., 2007). The same group recently showed that in reporter mouse podocytes TNF- α represses RAR activity and suggested that nephrin gene suppression by TNF- α is caused at least partly through inhibition of the cAMP-RAR pathway (Saito et al., 2010).

Two studies show the involvement of peroxisome proliferator-activated receptors (PPARs) in nephrin regulation. In cultured human podocytes and human embryonic A293 kidney cells, PPAR α stimulates nephrin mRNA and protein expression. The action of PPAR α was suggested to result from increased nephrin promoter activity and/or increased stabilization of nephrin mRNA (Ren et al., 2005). Benigni et al. (2006) showed that in human HK-2 cells, pioglitazone, a ligand of PPAR γ , increases the activity of human nephrin proximal promoter, which contains a putative peroxisome proliferator-responsive element (PPRE). Pioglitazone-induced binding of PPAR/RXR heterodimer to this PPRE was demonstrated. Further, *in vivo* studies using passive Heymann nephrosis (PHN) rats, an immune model of progressive nephropathy, demonstrated decreased nephrin mRNA and protein expression in glomeruli of PHN rats, but pioglitazone restored the expression, suggesting a renoprotective effect of PPAR γ via enhanced nephrin expression through PPRE (Benigni et al., 2006).

1.4.3.3 Transcription factors regulating nephrin and Neph3 genes in other tissues

Ptfla, a basic helix-loop-helix transcription factor that plays an indispensable role in neuronal development, controls nephrin and Neph3 gene expression in the developing central nervous system of mice. Ptfla and nephrin and Neph3 mRNA are expressed in overlapping regions and the same cells in the mouse developing cerebellum, hindbrain, spinal cord, retina and hypothalamus. In *Ptfla*-null mice, the expression of nephrin and Neph3 mRNA was lost in these regions. *In vivo* and *in vitro*, Ptfla was shown to bind to the Ptfla binding element located about 1.8 kb upstream from the major nephrin TSS in the shared 5'-flanking region of the nephrin and Neph3 mouse genes. Further, Ptfla drove the promoter activity of this region in both directions (Nishida et al., 2010).

1.4.4 Summary of transcription factors and agents of interest

A number of transcription factors expressed in developing and mature podocytes play a role in kidney development, kidney maintenance and/or glomerular disease development. Some of these regulate nephrin or other podocyte molecules. There are also many agents involved in nephrin regulation. Tables 1 and 2 summarize the transcription factors and agents of interest in this thesis.

Table 1. Summary of transcription factors of interest.

	Description
WT1	<ul style="list-style-type: none"> Crucial during kidney development and for maintaining the integrity of mature podocytes (Prichard-Jones et al., 1990; Pelletier et al., 1991; Kreidberg et al., 1993; Davies et al., 2004; Chau et al., 2011) Regulates nephrin and podocalyxin (Palmer et al., 2001; Guo et al., 2004; Wagner et al., 2004)
NF-κB	<ul style="list-style-type: none"> Activated in podocytes in human and experimental kidney diseases (Mudge et al., 2001; Mezzano et al., 2004; Martinka and Bruggeman, 2006; Zheng et al., 2006; Fujihara et al., 2007) May be important for activating WT1 expression (Dehbi et al., 1998; Chen and Williams, 2000)
Sp1	<ul style="list-style-type: none"> Proposed to play a role in nephrogenesis (Cohen et al., 1997) Regulates nephrin, WT1, CD2AP and podocalyxin (Cohen et al., 1997; Butta et al., 2006; Lu et al., 2008; Su et al., 2009; Beltcheva et al., 2010)
Pax2	<ul style="list-style-type: none"> Essential role in kidney development (Torres et al., 1995) Down-regulation during kidney development is suggested to be needed for proper podocyte maturation (Dressler et al., 1993; Ryan et al., 1995; Donovan et al., 1999).
LMX1B	<ul style="list-style-type: none"> Suggested to be important for the initial differentiation of podocytes and the maintenance of the podocyte phenotype (Chen et al., 1998; Miner et al., 2002; Rohr et al., 2002; Suleiman et al., 2007) Interacts physically with Pax2 (Marini et al., 2005) May regulate podocin and CD2AP (Miner et al., 2002; Rohr et al., 2002; Heidet et al., 2003; Suleiman et al., 2007; Harendza et al., 2009)
Kreisler	<ul style="list-style-type: none"> Essential for podocyte differentiation (Imaki et al., 2000)
Pod1	<ul style="list-style-type: none"> Important role in kidney morphogenesis and differentiation of podocytes (Quaggin et al., 1999)
Foxc2	<ul style="list-style-type: none"> Suggested to have a role in podocyte differentiation (Takemoto et al., 2006) Interplay with WT1 and Notch signalling is important for podocyte development in <i>Xenopus</i> pronephrons and zebrafish (White et al., 2010; O'Brien et al., 2011)
Snail	<ul style="list-style-type: none"> Down-regulates nephrin in injured rat podocytes (Matsui et al., 2007)
Ptf1a	<ul style="list-style-type: none"> Regulates nephrin and Neph3 genes in the developing central nervous system of the mouse (Nishida et al., 2010)

Table 2. Summary of agents regulating nephrin mRNA and protein expression.

	Description
ATRA	<ul style="list-style-type: none"> Increases the activity of human and mouse nephrin promoter activity through RARE (Suzuki et al., 2003; Yamauchi et al., 2006; Okamura et al., 2009) Prevents the decrease in nephrin mRNA and protein expression in murine models of kidney diseases (Suzuki et al., 2003; Vaughan et al., 2005)
1,25(OH)₂D₃	<ul style="list-style-type: none"> Induces mouse nephrin promoter through co-operation of VDR and RAR (Yamauchi et al., 2006; Okamura et al., 2009) Stimulates nephrin mRNA and protein expression in mouse podocytes by acting on a VDRE (Deb et al., 2011)
Dexamethasone	<ul style="list-style-type: none"> Induces mouse nephrin promoter activity and mRNA expression (Yamauchi et al., 2006)
TNF-α	<ul style="list-style-type: none"> Up-regulates nephrin mRNA and protein expression in A293 cells (Huwiler et al., 2003) Down-regulates nephrin promoter activity and mRNA expression in mouse podocytes (Yamauchi et al., 2006)
IL-1β	<ul style="list-style-type: none"> Up-regulates nephrin mRNA and protein expression in A293 cells and primary culture of human podocytes (Huwiler et al., 2003) Down-regulates nephrin promoter activity and mRNA expression in mouse podocytes (Yamauchi et al., 2006)
PPAR	<ul style="list-style-type: none"> PPARα stimulates nephrin mRNA and protein expression in cultured human podocytes and A293 cells (Ren et al., 2005) Pioglitazone, a ligand of PPARγ, increases the activity of human nephrin promoter and restores decreased nephrin mRNA and protein expression in glomeruli of PHN rats (Benigni et al., 2006)

ATRA, all-*trans* retinoic acid; RAR(E), retinoic acid receptor (element); 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; VDR, vitamin D receptor; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; PPAR, peroxisome proliferator-activated receptor; PHN, passive Heymann nephrosis

2 AIMS OF THE STUDY

Nephrin and the members of the Neph-protein family, Neph1-3, are essential structural and signalling components in the slit diaphragm (SD) of the kidney filtration barrier. This thesis aimed at determining whether Neph3 has similar functions as nephrin and other Neph-family members. The study also focused on the transcriptional regulation of nephrin and Neph3 genes. Based on the chromosomal location of nephrin and Neph3 genes and the similar functions of their protein products, the study aimed at identifying common mechanisms in the transcriptional regulation of nephrin and Neph3 genes. Specific objectives were as follows:

1. To investigate the interactions and cell adhesion activities of nephrin and Neph-family members
2. To identify and characterize the key regulatory region(s) involved in constitutive transcription of the Neph3 gene
3. To identify common transcriptional mechanisms and factors regulating nephrin and Neph3 genes
4. To evaluate the role of DNA methylation in regulating nephrin and Neph3 genes

3 MATERIALS AND METHODS

3.1 DNA constructs

3.1.1 Expression vectors (I-IV)

Full-length and different parts of nephrin, Neph1 and Neph3 genes were subcloned into mammalian expression vectors as detailed in Study I. These vectors were used in retrovirus production and to produce proteins for protein-protein interaction studies. Expression vectors for various transcription factors were obtained via collaboration and used to evaluate their role in nephrin and Neph3 gene regulation and their protein-protein interactions (Studies II-IV). The expression vectors are listed in Table 3.

Table 3. Summary of expression vectors used in Studies I-IV.

Gene (Vector backbone)	Species	Description	Vector backbone supplier / Construct provider	Study
EGFP (pMSCVpuro)	jellyfish	full-length	Clontech Laboratories (Mountain View, CA, USA)	I
Nephrin (pcDNA3.1/Myc-His)	rat	full-length	Invitrogen (Carlsbad, CA, USA)	I
(pMSCVneo)	rat	full-length	Clontech Laboratories	I
(derivative of pCDM8)	human	intracellular domain	(Tsiokas et al., 1997)	I
Neph1 (pBabe-hygro)	mouse	full-length, HA-tagged	(Morgenstern et al., 1990)	I
(derivative of pCDM8)	human	intracellular domain	(Tsiokas et al., 1997)	I
Neph3 (pcDNA3.1/Myc-His)	mouse	full-length	Invitrogen	I
(pMSCVpuro)	mouse	full-length, EGFP-tagged	Clontech Laboratories	I
(derivative of pCDM8)	human	intracellular domain	(Tsiokas et al., 1997)	I
(signal plgplus)	human	extracellular domain	R&D Systems (Minneapolis, MN, USA)	I
Sp1 (pPAC)	human	full-length	Guntram Suske, Philipps-Universität Marburg, Germany	II
NF-κB p50 (pcDNA3.1)	human	full-length	Alan Krensky, Stanford University School of Medicine, CA, USA	II, III
NF-κB p65 (pcDNA3.1)	human	full-length	Alan Krensky, Stanford University School of Medicine, CA, USA	II, III
WT1 (pcDNA3)	human	isoform WT1(+/-)	Charles Roberts, Oregon Health and Science University, OR, USA	III
GABPα (pCR3.1E)	human	full-length	Toshibumi Shimokawa and Chisei Ra, Nihon University Graduate School of Medical Sciences, Japan	IV
GABPβ (pCR3.1E)	human	full-length	Toshibumi Shimokawa and Chisei Ra, Nihon University Graduate School of Medical Sciences, Japan	IV

3.1.2 Luciferase reporter gene constructs (II-IV)

To identify and characterize the promoter regions important for the regulatory processes of nephrin and Neph3 genes, a series of nephrin and Neph3 luciferase reporter gene constructs was created. Site-directed mutagenesis of putative transcription factor binding sites was performed with the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) using mutated oligonucleotides. The detailed cloning processes of all constructs, including the primers used, are described in the original publications II-IV. The constructs are listed in Table 4.

Table 4. Reporter gene constructs used in Studies II-IV. Numbers from/to indicate the distance in base pairs related to the nephrin and Neph3 transcription start site.

Reporter gene construct	From	To	Mutation	Study
Neph3	-5070	+48		II
	-4543	+48		II
	-3877	+48		II
	-2260	+48		II
	-1072	+48		II
	-536	+48		II
	-366	+48		II
	-162	+48		II, III
	-105	+48		II
	-105	+48	Sp1 mut	II
	-105	+48	NF-κB mut	II
	-105	+48	Sp1 mut, NF-κB mut	II
	-57	+48		II
Nephrin	-837	+156		III, IV
	-617	+156		unpublished data
	-392	+156		IV
	-48	+156		IV
	-48	+156	Ets(1) mut	IV
	-48	+156	Ets(2) mut	IV
	-48	+156	Ets(3) mut	IV
	+25	+156		IV

3.2 Cell culture (I-IV)

Several cell lines were used to investigate the protein-protein interactions and the adhesive properties of nephrin, Neph1 and Neph3, and the transcriptional regulation of nephrin and Neph3 genes. The cell lines used and their culture media are shown in Table 5. 293T cells, L-cells, Phoenix amphi cells and A293 cells were maintained in 5% CO₂ at 37°C, and S2 cells at 25°C without CO₂. Human podocytes were cultured at 33°C (undifferentiated podocytes) and differentiated at 37°C for 14 days in 5% CO₂.

Table 5. Summary of cell lines used in Studies I-IV.

Cell line	Culture medium	Reference / supplier	Study
293T (Highly transfectable derivative of the human embryonic kidney 293 cell line)	Dulbecco's Modified Eagle's Medium (DMEM) (glucose 4500 mg/l) supplemented with 10% foetal bovine serum, 2 mM glutamine, 25 mM HEPES (pH 7.5), 100 U/ml penicillin and 100 µg/ml streptomycin	ATCC	I
L-cells (Mouse L fibroblasts; L-929)	DMEM (glucose 4500 mg/l) supplemented with 10% foetal bovine serum, 2 mM glutamine, 25 mM HEPES (pH 7.5), 100 U/ml penicillin and 100 µg/ml streptomycin	ATCC	I
Phoenix amphi packaging cells (Highly transfectable derivative of the 293T cell line for retrovirus production)	DMEM (glucose 4500 mg/l) supplemented with 10% foetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin	Garry Nolan, Stanford University, CA, USA	I
S2 cells (<i>Drosophila</i> Schneider 2 cells)	Express Five serum-free medium (SFM) supplemented with 10% foetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin	Jussi Taipale, University of Helsinki, Finland	II
A293 (Human embryonic kidney cells)	RPMI 1640 medium supplemented with 10% foetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin	(Wang et al., 2001)	II, III, IV
Human podocytes	RPMI 1640 medium containing L-glutamine supplemented with 10% foetal bovine serum and insulin, transferrin and selenite	(Saleem et al., 2002)	II, III, IV

To obtain L-cells stably expressing nephrin, Neph1, Neph3 and EGFP alone or nephrin and Neph1 or Neph3 in combination, packaging cell lines were used for retrovirus production. 293T cells were co-transfected with the retroviral constructs nephrin-pMSCVneo, Neph3-EGFP-pMSCVpuro or EGFP-pMSCVpuro together with packaging vector pKAT (Finer et al., 1994). Alternatively, Phoenix Amphi packaging cells were transfected with retroviral constructs Neph1-HA-pBabe-hygro or pBabe-hygro. L-cells were infected with the retroviral supernatants and hygromycin, puromycin and G418 were used for selection of cells stably expressing nephrin, Neph1, Neph3 or EGFP. To generate stable cell lines expressing nephrin and Neph1 or Neph3 in combination, L-cells stably expressing nephrin were infected with retroviruses carrying Neph1-HA or Neph3-EGFP and selected with the appropriate antibiotic.

3.3 Animals

3.3.1 Nephrin-deficient mouse line (I)

Nephrin TRAP mice were established in the GSF Centre for Environment and Health, Institute of Mammalian Genetics (Neuherberg, Germany), as described by Hill and Wurst (1993) and characterized by Rantanen et al. (2002). The localization and expression of

Neph1 and Neph3 were investigated in the kidneys of these mice. All experiments were approved by the Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland.

3.3.2 Sprague-Dawley rats (IV)

The kidneys of Sprague-Dawley rats were used to investigate GABP α expression in the kidney. Animal work was approved by the national Animal Experiment Board.

3.4 Antibodies (I-IV)

The primary antibodies used in Studies I-IV are listed in Table 6.

Table 6. Primary antibodies used in Studies I-IV.

Antibody	Description	Reference / supplier	Study
Nephrin #033 (extracellular domain)	rabbit polyclonal	(Holthöfer et al., 1999)	I
Nephrin #1034 (intracellular domain)	rabbit polyclonal	(Ahola et al., 2003)	I
Nephrin pY1193	rabbit polyclonal	(Jones et al., 2009)	I
Nephrin pY1217	rabbit polyclonal	(Jones et al., 2009)	I
Neph1	rabbit polyclonal	(Sellin et al., 2003)	I
Neph3 #1201	rabbit polyclonal	(Ihalmo et al., 2007)	I
Neph3	rabbit polyclonal	Alpha Diagnostic International (San Antonio, TX, USA)	I
Podocin	rabbit polyclonal	Sigma (St. Louis, MO, USA)	I
GFP	rabbit polyclonal	Invitrogen	I
HA	mouse monoclonal	Covance (Emeryville, CA, USA)	I
Myc	mouse monoclonal	Clontech Laboratories (Mountain View, CA, USA)	I
Phosphotyrosine	mouse monoclonal	Sigma	I
Tubulin	mouse monoclonal	Sigma	I
Actin	mouse monoclonal	Sigma	I, IV
Sp1 (H-225; sc-14027)	rabbit polyclonal	Santa Cruz Biotechnology (Santa Cruz, CA, USA)	II
NF- κ B p50	rabbit polyclonal	Abcam (Cambridge, UK)	II, III
NF- κ B p65	rabbit polyclonal	Abcam	II, III
WT1 (C-19; sc-192)	rabbit polyclonal	Santa Cruz	III
WT1 (6F-H2)	mouse monoclonal	Upstate (Lake Placid, NY, USA)	unpublished data
GABP α (G-1; sc-28312)	mouse monoclonal	Santa Cruz	IV
GABP α (H-180; sc-22810)	rabbit polyclonal	Santa Cruz	IV

3.5 Cell assays

3.5.1 Hanging drop aggregation assay (I)

To investigate whether nephrin, Neph1 and Neph3 can promote cell adhesion, the adhesive properties of L-cells stably expressing nephrin, Neph1 and/or Neph3 were examined by hanging drop aggregation assay. Single cell suspensions of L-cells were placed in drops of culture medium on the lids of culture dishes. After incubation cells and cell aggregates in the hanging drop cultures were counted so that cell aggregate containing more than four cells was determined as a single particle.

3.5.2 Reporter gene assay (II-IV)

To investigate the regulatory regions and transcription factors important for nephrin and Neph3 gene regulation, A293 cells were transiently transfected with nephrin and Neph3 luciferase reporter gene constructs together with *Renilla* luciferase reporter vector (phRL-TK; Promega, Madison, WI, USA), which was used as an internal control to correct the transfection efficiency. Transcription factor overexpression studies were performed either in A293 cells (NF- κ B, WT1 and GABP studies) or in *Drosophila* S2 cells (Sp1 studies). Expression vectors for transcription factors were co-transfected with reporter gene vectors (nephrin and Neph3 luciferase reporter gene construct and phRL-TK vector). A293 cells were lysed 24 h and *Drosophila* S2 cells 48 h after transfection, and luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega). The studied luciferase activities (firefly luciferase) were normalized by control luciferase activities (*Renilla* luciferase) in A293 cells or by the protein concentration measured using a Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA) in *Drosophila* S2 cells.

3.5.3 Transcription factor overexpression studies (II, III)

To investigate the effects of transcription factors on the expression of endogenous nephrin and Neph3 mRNA, A293 cells were transfected with expression vectors for transcription factors and RNA was extracted 48 h after transfections and studied by quantitative reverse transcription-PCR (qRT-PCR) as described in Section 3.9.3.

3.5.4 TNF- α , BAY 11-7082 and 5-aza-2'-deoxycytidine treatments (III)

To investigate the involvement of NF- κ B in the regulation of nephrin and Neph3 genes, differentiated podocytes were treated with NF- κ B activator TNF- α and NF- κ B pathway inhibitor BAY 11-7082, and RNA was extracted for qRT-PCR. To determine whether DNA

methylation plays a role in regulating nephrin and Neph3 gene expression, A293 cells and undifferentiated and differentiated podocytes were treated with the demethylating agent, 5-aza-2'-deoxycytidine, and RNA was studied by qRT-PCR. Treatments of the cell lines are explained in detail in Study III.

3.6 Protein studies

3.6.1 Preparation of E17 kidney lysates and glomerular and tubular lysates (I, IV)

Mouse kidneys from nephrin-deficient and nephrin wild-type embryos at E17 were lysed using a glass homogenizer in RIPA lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1% Nonidet P40, 0.5% sodium deoxycholate and 0.1% SDS supplemented with 1× Complete, EDTA-free proteinase inhibitor cocktail (Roche, Mannheim, Germany). Glomeruli and tubules were isolated from rat kidney cortices using a graded sieving method (sieves of decreasing pore sizes: 250, 150 and 75 μm) (Orlando et al., 2001). Tubules were collected from the 150-μm sieve and glomeruli from the final sieve, and both fractions were pelleted by centrifugation. Glomeruli and tubules were lysed in lysis buffer containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.5) and 1% Nonidet P-40, and supplemented with 1× Complete, EDTA-free proteinase inhibitor cocktail (Roche), 50 mM sodium fluoride and 1 mM sodium orthovanadate by rotation at 4°C for 30 min. Insoluble material of the lysates was removed by centrifugation, and the protein concentrations were measured by using a Bradford assay.

3.6.2 Preparation of total cell lysates (I, IV)

Total lysates from L-cells were prepared using RIPA lysis buffer supplemented with 1× Complete, EDTA-free proteinase inhibitor cocktail (Roche). To obtain total cell lysates from A293 cells and undifferentiated and differentiated podocytes, cells were lysed into the buffer containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.5) and 1% Nonidet P-40 supplemented with 1× Complete, EDTA-free proteinase inhibitor cocktail (Roche), 50 mM sodium fluoride and 1 mM sodium orthovanadate. Cell lysates were rotated at 4°C for 30 min, insoluble material was pelleted by centrifugation and protein concentrations were measured by using a Bradford assay.

3.6.3 Preparation of nuclear extracts (IV)

Nuclear extracts were prepared according to the protocol of Schreiber et al. (1989) from A293 cells and undifferentiated and differentiated podocytes. Cells were washed with PBS

and resuspended in buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA and 1× Complete, EDTA-free proteinase inhibitor cocktail. Cells were allowed to swell on ice for 15 min, and thereafter, cell membranes were disrupted by adding Nonidet P-40. The resulting homogenate was centrifuged and the nuclear pellet was resuspended in buffer containing 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA and 1× Complete, EDTA-free proteinase inhibitor cocktail, and rotated at 4°C for 15 min. The samples were centrifuged and supernatants containing the nuclear proteins were collected and their protein concentrations were measured by using a Bradford assay.

3.6.4 Immunoblotting (I, III, IV)

Proteins were separated by SDS-polyacrylamide gels, transferred to PVDF membranes (Millipore, Billerica, MA, USA), which were blocked with 5% skimmed milk or with Odyssey blocking buffer (LI-COR Biosciences, Bad Homburg, Germany). Membranes were incubated with specific primary antibodies and washed, and the bound antibodies were detected using secondary antibodies conjugated with fluorescent dyes Alexa Fluor 680 (Invitrogen, Carlsbad, CA, USA) or IRDye 680 or 800 (LI-COR Biosciences). The signal was detected using the Odyssey infrared imaging system (LI-COR Biosciences).

3.6.5 Immunofluorescence microscopy

3.6.5.1 Immunofluorescence microscopy of kidney tissue (I, IV)

Dissected kidneys from nephrin-deficient and nephrin wild-type embryos at E17 and from 2-month-old Sprague-Dawley rats were snap-frozen in liquid nitrogen and further processed for cryosectioning. Tissue sections (6 µm) were fixed with acetone, washed with PBS and blocked with Cas Block (Zymed Laboratories, South San Francisco, CA, USA) followed by overnight incubation with primary antibodies. Sections were washed with PBS and incubated with TRITC- or FITC-conjugated secondary antibodies and Hoechst 33342 for nuclear staining, followed by washing with PBS and mounting with Mowiol. Sections were examined using an Olympus AX70 (Olympus, Tokyo, Japan) or Zeiss Axiophot 2 microscope (Zeiss, Oberkochen, Germany).

3.6.5.2 Immunofluorescence microscopy of cultured cells (I, III, IV)

A293 cells, cultured podocytes and L-cells grown on glass coverslips were washed with PBS and fixed with PFA or acetone. L-cell hanging drop cultures were directly fixed with acetone. PFA-fixed cells were permeabilized with Triton X-100. Cells were then washed with PBS, blocked with the blocking solution (2% FBS, 2% BSA, 0.2% fish gelatine in PBS) or with CAS-block (Zymed Laboratories) and incubated with primary antibodies. After washing with PBS, cells were incubated with TRITC- or FITC-conjugated secondary

antibodies and Hoechst 33342 for nuclear staining, washed with PBS, mounted in Mowiol and examined using a Zeiss Axiophot 2 microscope (Zeiss).

3.6.5.3 Surface staining (I)

For surface staining of nephrin, L-cells grown on glass coverslips were incubated with an antibody directed against the extracellular domain of nephrin. After washes with PBS, stained cells were fixed with PFA, washed with PBS and blocked with 5% FBS, followed by incubation with TRITC-conjugated secondary antibodies, washes with PBS and mounting in Mowiol. Cells were viewed with a Leica TCS SP2 AOBS confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany).

3.6.6 Protein interaction studies

3.6.6.1 Co-immunoprecipitation assay (I, III)

A293 or 293T cells were transiently transfected with particular expression vectors and after 48 h cells were lysed in 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1% Nonidet P-40 supplemented with 1× Complete, EDTA-free proteinase inhibitor cocktail (Roche), 50 mM sodium fluoride and 1 mM sodium orthovanadate. Insoluble material was removed by centrifugation and total protein concentration was measured by using a Bradford assay. Cell lysates were pre-cleared with protein A or G sepharose (Zymed Laboratories), followed by incubation overnight with primary antibodies or purified IgG serving as controls. Protein A or G sepharose was used to capture antibody-protein(s) complexes, and unbound proteins were removed by washing. For immunoprecipitations with intracellular fusion proteins (Study I), the preclearing step was omitted and cell lysates were incubated overnight with protein G sepharose, followed by washing steps. Finally, all samples were boiled in Laemmli sample buffer and immunoblotted.

3.6.6.2 Pull-down assays with the extracellular domain of Neph3 (I)

Signal plgplus vector containing the extracellular domain of Neph3 was transfected into 293T cells, and after 48 h the Neph3 fusion protein was purified from the cell culture medium using protein A sepharose. Rat glomerular lysate was pre-cleared with protein A sepharose, followed by incubation with Neph3 fusion protein conjugated with protein A sepharose. Sepharose beads were washed and boiled in Laemmli sample buffer, and the samples were analysed by immunoblotting.

3.7 Computer-based promoter analysis

3.7.1 Promoter alignment and identification of transcription factor binding sites (II-IV)

Comparison of the nephrin and Neph3 promoter sequences between different species was performed using the alignment program DiAlign in the Genomatix software suite (www.genomatix.de). Putative binding sites for transcription factors in the nephrin and Neph3 promoter were identified using MatInspector software of Genomatix. The maximum core and matrix similarity is 1.0, and MatInspector considers a “good” match to the matrix to have similarity of > 0.80. The values of core and matrix similarities used to scan sequences for matches are reported in the Results section.

3.7.2 Identification of Alu elements and CpG islands (II, III)

Genomic repeats tool in Genomatix was used to localize Alu elements. To analyse the presence of CpG islands in the nephrin and Neph3 promoter, CpG Island Searcher (cpgislands.usc.edu/) and MethPrimer (www.urogene.org/methprimer/index1.html) were used.

3.8 Chromatin immunoprecipitation (II-IV)

To study the binding of transcription factors to the specific nephrin and Neph3 promoter regions, chromatin immunoprecipitation (ChIP) was performed with differentiated human podocytes using the ChIP-IT™ Express Kit (Active Motif, Carlsbad, CA, USA). Transcription factors were cross-linked to DNA by treating cells with 1% formaldehyde, after which cells were washed and the fixation reaction was terminated by adding glycine. Cells were collected and lysed, and thereafter, nuclei were pelleted and chromatin was sheared by sonication into fragments of approximately 200-600 bp. For immunoprecipitations, sheared chromatin was incubated overnight with specific antibodies or with IgG serving as a negative control. Immunocomplexes bound to protein G magnetic beads were collected and washed, the protein-DNA crosslinks were reversed and DNA was eluted for real-time PCR analysis performed in an iCycler system (Bio-Rad Laboratories). PCR reactions, primers and profiles are listed in the original publications II-IV. The specificity of the PCR products was verified by both melting curve analysis and agarose gel electrophoresis.

3.9 mRNA expression analysis

3.9.1 RNA preparation (II, III)

Total RNA was isolated from A293 cells and cultured podocytes with RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA was treated with DNase I (Promega) and reverse-transcribed into cDNA (complementary DNA) using Primer random p(dN)6 (Roche) and M-MLV Reverse Transcriptase enzyme (Promega) as suggested in the manufacturer's instructions.

3.9.2 Conventional reverse transcription-PCR (II)

Conventional reverse transcription-PCR (RT-PCR) was performed with HotStar Taq DNA polymerase (Qiagen). A detailed description of the PCR and primers can be found in Study II. PCR products were visualized on an agarose gel, and identity of the amplification products was confirmed by sequencing.

3.9.3 Quantitative reverse transcription-PCR (II, III)

Quantitative reverse transcription-PCR (qRT-PCR) was performed with an iCycler system (Bio-Rad Laboratories) using TaqMan gene expression assays (Applied Biosystems, Foster City, CA, USA) for human nephrin (Assay ID: Hs00190446_m1), Neph3 (Assay ID: Hs00375638_m1) and GAPDH (Hs99999905_m1) genes, and TaqMan Fast Universal PCR Master Mix (Applied Biosystems). The expression levels of nephrin and Neph3 mRNA were normalized against GAPDH using the comparative C_t ($\Delta\Delta C_t$) method.

3.10 Statistical analyses

Statistical analyses were performed using Student's *t*-test between two groups and using one-way ANOVA followed by the least significant difference (LSD) *post hoc* test for comparisons of several groups. *P*-values less than 0.05 were considered statistically significant.

4 RESULTS

4.1 Neph3 is a component of the nephrin protein complex (I)

Earlier studies have shown that Neph3 localizes to the SDs and shares high homology with other members of the Neph-protein family, Neph1 and Neph2 (Ihalmo et al., 2003; Sellin et al., 2003; Ihalmo et al., 2007). Neph1 and Neph2 interact with nephrin, and the extracellular domains of Neph1 and nephrin form homo- and heterodimers and these extracellular interactions are suggested to be involved in the formation of the SD (Barletta et al., 2003; Gerke et al., 2003; Liu et al., 2003; Gerke et al., 2005). We investigated whether Neph3 binds to nephrin and the nephrin protein complex and if so which parts of the proteins participate in the interactions. Co-immunoprecipitation assay using lysates from 293T cells overexpressing full-length nephrin and myc-tagged Neph3 revealed that nephrin and Neph3 interact with each other (Study I, Figure 1A). Pull-down assay using rat glomerular lysates and a fusion protein of the extracellular part of Neph3 showed that the extracellular domain of Neph3 mediates the interaction with nephrin (Study I, Figure 1B). Immunoprecipitation assays from 293T cells overexpressing full-length myc-tagged Neph3 and the intracellular part of nephrin, Neph1 or Neph3 showed that the intracellular domains of nephrin, Neph1 and Neph3 participate in the interactions with Neph3 (Study I, Figure 1C). These results establish that Neph3 homodimerizes and interacts with nephrin and Neph1, indicating that Neph3 is a component of the nephrin protein complex, and thus, may, similarly to nephrin and other Neph-family members, participate in the formation of the SD.

4.2 Adhesive properties of nephrin, Neph1 and Neph3 (I)

Since interactions between nephrin and Neph-family members are suggested to induce adhesion between neighbouring podocytes leading to the formation of the SD (Barletta et al., 2003; Gerke et al., 2003; Liu et al., 2003; Gerke et al., 2005), we used mouse L fibroblasts (L-cells) as a model to investigate the adhesion activity of nephrin, Neph1 and Neph3. For this purpose, we established stable L-cell lines expressing nephrin, EGFP-tagged Neph3 or HA-tagged Neph1 alone or in combination and performed a hanging drop aggregation assay using these cell lines. The results revealed that nephrin alone does not induce cell adhesion, whereas Neph1 and Neph3 are able to induce cell adhesion alone, and further, the adhesion activity of Neph1 and Neph3 was enhanced when they *trans*-interacted with nephrin. *Cis*-interactions between nephrin and Neph1 or Neph3 did not induce cell adhesion. A summary of the adhesion activities of nephrin, Neph1 and Neph3 in L-cells is presented in Figure 8. These results suggest that Neph1 and Neph3 are homophilic adhesion molecules and further indicate that *trans*-interactions of Neph1 and Neph3 with nephrin promote cell adhesion.

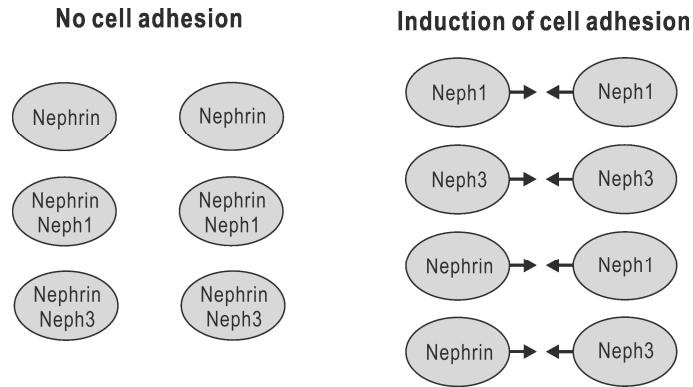


Figure 8. Summary of the adhesion activities of nephrin, Neph1 and Neph3 in L-cells stably expressing nephrin, Neph1 and/or Neph3. Homophilic interactions of nephrin or cis-interactions between nephrin and Neph1 or Neph3 did not induce cell adhesion. Neph1 and Neph3 alone as well as their trans-interactions with nephrin induced cell adhesion (Study I).

Tyrosine phosphorylation of nephrin mediates actin reorganization in podocytes (Jones et al., 2006; Verma et al., 2006; Zhu et al., 2008) and is increased during podocyte development and in experimental animal models showing podocyte injury and lack of proper SD structures (Li et al., 2004; Verma et al., 2006; Garg et al., 2007). We investigated whether cell adhesion induced by the *trans*-interaction of nephrin with Neph1 or Neph3 alters the tyrosine phosphorylation status of nephrin. To this end, immunoprecipitation with nephrin antibody and quantitative immunoblot assay were performed from hanging drop cultures expressing nephrin only, cultures expressing nephrin and EGFP-tagged Neph3 or HA-tagged Neph1 in the same cell, or a combination of cultures expressing nephrin and EGFP-tagged Neph3 or HA-tagged Neph1 in different cells. The results showed that nephrin tyrosine phosphorylation stayed at similar levels in hanging drop cultures expressing nephrin and Neph1 or Neph3 in the same cell as in the culture expressing nephrin only. However, nephrin tyrosine phosphorylation decreased markedly in hanging drop cultures expressing nephrin and Neph1 or Neph3 in different cells relative to the culture expressing nephrin only (Study I, Figure 4A). The results further demonstrated that the decrease in nephrin phosphorylation occurs at sites other than Tyr1204 and Tyr1228 (rat sequence) (Study I, Figure 4E-F). The results indicate that the *trans*-interaction between nephrin and Neph1 or Neph3 inducing cell adhesion results in nephrin dephosphorylation.

4.3 Neph3 is up-regulated in nephrin-deficient mouse kidneys (I)

Mice lacking nephrin are born without typical SDs, but the podocyte foot processes of these mice are connected by narrow tight junction-like structures (Rantanen et al., 2002). Since

we found that Neph1 and Neph3 are homophilic adhesion molecules mediating cell-cell contact formation, the localization and expression of Neph1 and Neph3 in nephrin-deficient mouse kidneys were investigated. Quantitative immunoblotting analysis revealed a significant increase in Neph3 expression in nephrin-deficient mouse kidneys, whereas the increase in Neph1 expression did not reach statistical significance (Study I, Figure 5A-B). Immunofluorescence microscopy showed a similar expression pattern of Neph3 in nephrin-deficient mouse kidney sections as in wild-type kidneys; Neph3 localized in the podocytes lining the capillary loops (Study I, Figure 5C). These results suggest that when nephrin is absent Neph3 may have a role in the formation of tight junction-like structures between podocytes.

4.4 Characterization of the chromosomal region of Nephrin-Neph3 gene pair

4.4.1 Nephrin-Neph3 promoter sequence comparison between species

The human nephrin and Neph3 genes, *NPHS1* and *KIRREL2*, respectively, are located on chromosome 19q13.12 in a head-to-head orientation separated by a 4914-bp intergenic region. The mouse and rat nephrin and Neph3 genes have a similar head-to-head arrangement, but in mouse 7qB1 and rat 1q21 chromosomes, genes are separated by shorter intergenic regions, 2542 bp and 2811 bp, respectively. This kind of bidirectional arrangement is often conserved at least among mammals, and the gene products of many bidirectional gene pairs are functionally related to each other (Adachi and Lieber, 2002; Trinklein et al., 2004; Li et al., 2006). Using UCSC Genome Browser and NCBI Map Viewer, the chromosomal regions flanking the nephrin-Neph3 gene pair in humans, mice and rats were investigated. The analysis revealed that in all species at least some of the genes near the nephrin-Neph3 gene pair are the same, e.g. *APLP1*, *PRODH2* and *UPK1A*. This suggests that not only the gene pair arrangement but also the whole chromosomal area may have been conserved throughout evolution. Sequences for the human, mouse and rat nephrin genes were from GenBank under accession numbers NM_004646, NM_019459 and AF161715, respectively, and for the human and mouse Neph3 genes NM_199180 and NM_130867, respectively. For the rat Neph3 gene, GenBank listed only the predicted sequence (XM_002725604). Genomic promoter sequences were obtained from the UCSC Genome Browser.

Genomatix alignment tool DiAlign was used to perform a comparison of the human, mouse and rat sequences covering the region between the first exons of nephrin and Neph3 genes. The comparison revealed that, excluding the primate-specific Alu repetition sequences in human sequence (see Section 4.4.2), there is a high degree of local sequence similarity among these species. The human sequence shared 42% and 46% nucleic acid identity with mouse and rat sequences, respectively. The rat sequence showed 83% identity to the mouse sequence. Similarities in promoter sequences suggest a conservation of important regulatory elements. Figure 9 shows the genomic arrangement of the human, mouse and rat nephrin and Neph3 genes and features in the intergenic region. Figure 10 shows the

alignment of the promoter regions considered interesting due to the presence of potential important regulatory elements; these are described in more detail in later sections of the results.

The mouse nephrin sequence contains an alternatively used exon 1B which is expressed only in the cerebellum. The transcription start site (TSS) for exon 1B is located 1808 bp upstream of the TSS for exon 1A (Beltcheva et al., 2003). Mouse exon 1B region, starting from the TSS, was compared with the same region in humans and rats. The sequences in this area showed 69% and 64% nucleic acid similarities for humans compared with mice and rats, respectively, and 90% similarity for the rat compared with the mouse. Whether the same alternatively used exon is present also in human and rat sequences remains to be elucidated.

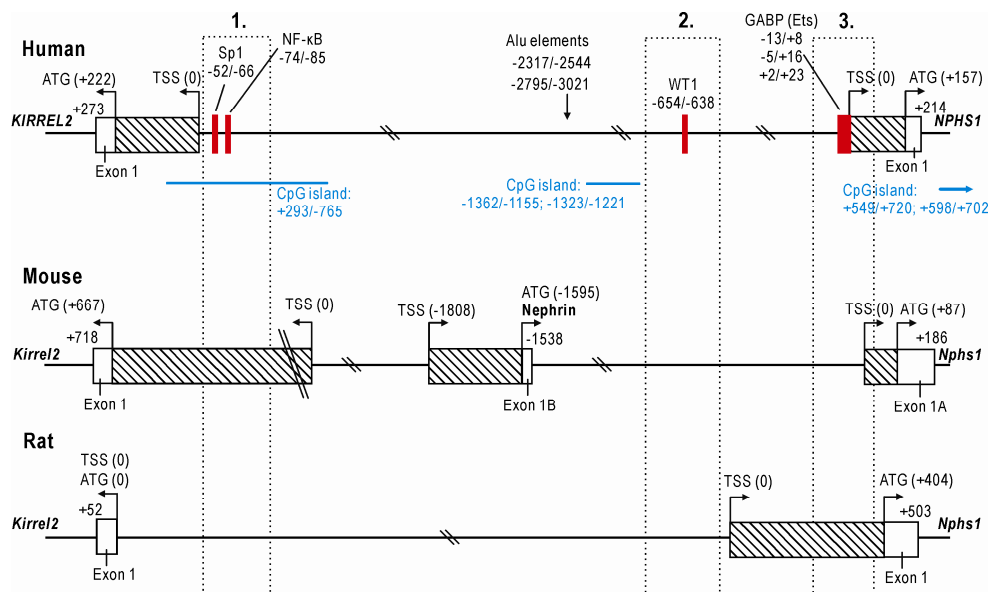


Figure 9. Genomic arrangement of nephrin and Neph3 genes in humans (*NPHS1* and *KIRREL2*, respectively) and in mice and rats (*Nphs1* and *Kirrel2*). TSS indicates the transcription start site and ATG the position of the translation initiation site. First exons are marked with white boxes and 5' untranslated regions with hatched boxes. The location of Alu elements related to the *Neph3* TSS is shown. The CpG islands are indicated with blue lines under the human sequence; the position of the CpG island in *Neph3* proximal promoter and coding region is shown related to the *Neph3* TSS and the positions of the two others related to the *neph3* TSS. Functional transcription factor binding sites are marked with red boxes and the dotted line shows regions (1-3) considered interesting due to the presence of potential important regulatory elements. The alignment of the sequences of these regions is shown in Figure 10. Numbers indicate the distance in base pairs from the major TSS. TSS, transcription start site.

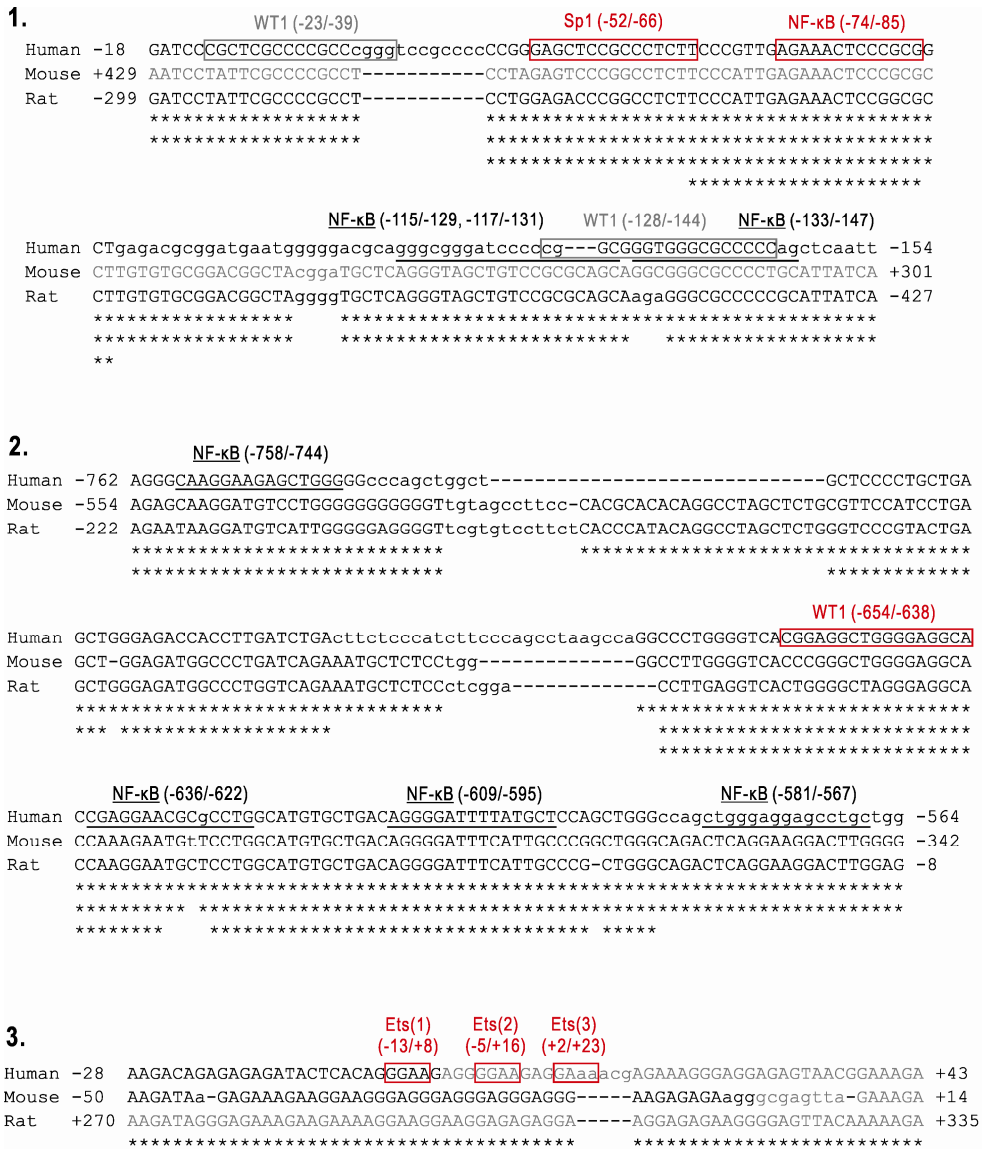


Figure 10. Alignment of sequences of regions 1-3 in Figure 9. The number of “*” characters below the alignment reflects the degree of local similarity; the maximum similarity is 5 “*” characters. Capital letters denote aligned nucleic acids and lower-case letters are considered not to be aligned. Lines are introduced to align the regions of maximal homology. Functional binding sites for indicated transcription factors are marked with red and putative binding sites for indicated transcription factors with black or grey. The numbers in region 1 indicate the distance in base pairs from the Neph3 TSS and in regions 2 and 3 from the nephrin TSS. Grey letters indicate the 5' untranslated and coding region. TSS, transcription start site.

4.4.2 Features of the upstream region of human Neph3 gene (II)

In the human genome, the TSSs of the nephrin and Neph3 genes are separated by a 4914-bp-long intergenic region. Several studies have investigated the regulatory region of the nephrin gene, and thus, we concentrated on the intergenic region of the nephrin and Neph3 genes mainly characterizing the 5' upstream region from the Neph3 TSS. Sequence analysis revealed that the Neph3 proximal promoter between -200 bp and the TSS is a highly GC-rich region, with a GC content of 73.5%. The region close to the start of transcription was noted to be devoid of typical TATA and CAAT boxes, as often seen with GC-rich promoters. Two primate-specific Alu repetition sequences were localized in the region from -3021 to -2795 and from -2544 to -2317 bp upstream from the TSS using Genomic repeats tool in Genomatix (Figure 9; page 50). Alternative promoters or TSSs were not found for Neph3 in literature or database searches using the UCSC Genome Browser.

4.5 NF- κ B and Sp1 regulate basal level expression of Neph3 gene (II)

To determine the minimal promoter region required for the basal transcriptional activity of the human Neph3 gene, a series of Neph3 luciferase reporter gene constructs containing various fragments of the Neph3 promoter were used. The constructs covered nucleotides from -5070, -4543, -3877, -2260, -1072, -536, -366, -162, -105 and -57 to +48 related to the Neph3 TSS. The proximal promoter region starting from -105 was revealed to be the major activation region for the Neph3 gene in A293 cells and the regulatory elements important for the constitutive transcriptional activity of the Neph3 gene were mainly located in the region between nucleotides -105 and -58 (Figure 11). Results also revealed the possible presence of a weaker activation site in the distal promoter region from -5070 to -3878, whereas repressing transcription factor binding sites may be present in the regions from -3877 to -2261 and from -162 to -106. Two Alu elements found in the human sequence were located within the region -3877/-2261, which may also be the reason for lower promoter activity.

To define the transcription factors responsible for Neph3 promoter activity, the Neph3 promoter region between nucleotides -105 and -58 was investigated in more detail. The sequence comparison between humans, mice and rats revealed a high degree of similarity (Figure 10, region 1; page 51). The location of transcription factor binding sites within this region in the human sequence was predicted by MatInspector software, and putative binding sites for NF- κ B and Sp1 were found at positions -85 to -74 (core/matrix similarity: 1.000/0.886) and -66 to -52 (1.000/0.930), respectively (Figure 10, region 1; page 51). According to site-directed mutations of the NF- κ B and Sp1 binding sites, both response elements are essential for basal level activity of the Neph3 -105 promoter in A293 cells (Study II, Figure 3C). Furthermore, both transcription factors bound in a ChIP to the Neph3 promoter region harbouring their respective binding sites (Study II, Figure 7).

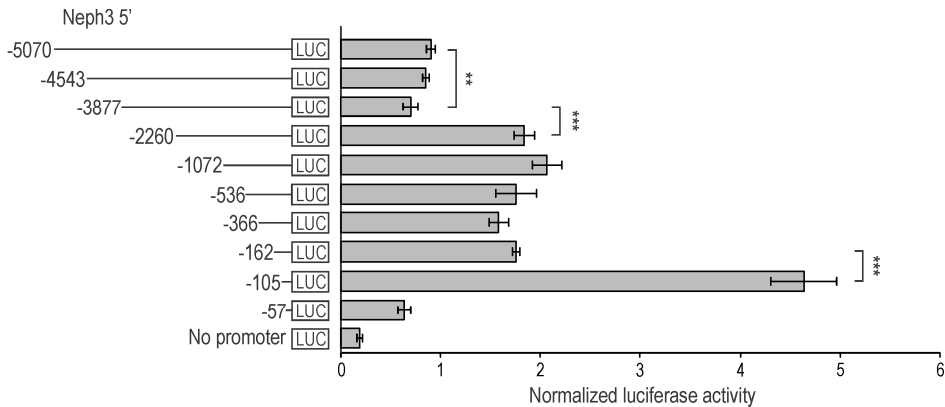


Figure 11. Transcriptional activity of the 5'-flanking regions of the human *Neph3* gene in A293 cells. The values represent means \pm SD of one experiment performed in triplicate. The significance between the samples indicated by brackets is shown (** $P < 0.01$, *** $P < 0.001$). LUC, luciferase gene (Study II and III).

The role of NF- κ B in *Neph3* gene regulation was further analyzed and NF- κ B p65 overexpression was found to increase *Neph3* mRNA level, and simultaneous overexpression of p50 and p65 led to a further increase (Figure 12A; page 55). This suggested that NF- κ B functions as a p50/p65 heterodimer in *Neph3* gene regulation. Reporter gene assay revealed that the activity of the *Neph3* -105 promoter region is increased by p65 overexpression, but simultaneous overexpression of p50 and p65 subunits did not lead to a further increase (Figure 12B; page 55). However, the longer *Neph3* promoter region, -162, reached higher promoter activity by simultaneous p50 and p65 overexpression than by p65 overexpression alone (Figure 12B; page 55). This supports NF- κ B acting as a p50/p65 heterodimer in regulating the *Neph3* gene. The results also suggests that the region between nucleotides -162 and -106 may harbour additional functional NF- κ B binding sites or other sequences needed for optimal binding and function of NF- κ B as a p50/p65 heterodimer. A search with MatInspector revealed several potential binding sites for NF- κ B within the region from -162 to -106. According to the core and matrix similarity values, the two best matches occupy the same nucleotides at positions -129 to -115 (1/0.877) and -131 to -117 (1/0.917). These sites are located in the region thought not to be conserved (lower-case letters in Figure 10, region 1; page 51), but a lack of conservation does not guarantee that the sites are not functional (Henikoff, 2007; Odom et al., 2007). By decreasing the core and matrix similarities, putative binding sites for NF- κ B were found also in the conserved regions, with the site with the highest core and matrix similarity values located at position -147 to -133 (0.75/0.805) (Figure 10, region 1; page 51).

Neph3 mRNA level was not increased by Sp1 overexpression in assays performed in A293 cells, probably due to a high endogenous level of Sp1 in this cell type. However, reporter gene assay performed in *Drosophila* Schneider 2 (S2) cells, a cell model widely used in Sp1 studies due to their lack of endogenous Sp1, revealed that Sp1 overexpression activates the *Neph3* -105 promoter (Study II, Figure 5). Further, when Sp1 binding site in the -105

construct was mutated, the activation by Sp1 was abolished, indicating that Sp1 activates Neph3 gene transcription through Sp1 binding site at position -66 to -52.

4.6 WT1 and NF- κ B synergistically activate nephrin and Neph3 genes (III)

Bidirectional gene pairs often have a shared promoter or common regulatory mechanisms that regulate both genes (Adachi and Lieber, 2002; Trinklein et al., 2004; Li et al., 2006). This prompted us to study whether nephrin and Neph3 genes share similar mechanisms in their transcriptional regulation. WT1 is a key regulator of podocyte function and it has previously been shown to activate transcription of the nephrin gene (Guo et al., 2004, Wagner et al., 2004). Thus, we hypothesized that WT1 might also regulate the Neph3 gene. Multiple putative binding sites for WT1 were found in at least partly conserved regions of the Neph3 promoter downstream and upstream from the functional NF- κ B and Sp1 binding sites. According to the core and matrix similarity values, the best matches were located at positions -39 to -23 (1/0.930) and -144 to -128 (1/0.887) (Figure 10, region 1; page 51). WT1 was observed to occupy the Neph3 proximal promoter shown by a ChIP assay (Study III, Figure 4B), but luciferase reporter assay did not show an increase in Neph3 -162 promoter activity with WT1 overexpression (Figure 12B). This led us to investigate whether WT1 collaborates with other transcription factor(s), and NF- κ B was considered a candidate. Interestingly, Neph3 -162 promoter activity reached the highest level when WT1 and NF- κ B subunits p50 and p65 were overexpressed at the same time, suggesting co-operativity between WT1 and NF- κ B in the transcriptional regulation of the Neph3 gene (Figure 12B). Contrary to the reporter gene assay, WT1 overexpression up-regulated Neph3 mRNA (Figure 12A), which might be explained by the artificial nature of promoter DNA in reporter constructs compared with the chromosomal structure of the endogenous gene. Synergism between WT1 and NF- κ B was further supported by the finding that the NF- κ B p50/p65 heterodimer co-operates with WT1 in up-regulating Neph3 mRNA expression (Figure 12A).

In the search for common regulatory mechanisms for nephrin and Neph3 genes, potential co-operation of WT1 and NF- κ B underlying also the regulation of the nephrin gene was investigated. Previous findings demonstrate that the functional WT1 binding site in the human nephrin promoter is located at position -654/-638 bp relative to the nephrin TSS (Guo et al., 2004). The use of MatInspector revealed several putative binding sites for NF- κ B (Figure 10, region 2; page 51). One putative binding site for NF- κ B was located next to the WT1 site in a conserved area of the nephrin promoter, occupying nucleotides -636 to -622 (1/0.818). Another putative NF- κ B binding site occupied nucleotides -581 to -567 (1/0.838) and was located downstream of the WT1 site in a region whose sequence was not considered to be conserved in humans, mice and rats. By decreasing the core and matrix similarity values, more putative binding sites for NF- κ B were found in the conserved regions downstream and upstream of the WT1 site. The best matches were located at positions -609 to -595 (0.75/0.747) and -758 to -744 (0.75/0.777).

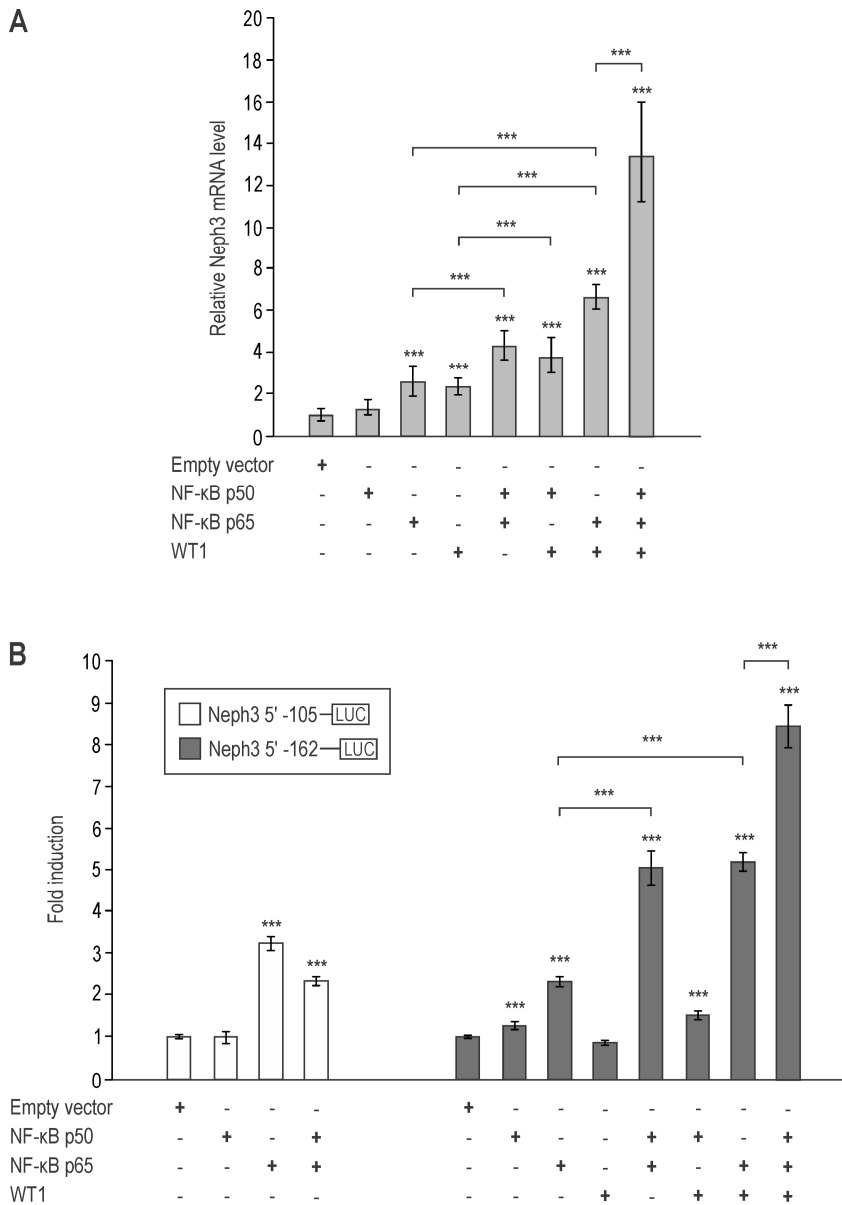


Figure 12. Summary of the effect of NF-κB and WT1 overexpressions on Neph3 mRNA level (A) and promoter activity (B). (A) Data are presented relative to the control transfection (empty vector), and the values represent means and range of three measurements from two biological replicate experiments. (B) The results are presented as fold induction of the control experiment (empty vector), and the values represents means \pm SD from three independent experiments performed in triplicate. The significance relative to the control transfection or between the samples indicated by brackets is shown (***) $P < 0.001$. LUC, luciferase gene (Studies II and III).

By a ChIP assay, WT1 and NF- κ B p50 and p65 were shown to bind to the respective region of their putative binding sites in the nephrin promoter (Study III, Figure 4A). Overexpression of WT1 and NF- κ B followed by analysis of nephrin mRNA level revealed that NF- κ B p50 up-regulates nephrin mRNA expression and functions synergistically with WT1 (Figure 13A). Reporter gene assay of the nephrin -837 promoter also showed a synergistic effect of WT1 and p50, but also of p50 and p65. Individual p65 overexpression had no effect on nephrin promoter activity (Figure 13B), however, it did cause a massive increase in nephrin mRNA level (Figure 13A). These results suggest that WT1, p50 and p65 are involved in nephrin gene regulation, but it is difficult to determine whether p65 co-operates together with p50 and WT1. The nephrin promoter region may have multiple functional binding sites for NF- κ B since in reporter gene assay, NF- κ B overexpression induced also shorter fragment of the nephrin promoter (nephrin -617 promoter; Figure 13B).

Collectively, these results indicate that WT1 and NF- κ B co-operatively regulate nephrin and Neph3 genes, which is further supported by an interaction between WT1 and NF- κ B p65 as revealed by co-immunoprecipitation (Study III, Figure 5).

4.7 TNF- α induces nephrin and Neph3 mRNA expression via the NF- κ B pathway (III)

NF- κ B is an inducible transcription factor that is activated and translocated into the nucleus as a result of phosphorylation, ubiquitination and degradation of the cytoplasmic inhibitor I κ B- α bound to NF- κ B (reviewed by Hayden and Ghosh, 2008). The involvement of NF- κ B in the transcriptional regulation of nephrin and Neph3 genes prompted us to investigate the pathway leading to NF- κ B-dependent nephrin and Neph3 gene expression. Previous findings have demonstrated that NF- κ B activator TNF- α induces nephrin mRNA expression in human embryonic kidney cells (Huwiler et al., 2003). We took advantage of cultured human podocytes and treated them with TNF- α and further with the NF- κ B pathway inhibitor BAY 11-7082, an inhibitor of cytokine-induced I κ B- α phosphorylation, to confirm the involvement of NF- κ B activation after TNF- α induction. Both nephrin and Neph3 mRNA expression was induced in response to TNF- α ; nephrin mRNA expression was induced time- and dose-dependently (Figure 14A, page 58), and for Neph3, time- and dose-dependent responses were observed, but the responses were not statistical significant. Treatment with BAY 11-7082 attenuated TNF- α -induced nephrin and Neph3 mRNA expression (Figures 14B-C, page 58), indicating that NF- κ B activation mediates the effect of TNF- α . Immunofluorescence stainings with NF- κ B p65 antibody showed that TNF- α induced nuclear translocation of NF- κ B and BAY 11-7082 inhibited this translocation, confirming that NF- κ B is indeed inhibited (Study III, Figure 2E). Collectively, the results demonstrate that in cultured human podocytes NF- κ B regulates nephrin and Neph3 mRNA in response to TNF- α .

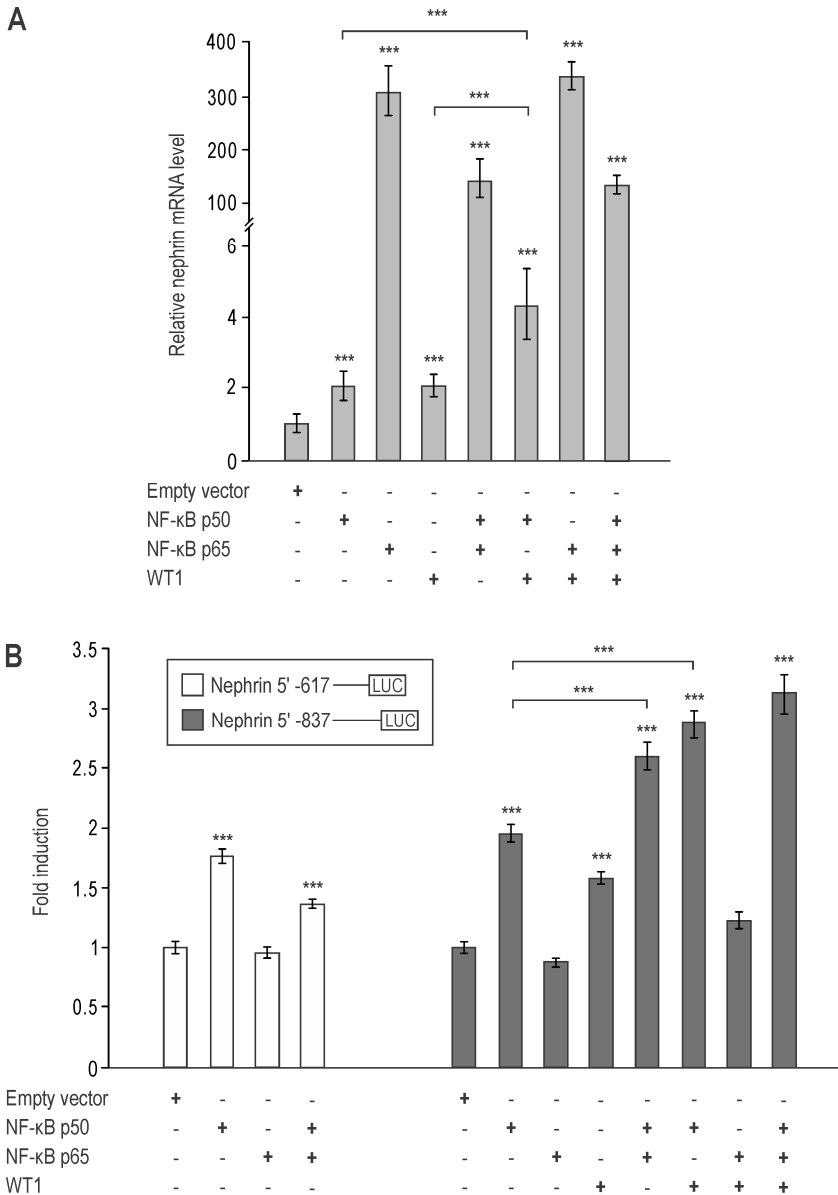


Figure 13. Summary of the effect of NF-κB and WT1 overexpressions on nephrin mRNA level (A) and promoter activity (B). (A) Data are presented relative to the control transfection (empty vector), and the values represent means and range of three measurements from two biological replicate experiments. (B) The results are presented as fold induction of the control experiment (empty vector), and the values represents means \pm SD from three independent experiments performed in triplicate. The significance relative to the control transfection or between the samples indicated by brackets is shown (***) $P < 0.001$. LUC, luciferase gene (Study III).

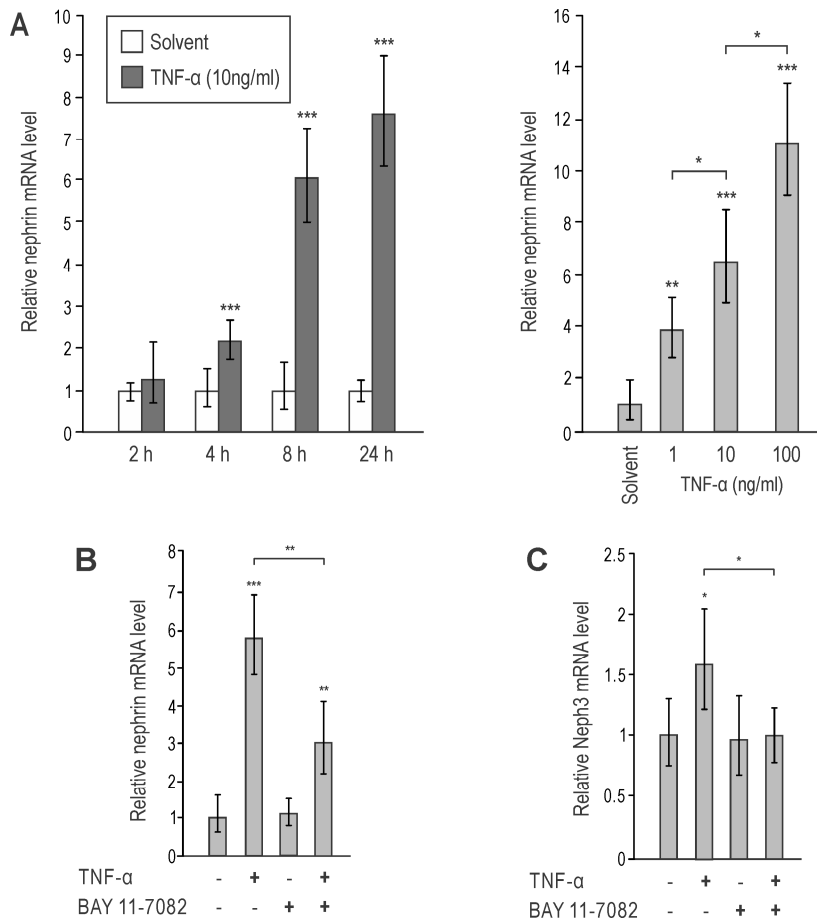


Figure 14. (A-C) TNF- α induces the expression of endogenous nephrin and Neph3 mRNA in podocytes via the NF- κ B pathway. Data are presented relative to the cells treated with solvent only, and the values represent means and range of three measurements from two biological replicate experiments. The significance relative to the control transfection or between the samples indicated by brackets is shown (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) (Study III).

4.8 DNA methylation plays a role in regulating nephrin and Neph3 genes (III)

Characterization of the Neph3 promoter revealed a highly GC-rich region in the Neph3 proximal promoter (Section 4.4.2). In GC-rich regions, clusters of CpG dinucleotides are called CpG islands, and they represent an important feature of mammalian genomes. Methylation of CpG islands is involved in the regulation of transcription by leading to silencing of the associated gene (Siegfried et al., 1999; Strathdee et al., 2004). Interestingly, bidirectional gene pairs feature a significant enrichment of CpG islands, and their hypermethylation has been shown to silence the expression of both genes simultaneously (Adachi and Lieber, 2002; Trinklein et al., 2004; Shu et al., 2006). The presence of CpG islands in the intergenic region between the nephrin and Neph3 genes and in the coding regions of the nephrin and Neph3 genes was sought using two different CpG island searcher programs. The search criteria for a CpG island were defined as at least 50% GC content and an observed/expected CpG ratio greater than 0.6. CpG Island Searcher identified one CpG island covering the Neph3 proximal promoter and coding region between nucleotides -765 and +293, one CpG island closer to the nephrin TSS at position -1362/-1155 related to the nephrin TSS and one CpG island in the nephrin coding region at position +549/+720. MethPrimer program identified two CpG islands at similar locations in the nephrin promoter and coding region, at positions -1323/-1221 and +598/+702. The location of CpG islands in the human sequence is shown in Figure 9 (page 50).

The presence of CpG islands in the regulatory regions of nephrin and Neph3 genes suggests that DNA methylation could play a role in regulating these genes. To investigate this, different types of cells, undifferentiated and differentiated podocytes and A293 cells, were treated with demethylating agent 5-aza-2'-deoxycytidine (5-azadC). In undifferentiated podocytes, which do not express nephrin or Neph3 mRNA at the basal level, the treatment resulted in dose-dependent increase in the mRNA expression of both nephrin and Neph3 (Figure 15A). In differentiated podocytes (Figure 15B) and A293 cells (Figure 15C), both expressing nephrin and Neph3 mRNA endogenously, the treatment led to at least a partly dose-dependent up-regulation of nephrin and Neph3 mRNA. In A293 cells, however, the effect of 5-azadC was much stronger than in differentiated podocytes, an *in vitro* cell model currently considered the best for podocyte studies. Collectively, these data indicate that silencing by DNA methylation plays a similar role in nephrin and Neph3 gene regulation depending on the cell type and the differentiation stage of podocytes. DNA methylation may be involved in the regulation of tissue-specific expression of nephrin and Neph3 genes.

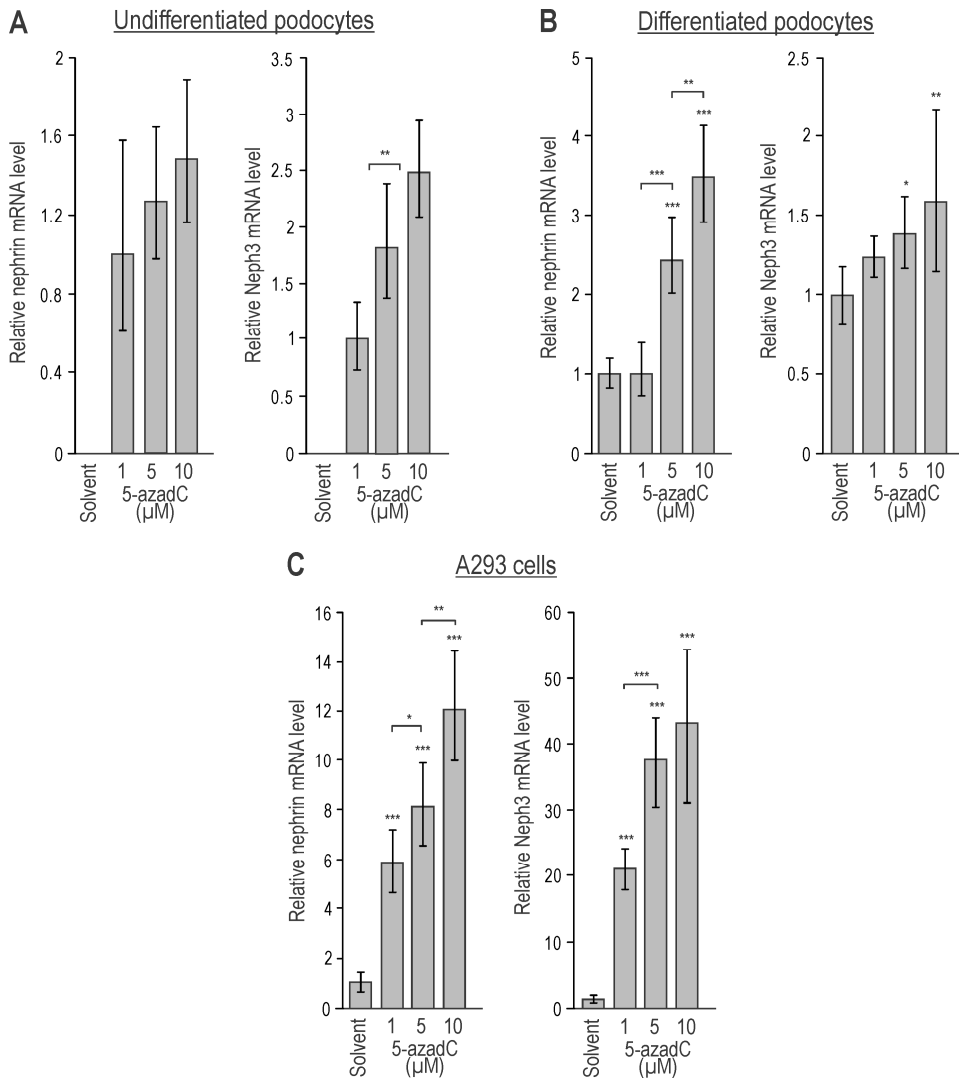


Figure 15. (A-C) Effect of demethylating agent 5-aza-2'-deoxycytidine (5-azadC) on endogenous nephrin and Neph3 mRNA in undifferentiated and differentiated podocytes and A293 cells. Data are presented relative to the cells treated with solvent only, and the values represent means and range of three measurements from two biological replicate experiments. The significance relative to the control transfection or between the samples indicated by brackets is shown (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) (Study III).

4.9 GABP regulates nephrin gene (IV)

Transcription factor GA-binding protein (GABP) is suggested to be involved in regulating the cell-cell and cell-matrix interactions in, for example, leukocytes and myeloid cells (Bottinger et al., 1994; Rosen et al., 1994; Nickel et al., 1995; Rosmarin et al., 1995; Nuchprayoon et al., 1997) as well as in the formation and function of neuromuscular junctions (Fromm and Burden, 1998; Khurana et al., 1999; Mejat et al., 2003). These studies prompted our interest in analysing the role of GABP in podocytes and in nephrin gene regulation. Since previous findings demonstrate that GABP is expressed in the adult mouse kidney at the mRNA level (O'Leary et al., 2005), but a more precise expression pattern is unknown, immunofluorescence stainings of rat kidney sections and Western blotting of rat glomerular and tubular fractions were performed. Western blotting revealed that GABP is primarily expressed in glomeruli (Study IV, Figure 1E). Immunofluorescence stainings showed that GABP and WT1 co-localize in glomeruli, indicating expression of GABP in the podocyte nucleus (Figure 16). As expected due to the ubiquitous nature of GABP expression (reviewed by Rosmarin et al., 2004), GABP was also detected in cells other than podocytes, apparently mesangial cells. In A293 cells and cultured undifferentiated and differentiated human podocytes, GABP is highly enriched in the nucleus, as indicated by Western blotting and immunofluorescence staining (Study IV, Figures 2 and 3).

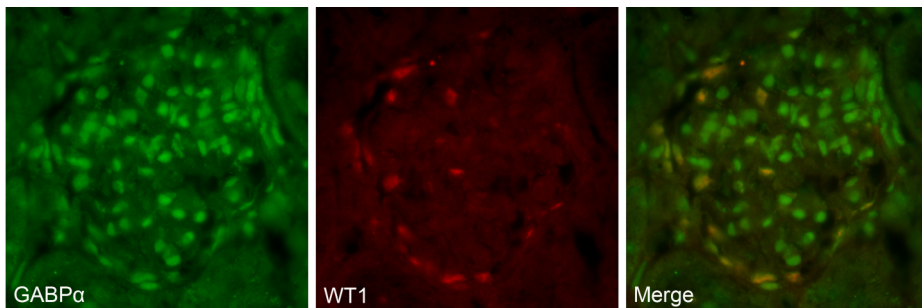


Figure 16. Double staining of GABP α and WT1 indicates that GABP is expressed in podocyte nuclei in the rat kidney (Study IV).

Because computation analysis revealed several putative binding sites for GABP distributed along the nephrin regulatory region, a series of deletion promoter fragments of nephrin (from nucleotides -837, -392, -48 and +25 to +156 related to the major nephrin TSS) in luciferase reporter vector were used to map the region where GABP might bind. Reporter gene assay with GABP α and GABP β overexpression revealed that GABP as a heterotetramer increases nephrin promoter activity (Study IV, Figure 4). Since the -48 region was activated by GABP overexpression, the nephrin promoter region between the nucleotides -48 and +24 apparently harbours the functional element(s) for GABP. GABP binds to DNA sequences called Ets motifs (5' GGAA 3'). Within the region -48/+24, MatInspector software revealed three putative GABP binding sites partially overlapping

and close to the TSS (named Ets(1), Ets(2) and Ets(3)) (Figure 10, region 3; page 51). These sites were found to be at least partly located in the conserved region, occupying nucleotides -13 to +8 (1/0.860), -5 to +16 (1/0.964) and +2 to +23 (1/0.920). A ChIP assay revealed that GABP binds to the region covering these three Ets sites (Study IV, Figure 6). According to site-directed mutations of the Ets binding sites, only Ets(1) site was essential for the basal level activity of the nephrin -48 promoter (Figure 17). However, GABP overexpression experiments with constructs harbouring mutated and non-mutated Ets binding sites showed that Ets(2) and Ets(3) mutations abolish the activation by GABP overexpression, confirming that at least these two sites are functional GABP binding sites (Figure 17). These results suggest that GABP is able to stimulate nephrin gene transcription through Ets sites located in the proximal nephrin promoter.

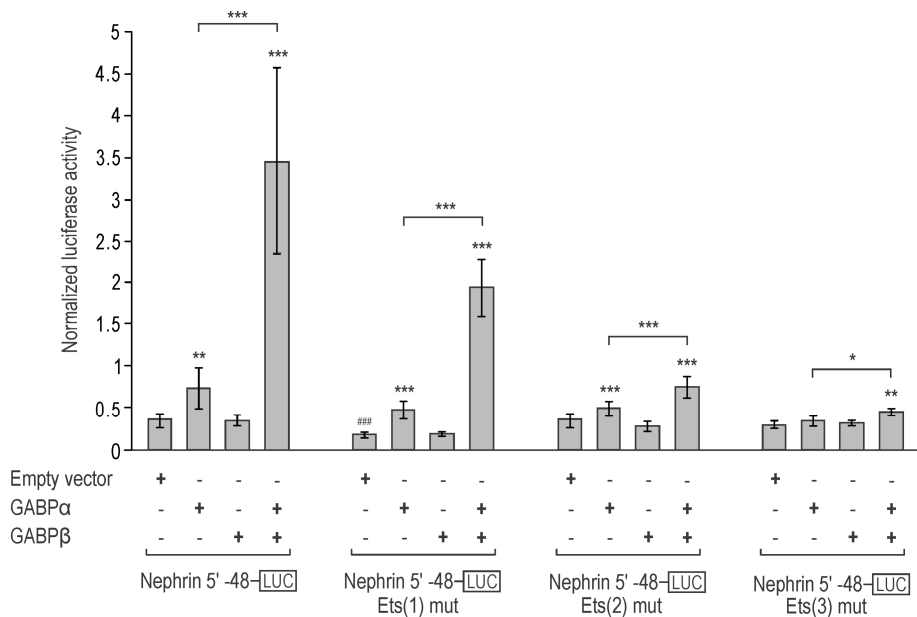


Figure 17. Effect of GABP overexpression on nephrin promoter activity. The results are presented as fold induction of the control experiment (empty vector), and the values represent means \pm SD from three independent experiments performed in triplicate. The significance relative to the control transfection (empty vector) or between the samples indicated by brackets is shown with asterisks, and hashes show the significance relative to the non-mutated construct (* $P < 0.05$, * $P < 0.01$, *** $P < 0.001$, #### $P < 0.001$). LUC, luciferase gene (Study IV).

5 DISCUSSION

In the original publications of this thesis, Neph3 was shown to be part of the nephrin protein complex and to have similar functions as nephrin and Neph1-2 in forming homo- and heterodimers to participate in cell adhesion. The work also established transcriptional mechanisms behind nephrin and Neph3 gene regulation in podocytes and revealed a role for transcription factors WT1, NF- κ B and Sp1 and DNA methylation in regulating the transcriptional activity of nephrin and Neph3 genes. Finally, transcription factor GABP was located in podocytes and shown to participate in nephrin gene regulation. The significance of these findings as they relate to existing data as well as some methodological aspects will be discussed in the following sections.

5.1 Neph3 shares similar functions with nephrin and Neph-family members

5.1.1 Neph3 is a component of the nephrin protein complex

Neph3, similarly to nephrin and the two other members of the Neph-protein family, Neph1 and Neph2, localizes to the SD (Holthöfer et al., 1999; Holzman et al., 1999; Ruotsalainen et al., 1999; Barletta et al., 2003; Liu et al., 2003; Gerke et al., 2005; Ihalmo et al., 2007). The existing data demonstrate that nephrin, Neph1 and Neph2 are present in the same protein complex (Barletta et al., 2003; Gerke et al., 2003; Liu et al., 2003; Gerke et al., 2005) and interact with the SD proteins podocin and ZO-1 (Huber et al., 2001; Huber et al., 2003b; Sellin et al., 2003; Lehtonen et al., 2004). Since Neph3 has also been shown to interact with podocin and ZO-1 (Huber et al., 2003b; Sellin et al., 2003), it was speculated to be a component of the nephrin protein complex. We showed that Neph3 interacts with nephrin and Neph1, and similarly to nephrin, Neph1 and Neph2 (Gerke et al., 2003; Khoshnoodi et al., 2003; Gerke et al., 2005), it forms homodimers (Study I). Thus, Neph3 belongs to the nephrin protein complex and most probably shares similar functions and behaviour with nephrin and Neph-family members and participates in the formation of the SD.

5.1.2 Nephrin, Neph1 and Neph3 in cell adhesion

Nephrin and Neph-family members are suggested to play a key role through their interactions in cell adhesion between neighbouring podocytes. In *Drosophila* and *C. elegans*, the interactions between nephrin and Neph1-3 orthologues that occur between opposing cell surfaces promote cell adhesion during muscle, eye and synapse development (Bour et al., 2000; Ruiz-Gomez et al., 2000; Dworak et al., 2001; Strunkelberg et al., 2001; Shen and Bargmann, 2003; Shen et al., 2004; Bao and Cagan, 2005). We revealed in Study I that both Neph1 and Neph3 induce cell adhesion in a homophilic fashion, whereas

nephrin does not. To form cell-cell contacts, nephrin was shown to require co-operativity with Neph1 or Neph3. However, cell-cell contacts were formed only when nephrin and Neph1 or Neph3 interacted *trans* on the opposite cell surfaces. *Cis*-interactions of nephrin and Neph1 or Neph3 in the same cell were unable to induce cell adhesion but rather inhibited the adhesion activity. If nephrin, Neph1 and Neph3 are all expressed in podocytes and localize in the SD, why does the presence of nephrin and Neph1/Neph3 in the same cell then inhibit the adhesion activity? Possibly, the intracellular interactions between nephrin and Neph1/Neph3 enhance structural and/or functional changes in the extracellular binding domains of Neph1 and Neph3, thus blocking their interactions. Further, mouse L fibroblasts (L-cells) used in these adhesion assays represent a simple cell culture model and may lack important molecules, such as certain SD components, or signalling pathways needed for proper behaviour of nephrin and Neph-family members.

L-cells, generally, do not form intercellular connections due to lack of endogenous expression of adherens junction proteins like cadherins. L-cells have therefore been widely used in studies related to adhesion and attachment; for example, in evaluating adhesion activity of some Ig superfamily proteins such as nectins (Sato-Horikawa et al., 2000). L-cells are a relevant *in vitro* model also for our purposes. However, the use of different cell models and techniques in adhesion assays may produce discrepant results. Our findings demonstrated that nephrin is unable to induce cell adhesion in a homophilic fashion, but others have using adherent HEK293 cells shown that nephrin expression induces formation of cell aggregates (Khoshnoodi et al., 2003). These results suggest that nephrin alone cannot promote cell adhesion in adhesion-deficient cells, but in adherent cells expressing proteins that most probably contribute to cell-cell contact formation, nephrin induces cell adhesion. In terms of nephrin-Neph3 interaction, we demonstrated that nephrin and Neph3 induce cell adhesion via their heterophilic *trans*-interactions, whereas Nishida et al. (2010) did not observe the same effect in Jurkat cells, which are T-lymphocytes.

5.1.3 Relationship between nephrin-Neph1/Neph3 interaction and nephrin phosphorylation

Nephrin is a signalling molecule and through the tyrosine phosphorylation of its intracellular domain nephrin induces signalling cascades that lead to actin polymerization (Jones et al., 2006; Verma et al., 2006; Zhu et al., 2008), elevated cytosolic calcium levels (Harita et al., 2009) and raft-mediated endocytosis of nephrin (Qin et al., 2009). An increase in nephrin phosphorylation is detected during podocyte development as well as in experimental animal models, such as passive Heymann nephritis, protamine sulphate nephrosis and puromycin aminonucleoside-induced nephrosis, all of which show podocyte injury and a lack of proper SD structures (Li et al., 2004; Verma et al., 2006; Garg et al., 2007). During podocyte foot process development and podocyte injury, podocytes undergo morphological alterations suggested to be linked to, for example, changes in podocyte actin cytoskeleton structure via actin polymerization. Once the SD is formed, less nephrin signalling is speculated to be needed to regulate actin cytoskeleton. In line with this, our data showed that nephrin tyrosine phosphorylation decreases when cell-cell contacts were formed through nephrin and Neph1/Neph3 *trans*-interactions (Study I). As mentioned, nephrin phosphorylation is increased during podocyte development and in experimental

animal models of podocyte injury (Li et al., 2004; Verma et al., 2006; Garg et al., 2007). However, nephrin is phosphorylated also in mature podocytes (Jones et al., 2009), and a decrease in nephrin phosphorylation is detected in patients with minimal change nephrosis and in rats with puromycin aminonucleoside-induced nephrosis (Uchida et al., 2008). Thus, nephrin phosphorylation and de-phosphorylation occurring at multiple sites in the nephrin intracellular domain and the balance between the phosphorylated and dephosphorylated stages appear to be important factors in maintaining the integrity of the SD as well as podocyte morphology and function.

5.2 Lack of nephrin leads to up-regulation of Neph3

In mice lacking nephrin, podocyte foot processes are not connected to the SD, but the filtration slits are narrowed and connected by tight junction-like structures and show up-regulation of tight junction protein claudin-3 (Rantanen et al., 2002; Done et al., 2008). We established that Neph3 expression is up-regulated in nephrin-deficient kidneys; the expression of Neph1 was also increased, but did not reach statistical significance (Study I). Even though Neph3 is structurally related to nephrin, apparently it and its up-regulation in the absence of nephrin cannot compensate for the function of nephrin in the SD formation. Neph1 and Neph3 were shown to be homophilic adhesion molecules and to mediate cell-cell contact formation (Study I). Thus, in the absence of nephrin, Neph1/Neph3 might participate in the formation of tight junction-like structures present in nephrin-deficient mice. Notably, the extracellular domains of Neph1/Neph3 are shorter than that of nephrin, which could explain the formation of a narrower junction than the SD (Ihalmo et al., 2003; Sellin et al., 2003).

During glomerular development in zebrafish, Neph3 expression peaks in developing podocytes, but is then down-regulated upon glomerular maturation (Wang et al., 2012). Similarly, in the developing spinal cord of the mouse, Neph3 is first up-regulated in the post-mitotic neural precursor cells, but becomes down-regulated during maturation of neurons (Minaki et al., 2005). The expression of Neph3 in developing and mature podocytes in the mouse is not known. However, podocytes arise from tight junctions (Reeves et al., 1978) and the loss of podocyte foot process architecture and the appearance of tight junctions in nephrin-deficient mice indicate that podocytes regress to more primitive developmental stages. Since Neph3 is up-regulated in these mice, it could be that similar down-regulation of Neph3 as observed in zebrafish glomeruli and mouse neurons during maturation also occurs in mouse podocytes.

5.3 Nephrin and Neph3 genes are regulated by common mechanisms

5.3.1 Bidirectional arrangement of nephrin and Neph3 genes

The human nephrin and Neph3 genes, *NPHS1* and *KIRREL2*, reside on chromosome 19q13.12, where the TSSs of the genes are separated by a 4914-bp intergenic region and the genes are transcribed into opposite directions. The mouse and rat nephrin and Neph3 genes have a similar head-to-head (bidirectional) arrangement, but in mouse 7qB1 and rat 1q21 chromosomes, the genes are separated by shorter intergenic regions, 2542-bp and 2811-bp, respectively. We compared the intergenic regions of the nephrin-Neph3 gene pair among humans, mice and rats and found that, excluding the additional approximately 2-kb region in the human sequence, the sequences are highly conserved. The additional region in the human sequence was found to contain Alu elements, which are primate-specific repetitive DNA elements. Alu elements have potential regulatory functions and have been shown to contribute actively to primate evolution (reviewed by Hasler and Strub, 2006). The presence of Alu elements may explain the difference in the length of the intergenic regions of the nephrin-Neph3 gene pair between humans and rodents.

Human chromosome 19 and related regions in the mouse show conservation of general gene arrangement (Dehal et al., 2001; Grimwood et al., 2004). In line with this we revealed that the chromosomal regions flanking the nephrin-Neph3 gene pair in humans and mice as well as in rats contain the same genes. Human chromosome 19 is the most gene-dense human chromosome (Venter et al., 2001), and more than 25% of its genes belong to clustered gene families. A marked prevalence of two kinds of duplication events is present in chromosome 19: tandemly clustered gene families and large segmental duplications (Grimwood et al., 2004). Based on this and conservation of the bidirectional arrangement as well as the flanking regions of nephrin and Neph3 genes, it may be that not only the nephrin-Neph3 gene pair but the whole chromosomal area flanking it has been conserved throughout evolution and has evolved through chromosomal rearrangement or duplication.

Bidirectional arrangement of genes is a relatively common feature of the genome and is often conserved among mammals, suggesting an important biological function. Despite substantial interest in bidirectional gene pairs, their transcriptional regulatory mechanisms remain poorly understood. Many bidirectional gene pairs, e.g. *SERPINI1-PDCD10* and *PREPL-C2ORF34* gene pairs, share regulatory elements that control both genes (Chen et al., 2007; Huang and Chang, 2009). Bidirectional gene pairs typically demonstrate an overlapping, but not identical, expression pattern in different cell and tissue types. Bidirectional gene pairs coding for homologous proteins, like nephrin and Neph3, are uncommon. Instead, the gene products of bidirectional gene pairs are often functionally related to each other or they operate in the same biological pathway for which the shared regulatory region(s) provide coordinated temporal and spatial expression (Adachi and Lieber, 2002; Trinklein et al., 2004; Li et al., 2006). Computational genome-wide analysis has demonstrated that more than 10% of the human genes represent bidirectionally arranged gene pairs whose TSSs are separated by less than 1000-bp (Trinklein et al., 2004).

However, the optimal length of a bidirectional promoter is not known. For example, it has been reported that a bidirectional gene pair with even a 25-kb intergenic region shows simultaneous induction of both genes through common regulatory elements (Ueda et al., 2006). Other features describing bidirectional gene pairs include lack of TATA boxes and the presence of GC-rich promoters. Neph3 and Neph3 have all of the features suggested for bidirectional gene pairs, and it is therefore likely that neph3 and Neph3 genes share key features in their regulation.

5.3.2 WT1, NF- κ B and Sp1 in the regulation of neph3 and Neph3 genes

The genomic arrangement of neph3 and Neph3 genes and the high degree of similarity in their promoter sequences among humans, mice and rats indicate conservation of important regulatory elements and common regulatory mechanisms for neph3 and Neph3 genes.

WT1 is an important transcription factor in neph3 gene expression (Guo et al., 2004; Wagner et al., 2004), but several different transcription factors apparently participate in the constitutive regulation of the neph3 gene in podocytes. A 1.25-kb region of the human neph3 proximal promoter is essential for the podocyte-specific expression of neph3 (Wong et al., 2000), and within this region, a highly conserved 186-bp fragment, which contains a functional binding site for WT1, alone drives the expression in podocytes (Guo et al., 2004). However, some of the transgenic lines generated with this 186-bp fragment showed mosaic and ectopic expression of the neph3 promoter-driven lacZ gene. This suggests that, in addition to WT1, other factors binding to the elements outside this fragment are needed for the proper spatial and temporal expression of neph3 in podocytes. To support this hypothesis, we demonstrated that NF- κ B is involved in the regulation of the human neph3 gene (Study III). Transcription factor Sp1 is suggested to have a role in neph3 gene regulation in mice (Beltcheva et al., 2010). Since the Sp1 binding region was highly conserved in mice and humans, it is possible that Sp1 also interacts with the human sequence.

The transcriptional regulation of the Neph3 gene in podocytes had not been studied prior to this work. We found that the main activating regulatory region of the Neph3 gene is located in its proximal promoter and that transcription factors NF- κ B and Sp1 are involved in the constitutive expression of the Neph3 gene in podocytes (Study II). Study III added WT1 to the group of Neph3 gene regulators. Collectively, the same transcription factors participate in the regulation of both Neph3 and neph3 genes. It is a common phenomenon that transcription factors regulate transcription through protein-protein interactions and co-operation with other transcription factors. Thus, it was tempting to speculate that transcription factors WT1, NF- κ B and Sp1 may work together via their interactions in the regulation of neph3 and Neph3 genes. Study III indeed showed that WT1 and NF- κ B physically interact and co-operatively regulate both neph3 and Neph3 genes. Others have reported an interaction between Sp1 and NF- κ B as well as their co-operative function in, for example, HIV-1 enhancer activation (Perkins et al., 1993; Perkins et al., 1994; Sif and Gilmore, 1994). We could not reliably test the co-operativity between NF- κ B and Sp1 in the regulation of neph3 and Neph3 genes due to cell line-specific issues. A293 cells are a relevant model for most of the studies investigating the transcriptional regulation of neph3

and Neph3 genes. However, A293 cells, like most mammalian cell lines, express Sp1 endogenously at very high level and therefore, Sp1 overexpression studies were not feasible with these cells. For the same reason, the co-operative function of WT1 and Sp1, or NF-κB, WT1 and Sp1 in the regulation of nephrin and Neph3 genes remains unclear.

Transcription factors can mediate their transactivation effects either directly by binding to their response elements in the promoter or indirectly via other transcription factors. Study II showed that NF-κB and Sp1 mediate their effects on the Neph3 gene through their binding sites located in close proximity to each other in the Neph3 proximal promoter. We, however, noted that possibly additional functional NF-κB binding sites may be located just upstream from the functional NF-κB and Sp1 elements. In the Neph3 promoter, we identified one putative binding site for WT1 downstream and one upstream from the functional NF-κB and Sp1 binding sites. Since the functionality of the WT1 binding sites was not tested, it remains uncertain whether the effect of WT1 on Neph3 gene regulation is direct or indirect. Human nephrin proximal promoter harbours the functional WT1 binding site (Guo et al., 2004). We detected several putative binding sites for NF-κB both upstream and downstream of this WT1 element, and our results suggest that there are most probably multiple functional NF-κB binding sites in the nephrin promoter (Study III). However, their functionality remains to be clarified, and moreover, an indirect effect of NF-κB via WT1 on the nephrin promoter is also possible.

Taken together, human nephrin and Neph3 genes are both regulated by transcription factors WT1 and NF-κB, and the Neph3 gene also by Sp1. At least partly, these transcription factors interact with each other and function co-operatively in the regulatory processes of nephrin and Neph3 genes. There might be multiple functional binding sites for WT1 in the Neph3 promoter and for NF-κB in both nephrin and Neph3 promoters. WT1 and NF-κB can also indirectly participate in the regulation of the Neph3 and nephrin genes, respectively. The results suggest that there are two or more separate regulatory regions for nephrin and Neph3 genes, and it may be that these regulatory regions form a common transactivation complex through looping of the DNA (Figure 18).

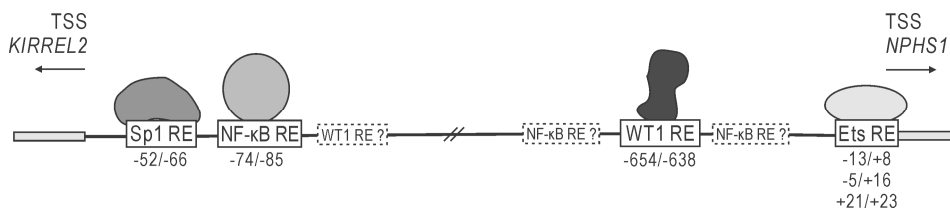


Figure 18. Schematic illustration of the studied transcription factors regulating human nephrin (*NPHS1*) and Neph3 (*KIRREL2*) genes. Functional response elements are boxed and putative response elements are shown with a dotted box and a question mark. The numbers below the functional response elements indicate the position related to nephrin/Neph3 TSS. RE, response element; TSS, transcription start site.

5.3.3 Role of different NF- κ B subunits in regulation of nephrin and Neph3 genes

The NF- κ B family consists of five members that normally exist as homo- and heterodimers. All of them are reported to be present in podocytes, and p65 homodimer and p50/p65 heterodimer are the most abundant dimers in the regulatory processes of many cell types (Baldwin, 1996; Karin and Ben-Neriah, 2000; Mudge et al., 2001; Martinka and Bruggeman, 2006; Hussain et al., 2009). We showed that NF- κ B functions as a p50/p65 heterodimer in Neph3 gene regulation (Studies II and III). Study III further demonstrated that the p50/p65 heterodimer co-operates with WT1 in the regulation of the Neph3 gene. The precise role of p50 and p65 subunits in nephrin regulation remained somewhat ambiguous, as Study III revealed controversial results between experiments performed with promoter constructs and the endogenous gene. The reasons for the discrepancies may be due to the artificial nature of promoter DNA constructs versus the chromosomal structure of the endogenous gene. In addition, endogenous NF- κ B may have had an effect on overexpressed subunits. Thus, it is difficult to determine which homo- or heterodimer(s) of the p50 and p65 subunits are involved in nephrin gene regulation and further co-operate with WT1. It is likely, however, that different NF- κ B homo- and heterodimers regulate and co-operate with other transcription factors in different ways under various (patho)physiological conditions.

5.3.4 TNF- α and NF- κ B pathway in regulating nephrin and Neph3 genes

NF- κ B is an inducible transcription factor whose activation is associated with multiple stimuli and responses, and it is known to be rapidly activated and translocated into the nucleus by, for example, cytokines (reviewed by Hayden and Ghosh, 2008). TNF- α has been demonstrated to up-regulate nephrin mRNA and protein expression in human A293 kidney cells (Huwiler et al., 2003). In line with this, Study III revealed that TNF- α specifically activates NF- κ B and increases nephrin mRNA level in cultured human podocytes. Similarly, Neph3 mRNA level was shown to be increased by TNF- α . In cultured mouse podocytes, TNF- α activates NF- κ B, but, in contrast to human cells, TNF- α down-regulates nephrin mRNA expression (Yamauchi et al., 2006; Saito et al., 2010). The discrepancy between human and mouse cell lines regarding nephrin expression may be explained by differences in cell models or differential regulation of nephrin by TNF- α in different species.

NF- κ B has been shown to be activated in podocytes in experimental kidney disease models (Mudge et al., 2001; Martinka and Bruggeman, 2006; Fujihara et al., 2007) and in the pathogenesis of various human kidney diseases, including diabetic nephropathy (DN) (Mezzano et al., 2004; Zheng et al., 2006). As cytokines, including TNF- α , are involved in the development and progression of DN (reviewed by Navarro-Gonzalez and Mora-Fernandez, 2008), it is possible that also in DN the stimulus for NF- κ B activation may originate from cytokines. Notably, in the kidneys of patients with DN, nephrin and Neph3 mRNA show down-regulation (Toyoda et al., 2004; Ihalmo et al., 2007). However, the mRNA expression of nephrin in DN, seems to be complex since during the development of

DN in a rat model, nephrin mRNA expression changes dynamically, showing first increased expression and later down-regulation (Forbes et al., 2002). Further studies are needed to clarify the role of NF- κ B regulating nephrin and Neph3 genes under pathophysiological conditions.

The link between the nephrin/Neph3 gene and NF- κ B is likely to be complex. The lack of functional nephrin in cultured human podocytes is associated with NF- κ B activation (Hussain et al., 2009). This, together with our results showing up-regulation of the nephrin gene by NF- κ B (Study III), suggests feedback regulation between nephrin and NF- κ B. In mice in which NF- κ B was activated by disruption of the natural NF- κ B pathway inhibitor Par4, nephrin protein level showed down-regulation in podocytes (Hussain et al., 2009). Down-regulation was, however, consistent with podocyte injury and loss, therefore likely representing an end-stage finding and is not in conflict with our results. In conclusion, distinct (patho)physiological conditions may alter the expression or activity of NF- κ B in podocytes, thereby contributing to the regulation of nephrin and Neph3 genes in different ways under various circumstances.

5.3.5 Role of DNA methylation in regulation of nephrin and Neph3 genes

DNA methylation represents an important mechanism of epigenetic regulation and is involved in the regulation of transcription. DNA methylation usually occurs in CpG islands, and in certain cells, most CpG islands are unmethylated, keeping the associated genes active. However, in various cell types, during development and differentiation and in some pathological situations, CpG islands are methylated, leading to silencing of the associated genes (Siegfried et al., 1999; Strathdee et al., 2004). One of the features observed in bidirectional gene pairs is the presence of CpG islands in their promoter region (Adachi and Lieber, 2002; Trinklein et al., 2004). In line with this, computer-based analysis detected three CpG islands within the intergenic region between nephrin and Neph3 genes and their coding regions (Studies II and III).

Study III demonstrated that a treatment with a demethylating agent, 5-aza-2'-deoxycytidine, of undifferentiated cultured human podocytes, which do not express nephrin or Neph3 mRNA endogenously, led to an increase in nephrin and Neph3 mRNA expression. In differentiated cultured human podocytes, an *in vitro* cell model currently considered the best for podocyte studies, the treatment increased only slightly the expression of nephrin and Neph3 mRNA. In the third tested cell line, A293, which represents non-podocyte cells but expresses nephrin and Neph3 mRNA endogenously, nephrin and Neph3 mRNA expressions were increased markedly by the demethylating agent treatment. The results suggest that silencing the transcription of nephrin and Neph3 genes by DNA methylation depends on the cell type and the differentiation stage of podocytes and that nephrin and Neph3 genes behave similarly. Interestingly, hypermethylation of CpG islands associated with bidirectional gene pairs, e.g. *WNT9A-CD558500* gene pair, can silence the expression of both genes simultaneously (Shu et al., 2006). This may also be the case for nephrin and Neph3 genes. Since specific regions of DNA methylation in nephrin and Neph3 sequence were not clarified, it is also possible that the effect of demethylating agent is indirect and modulates the expression of the

transcription factors or the other components of the signalling pathways (Karpf and Jones, 2002; Schmelz et al., 2005).

The mechanism of how methylation affects gene expression may be reduced accessibility of transcription factors to the methylated promoter (Watt and Molloy, 1988; Tate and Bird, 1993). However, demethylation is not always a prerequisite for the binding of the transcription factor; sometimes demethylation follows the binding of the transcription factor. Of the known nephrin and Neph3 gene regulators, Sp1 is linked to the maintenance of the methylation-free status of the CpG island (Brandeis et al., 1994; Macleod et al., 1994), and, interestingly, a functional Sp1 binding site in the Neph3 promoter is located on the CpG island (Study II). NF- κ B is also suggested to play a role in protecting DNA from methylation (Kirillov et al., 1996), and similarly to Sp1, its binding site in the Neph3 promoter is present on the CpG island (Study II). Since the precise location of functional binding site(s) for NF- κ B in the nephrin promoter has not been determined, it remains unclear whether they occupy a CpG island. It is possible, however, that Sp1 and NF- κ B can co-operate with DNA methylation in the regulation of nephrin and Neph3 genes.

5.4 GABP regulates nephrin gene

GA-binding protein (GABP) (also known as adenovirus E4 transcription factor [E4TF-1] or nuclear respiratory factor 2 [Nrf-2]) belongs to the Ets family of transcription factors. It is a heterotetramer composed of two subunits, GABP α and GABP β , both of which are needed to generate a functional complex (Sawa et al., 1996). GABP α contains an Ets DNA-binding domain that binds to DNA sequences rich in the nucleotides guanine and adenine. GABP β harbours a nuclear localization signal and also ankyrin repeats, which mediate the dimerization to α -subunit (reviewed by Rosmarin et al., 2004). Mammalian GABP is ubiquitously expressed and has also been shown to be distributed in adult mouse kidney at the mRNA level (O'Leary et al., 2005). Study IV demonstrated that GABP is expressed in glomeruli and localizes in podocyte nuclei and also in nuclei of other cell types in the glomerulus.

5.4.1 GABP and cell adhesion

GABP is essential for early embryogenesis, as deletion of GABP α in mice results in embryonic lethality (Ristevski et al., 2004). GABP is implicated in several critical cellular processes, including cell growth, cellular respiration, ribosome synthesis, differentiation and cell cycle. Despite GABP being expressed in a wide variety of tissues and controlling the expression of housekeeping genes, GABP also targets many lineage-restricted genes (reviewed by Rosmarin et al., 2004). Interestingly, GABP regulates the expression of α 4-integrin gene, whose protein product is involved in mediating cell-cell and cell-matrix interactions in leukocytes (Rosen et al., 1994). GABP is also suggested to be essential in regulating myeloid cell adhesion to the endothelium by targeting several key myeloid genes (Bottinger et al., 1994; Rosen et al., 1994; Nickel et al., 1995; Rosmarin et al., 1995; Nuchprayoon et al., 1997), and further, GABP is linked to the formation and function of

neuromuscular junctions by regulating the expression of synaptic genes (Fromm and Burden, 1998; Khurana et al., 1999; Mejat et al., 2003). One of these synaptic genes is utrophin, which is also expressed in podocytes. Utrophin localizes in the cytoplasm of the foot processes and is involved in linking the podocyte cytoskeleton to the glomerular basement membrane (Raats et al., 2000). Study IV revealed that GABP participates in the regulation of the nephrin gene in podocytes (Figure 18). As nephrin is a component of the SD junction (Holthöfer et al., 1999; Holzman et al., 1999; Ruotsalainen et al., 1999) and existing data (Liu et al., 2003; Gerke et al., 2005; Nishida et al., 2010) and Study I demonstrate that nephrin has a role in cell adhesion, it is tempting to speculate that GABP may have an important role in the formation or maintenance of the SD by regulating the expression of the nephrin gene and possibly also other podocyte genes.

5.4.2 Combinatorial activity of GABP and other transcription factors

GABP is often found in large complexes with other transcription factors, and it appears that the physical and functional interactions between GABP and other transcription factors and co-activators play a key role in regulating the expression of lineage-restricted genes (reviewed by Rosmarin et al., 2004). Thus, the regulation of nephrin gene transcription by GABP is most probably influenced by other transcription factors. Of the known nephrin gene regulators, Sp1 is reported to co-operatively activate transcription of several types of genes together with GABP (Rosmarin et al., 1998; Shirasaki et al., 1999; Gyrð-Hansen et al., 2002; Jiang et al., 2002; Rudge and Johnson, 2002; Takahashi et al., 2008). Further, co-operative interactions between RAR, GABP and Sp1 are proposed to be required for transcriptional activation of the CD18 gene by retinoic acid in myeloid cells (Bush et al., 2003). Whether co-operativity between GABP and Sp1 is important in nephrin gene regulation or interaction between RAR, GABP and Sp1 is involved in retinoic acid-induced transcription of the nephrin gene remains to be clarified.

5.4.3 Other features of GABP as a transcriptional regulator

Several interesting features have been linked to GABP. First, GABP binding site has been shown to be overrepresented in promoters of head-to-head oriented genes and to mediate bidirectional transcriptional activity. For this, co-operative binding of multiple transcription factors has been proposed (Collins et al., 2007; Lin et al., 2007; Huang and Chang, 2009). Since nephrin and Neph3 genes form a bidirectional gene pair, we investigated the effect of GABP also on Neph3 regulation, but GABP overexpression activated neither the Neph3 promoter in a reporter gene assay nor the endogenous Neph3 mRNA measured by quantitative PCR (unpublished data). It may be, however, that GABP participates in Neph3 gene regulation, not necessarily by itself, but by co-operating with other transcription factors involved in the regulation of the nephrin and/or Neph3 gene, such as WT1, NF- κ B and Sp1. A second interesting feature of GABP is its methylation-sensitive character. GABP has been shown to be recruited to the DNA when the locus is unmethylated (Yokomori et al., 1995; Lucas et al., 2009). Study III demonstrated that DNA methylation plays a role in silencing nephrin expression, and thus, methylation may contribute to in

GABP-mediated nephrin gene regulation. Lastly, in addition to being a transcriptional activator, GABP has been shown to function also as an initiator factor for genes with a TATA-less promoter (Yu et al., 1997). Nephrin does not have a TATA box and GABP binding sites in the nephrin promoter are located very near the TSS (Study IV), and thus, GABP-directed transcription initiation might be involved in regulation of the nephrin gene.

6 CONCLUSIONS AND FUTURE PERSPECTIVES

Nephrin is a structural backbone and signalling protein of the SD that is crucial in the maintenance of glomerular filtration barrier permselectivity. Nephrin homologues Neph1-3, comprising the Neph-protein family, are also components of the SD and participate in glomerular filtration. It is thus important to understand how these molecules participate in the formation of the SD and how they are structurally and functionally regulated. This work revealed that nephrin and Neph3 have similar functions by sharing similar binding properties and participating in cell adhesion. Further, nephrin and Neph3 genes were shown to share similar transcriptional regulatory mechanisms.

The data in this thesis show that Neph3 belongs to the nephrin protein complex and forms homodimers. The results also provide novel information about the function of nephrin, Neph1 and Neph3 in the formation of cell-cell contacts, indicating that Neph1 and Neph3 are homophilic adhesion molecules. Heterophilic *trans*-interactions between nephrin and Neph1 or Neph3 also promoted cell adhesion and induced de-phosphorylation of nephrin. These results suggest that Neph3 shares similar functions with nephrin and Neph-family members and may participate together with them in the formation and function of the SD. Neph3 was found to be up-regulated in the kidneys of nephrin-deficient mice with podocyte foot process effacement and displacement of the SDs with tight junction-like structures. Further investigations are needed to identify the molecular pathways and mechanisms associated with nephrin and Neph1/Neph3 interactions to induce cell adhesion and that lead to morphological changes in podocytes of nephrin-deficient kidneys. Specific kinases and phosphatases involved in regulating nephrin phosphorylation upon cell adhesion also remain to be identified. Further, to confirm the role of Neph3 in the formation and function of the SD, a podocyte-specific Neph3 knock-out mouse should be produced.

Glomerular transcription factors have been investigated extensively, but fewer studies have, however, concentrated on elucidating the transcriptional regulatory mechanisms of podocyte molecules, including nephrin and Neph3 genes. This work provides novel discoveries in the transcriptional regulation of the constitutive expression of human nephrin and Neph3 genes. Based on the bidirectional arrangement of nephrin and Neph3 genes and the similar structure and location of their protein products, nephrin and Neph3 genes have been proposed to share key features in their regulation. In the developing central nervous system of the mouse, both nephrin and Neph3 genes have been shown to be regulated by transcription factor Ptf1a (Nishida et al., 2010). The results of this thesis provide the first evidence that nephrin and Neph3 genes are regulated by common mechanisms also in podocytes. Transcription factors WT1 and NF- κ B were shown to participate in the regulation of nephrin and Neph3 genes co-operatively. In addition, DNA methylation played a similar role in silencing nephrin and Neph3 gene expression. Neph3 gene was further shown to be regulated by Sp1 and the nephrin gene by GABP. Nephrin and Neph3 genes are most probably regulated by several factors acting together to produce their exact cell and developmental stage-specific expression. It is important to pursue investigating these mechanisms in-depth to clarify how they affect the function of nephrin and Neph3 in the SD in the basal state, during development and during progression of glomerular diseases. For example, it would be interesting to clarify whether similar mechanisms in the transcriptional regulation of nephrin and Neph3 genes regulate the similar functions of their protein products.

Understanding how transcription of nephrin and Neph3 genes is regulated may also shed light on transcriptional regulation in podocytes in general and provide novel insights into the differentiation and function of podocytes. One important question remains to be answered – are there similar regulatory mechanisms also for other podocyte genes? Transcription factors WT1 and Sp1 have been shown to regulate podocalyxin (Palmer et al., 2001; Butta et al., 2006). Sp1 also regulates CD2AP (Lu et al., 2008; Su et al., 2009). Further, DNA methylation plays a role in the cell type-specific expression of podocalyxin (Butta et al., 2006). Studies with renal biopsies from patients with acquired human glomerular diseases show correlations in the mRNA expression of nephrin and Neph3 as well as in the mRNA expression of other genes expressed in podocytes, suggesting that common regulatory mechanisms may be activated in proteinuric glomerular diseases (Schmid et al., 2003; Ihalmo et al., 2007). However, the transcriptional regulatory mechanisms that lead to these down-regulations remain to be elucidated. Mutations in the intergenic region of nephrin and Neph3 genes found in CNF patients are not located in the regions that we focused on in this thesis, but it would be of interest to determine whether these mutations affect the expression of nephrin and Neph3 genes by disrupting the binding of some other critical transcription factors.

In conclusion, this work has extended current knowledge of nephrin and Neph3, revealing that they have similar functions and that the transcription of their genes is regulated by similar mechanisms. Nephrin and Neph3 were shown to play a role in cell adhesion, which may be important in the formation of the SD. Further, novel transcriptional regulatory mechanisms for nephrin and Neph3 genes were identified. This may shed light on the transcriptional regulatory processes in podocytes in general and provide novel insights into the function of podocytes.

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