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Nephrin: cellular trafficking and intracellular interactions

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For the Memory of My Father

Summary

Nephrin is a 180-kDa transmembrane glycoprotein belonging to the immunoglobulin family of cell adhesion molecules. Nephrin has an extracellular part with eight Ig-like motifs, one fibronectin-like domain, a transmembrane region and a short cytoplasmic tail. In the kidney, nephrin is exclusively expressed by glomerular podocytes, the protein being located in the slit diaphragm (SD) between podocyte foot processes. The nephrin gene *(NPHS1)* is mutated in congenital nephrotic syndrome of the Finnish type (CNF), a rare autosomal recessive disease with an incidence in Finland of approximately 1 in 10,000 newborns. CNF patients exhibit massive proteinuria already in utero. In most cases, nephrin is not detected in the SD.

Over 60 different mutations (deletions, insertions, splicing mutations, nonsense and missense mutations) have been reported so far, most of them being missense mutations leading to one amino acid change. In the first part of the project, we investigated the fate of 21 missense mutants identified in CNF patients using transfected human embryonic kidney (HEK293) cells. Immunostaining of stable transfected cells expressing these nephrin mutants demonstrated that most of them were retained in the endoplasmic reticulum (ER) with no detectable cell surface localization. Immunoelectron microscopy of cells expressing the wild type and a mutant nephrin further confirmed that the mutant nephrin could be abundantly found in the ER, but not on the plasma membrane. Subcellular fractionation of wild type and a mutant cell line clearly showed an altered subcellular distribution and molecular mobility of the mutant nephrin. Our data suggested that missense nephrin mutants lead to misfolding of the newly synthesized nephrin which is retained and degraded in the ER, instead of being transported to the plasma membrane. In conclusion, the misfolding and defective intracellular transport with consequent absence of the mutant nephrin on the plasma membrane might contribute to the pathomechanism of disease in patients carrying missense mutations resulting in the development of nephrotic syndrome.

Nephrin has a typical domain and sequence structure of a cell-cell or cell-matrix adhesion protein with nine intracellular tyrosines, some of which are phosphorylated

after podocyte-specific antiganglioside antibody-induced proteinuria. Signaling by cell adhesion molecules of the Ig superfamily, integrins, and certain receptor tyrosine kinases is initiated by the clustering of receptors with multivalent ligands or antibodies. In the second part of the project, we showed that nephrin is a signaling molecule by clustering of nephrin with antibodies on the cell surface, thus mimicking the situation where the interaction between nephrin and its extracellular ligand (s) is altered. As a first step in the elucidation of signaling functions of nephrin, we demonstrated that Src family kinase activity is crucial for tyrosine phosphorylation of nephrin, and that apparently several members of the Src family of kinases are able to catalyze nephrin phosphorylation. Furthermore, activation of one or more Src family kinases results not only in nephrin phosphorylation, but also in tyrosine phosphorylation of a 46 kD protein. In summary, we developed an experimental system for investigating signaling in the slit diaphragm and providing useful tools with a cell culture model. The results also suggested that the activity of Src family kinases as potential indicators as a signaling status of the slit diaphragm.

The podocin gene (*NPHS2),* that is mutated in patients with autosomal recessive steroid-resistant nephrotic syndrome (SRN), encodes a podocyte-specific protein termed podocin. Podocin is located at the slit diaphragm area where it directly interacts with the cytoplasmic domain of nephrin. Previously, we reported that the majority of nephrin missense mutants are retained in the ER and are not transported to the plasma membrane. The aim of the the third part of the project was to find out whether the disease-causing missense mutations in the *NPHS2* gene identified in patients with SRN might be due to dis- localization of the mutant podocin molecule, similarly to nephrin (first paper). Here, we analyzed 5 different disease-causing missense mutations found in SRN patients and their subcellular localization in HEK293 cells. Three out of these five mutations, located at the proximal C-terminal part of the podocin molecule, failed to localize to the plasma membrane. Interestingly, we also found that the normal localization of wild-type nephrin at the plasma membrane was also altered in cells co-expressing these three podocin mutants. Our data suggest that some disease-causing missense mutations in the *NPHS2* gene may abolish proper nephrin localization to the plasma membrane and lead to disruption of normal slit diaphragm structure and function in patients with SRN.

Sodium 4-phenylbutyrate (4-PBA) has been shown to function as a chemical chaperone and correct the cellular trafficking of several mislocalized or misfolded mutant plasma membrane proteins, like cystic fibrosis transmembrane conductance

regulator and α1-antitrypsin. We, therefore, in the last part of the project, wished to explore the potential effects of 4-PBA on the missense mutants found in CNF patients. The work was performed using HEK 293 cells stably expressing wild-type or the mutant forms of nephrin. Immunofluorescence microscopy and cell surface biotinylation showed that treatment with 4-PBA rescued several of the missense mutants from the ER to the cell surface. All six rescued mutants were found to be able to interact with Neph1, another interacting partner of nephrin in the slit diaphragm protein complex. Furthermore, tyrosine phosphorylation of the rescued mutants was rapidly induced by clustering with anti-nephrin antibodies as the wild type, implying that the rescued mutant nephrins are possible biologically functional. Consequently, this study suggests that the use of 4-PBA or corresponding chemical chaperones may be in the future a potential therapeutic approach for the treatment of CNF or other similar diseases affecting on renal filtration.

List of publications and manuscripts

I. **Liu, L.**, Cotta Doné, S., Khoshnoodi, J., Bertorello, A., Wartiovaara, J., Berggren, P-O., Tryggvason, K (2001) Defective nephrin trafficking caused by missense mutations in the *NPHS1* gene: insight into the mechanisms of congenital nephrotic syndrome. *Hum Mol Genet* 10: 2637-2644

II. Lahdenperä*, J., Kilpeläinen*, P., **Liu*, XL.**, Pikkarainen, T., Tryggvson, K (2003) (*equal contribution) Clustering-induced phosphorylation of nephrin by Src-family kinases. *Kidney Int* 64: 404-413

III. Nishibori, Y., **Liu**, **L.**, Hosoyamada, M., Endou, H., Kudo, A., Takenaka, Eiji Higashihara, H., Bessho, F., Kershaw, D., Ruotsalainen, V., Khoshnoodi, J., Tryggvason, K., Yan K: Disease-causing missense mutations in *NPHS2* gene alter normal nephrin trafficking to the plasma membrane. (Manuscript Submited)

IV. **Liu, XL.**, Cotta Doné, S., Yan, K., Kilpeläinen, P., Pikkarainen, T.,Tryggvason, K: Defective nephrin trafficking in congenital nephrotic syndrome corrected by a chemical chaperone. (Manuscript Submited)

Contents

Abbreviations

α-ACTN4	α-actinin-4
α1-AT	α1-antitrypsin
CD2AP	CD2-associated protein
CAMs	cell adhesion molecules
CFTR	cystic fibrosis transmembrane conductance regulator
CNF	congenital nephrotic syndrome of the Finnish type
CNS	congenital nephrotic syndrome
EM	electron microscopy
ER	endoplasmic reticulum
FSGS	focal segmental glomerulosclerosis
GLEPP1	glomerular epithelial protein 1
GBM	glomerular basement membrane
Ig	immunoglobulin
HSPG	heparan sulfate proteoglycan
kDa	kilodalton
mAb	monoclonal antibody
MCNS	minimal change nephrotic syndrome
NS	nephrotic syndrome
4-PBA	sodium 4-phenylbutyrate
PBS	phosphate buffered saline
PCR	Polymerase chain reaction
SD	slit diaphragm
SH3	src homology 3
SRN	autosomal recessive steroid-resistant nephrotic syndrome
ZO-1	zonula occludens–1

Review of literature

1 Background

1.1 Kidney

Together with the ureters and bladder, the kidney removes extra water as well as metabolic waste products from the blood. The kidney also participates in the regulation of bood pressure as well as in the absorption of anions and cations like chloride, calcium, and sodium. The kidney is also important for bones development by producing 1,25-dihydrooxyvitamine D_3. Over 24 hours, almost 180 liters of the cardiac output flows through the kidney, most of it being reabsorbed by the renal tubular networks with the final result of 1 to 1.5 liters of urine (Berry *et al*. 2000).

1.2 Glomerulus

A central role of the kidney (Fig.1) is to filter the blood plasma from circulating small toxins such as urea, while blood cells and essential macromolecules of the size of albumin and larger return to the circulatory system (Tisher and Madsen 2000). Plasma filtration occurs in the walls of capillaries located in the tuft of the kidney filtration units, the glomerulus (Fig. 1). The structurally complex glomerular capillary wall (glomerular filtration barrier) is composed of three layers: 1. fenestrated endothelium on the inner surface; 2. glomerular basement membrane (GBM), and 3. podocyte foot processes. Each capillary is covered by more than one podocyte (Fig.1). Podocytes project primary processes that branch into secondary interdigitated foot processes covering the entire outer aspect of the capillaries. The podocyte foot processes are interconnected by a thin extracellular membrane–the slit diaphragm (SD).

The fenestrae (holes) of innermost vascular endothelium of the glomerular filtration are about 70-100 nm in diameter and the plasma can reach the GBM properly without resistance (Savage 1994). Thus, the endothelium does not seem to be a direct barrier for the passage of macromolecules (Daniels 1993), but it prevents the blood cells from reaching the subendothelial space.

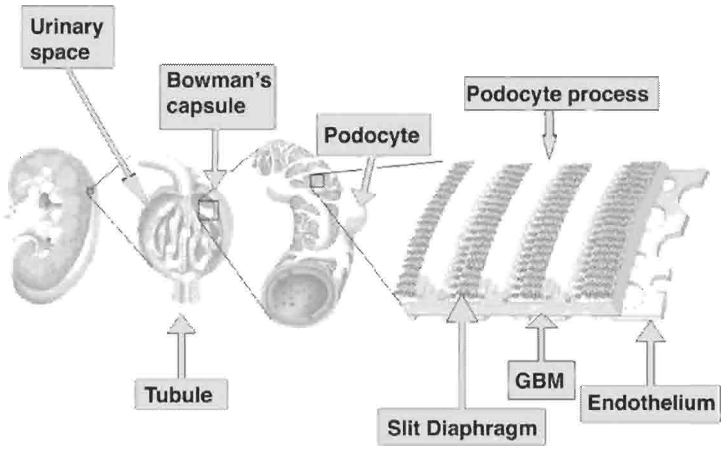

Figure 1. Each human kidney contains about one million glomeruli. An afferent arteriole branches into several capillaries (glomerular tuft), the walls of which constitute the filter system. The plasma filtrate (primary urine) is led to the proximal tubule, whilst the unfiltered blood returns to the blood circulation through the renal vein. The filtration barrier of the capillary wall contains the innermost fenestrated endothelium, the GBM, and the podocyte foot processes. Podocyte cell bodies emit thick extensions to form major and foot processes that cover the surface of glomerular capillary loops like interdigitating fingers. The slit diaphragm is a uniformly wide porous filter structure located between the foot processes. Modified from (Tryggvason and Pettersson 2003)

1.3 GBM

The GBM provides a complex physical surface where podocytes reside on its surface and the endothelium lines the inner one (Fig1.). The GBM originates in development from the fusion of a dual basement membrane between endothelial cells and primitive epithelial podocytes (Abrahamson 1991). Due to this fusion, the GBM has approximately twice the thickness (about 350 nm) of most basement membranes found elsewhere (Inoue 1994). The GBM is a molecular network composed of tightly cross-linked type IV collagen, laminin, nidogen, and heparan sulfate proteoglycans (HSPGs) (Timpl 1989; Yurchenco *et al.* 2002). It functions as a structural support for the capillary wall, as well as a size and charge selective barrier (Rennke *et al.* 1975; Kanwar *et al.* 1980 ; Kanwar *et al.* 1991).

Type IV collagen and laminin form two independent structural networks that are linked through nidogen molecules (Miner 1998). In addition to providing strength to the GBM, type IV collagen and laminin also function as adhesion molecules for the endothelial cells and podocytes (Aumailley and Gayraud 1998; Aumailley and Smyth 1998). Type IV collagen belongs to a large collagen family of about 25 members. Six different chains are known in mammals numbered $\alpha 1$ through $\alpha 6$. These chains can assemble together to form heterotrimeric protomers such as $\alpha 1$-$\alpha 1$-$\alpha 2$, $\alpha 3$-$\alpha 4$-$\alpha 5$ and $\alpha 5$-$\alpha 5$-$\alpha 6$ (Hudson *et al.* 1993; Hudson *et al.* 2003). The expression of each chain is

subjected to spatial and temporal regulation during the embryonic development. For example, in the glomerulus, α1-α1-α2 network is an embryonic collagen network which is gradually replaced by the α3-α4-α5 network later on in the development. Mutations in collagen IV genes (COL4A3, COL4A4, COL4A5) are the molecular pathological basis of the hereditary Alport syndrome, which clinically manifests itself as a progressive nephropathy alone or with hearing loss (Barker *et al.* 1990; Hostikka *et al.* 1990; Zhou *et al.* 1993) whereas the Goodpasture syndrome, that is characterized by hematuria and lung hemorrhages, is an autoimmune disease caused by antibodies against the α3 chain (Saus J 1988; Gunwar S 1991; Kalluri *et al.* 1996). Laminins are trimeric proteins consisting of α, β, and ϒ chains. These chains interact with each other to form as T-shaped or cross-like molecules. During development, the expression of laminins gradually shift from laminin-10 (α5β1ϒ1) to laminin-11 (α5β2ϒ1) (Virtanen *et al.* 1995). Laminin β2 chain gene knockout mice exhibit both neuromuscular junction and GBM defects only a few days after birth (Noakes *et al.* 1995) and absence of the laminin α5 chain results in abnormal glomerulogenesis and GBM (Miner and Li 2000). HSPGs consist of core proteins with glycosaminoglycan side chains, the negative charge of which possibly plays a role in controlling permeability of the anionic filtration barrier (Raats *et al.* 2000b). Digestion of the HS side chains by heparinase, and the binding of antibodies to HS leads to proteinuria in rats (Kanwar *et al.* 1980). However, mice with heparan sulfate deficient perlecan do not exhibit proteinuria (Rossi *et al.* 2003).

1.4 Podocytes

Podocytes are unique differentiated polarized epithelial cells with specialized apical and basal cell membrane domains. The term "Podocyte" refers to their complex shape with foot processes. The foot processes of podocyte is embedded in the GBM through α3β1-integrin (Cybulsky *et al.* 1992; Pavenstadt *et al.* 2003), which is the predominant integrin complex expressed by the glomerular podocyte (Baraldi *et al.* 1992; Baraldi *et al.* 1994). Intergrins are heterodimeric transmembrane glycoprotein receptors consisting of alpha and beta subunits that mediate adhesion and interactions between cells and extracellular matrix. The extracellular domain of integrins interacts specific sites on the extracellular matrix molecules in the GBM (type IV collagen, laminin, nidogen and fibronectin) (Adler 1992) (Dedhar *et al.* 1992), and their cytoplasmic domain is associated with the actin-cytoskeleton via talin, vinculin, and

paxillin (Otey *et al.* 1993)(Fig.2). Importance of α3β1-integrin in podocyte differentiation has been demonstrated by that α3 integrin knock-out mice were unable to assemble foot processes and the mice die within a few hours after birth most likely due to glomerular dysfunction (Kreidberg *et al.* 1996; Kreidberg 2000). Dystroglycan is another matrix receptor located in the basal domain of the foot processes. Its extracellular matrix ligands are laminin, agrin and perlecan, an intracellular binding partner being utrophin. Decreased dystroglycan expression correlates with proteinuria in animal models and minimal change nephrotic syndrome (MCNS) in humans (Raats *et al.* 2000a; Regele *et al.* 2000). Both integrin and dystroglycan complexes link the GBM matrix to the podocyte cytoskeleton (Fig.2) (Pavenstadt *et al.* 2003).

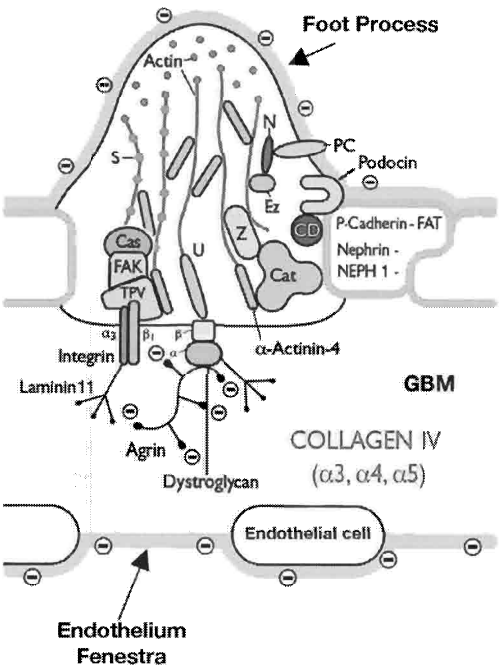

Figure 2. Schematic drawing of the molecular equipment of the podocyte foot processes.
Cas, p130Cas; Cat, catenins. CD, CD2-associated protein. Ez, ezrin. FAK, focal adhesion kinase. PC, podocalyxin; S, synaptopodin; TPV, talin, paxillin, vinculin; U, utrophin; Z, ZO-1. Modified from (Pavenstadt *et al.* 2003)

The apical membrane is covered by a thick surface coat (glycocalyx) which is composed of podoendin (Huang and Langlois 1985) and sialoglycoproteins, including podocalyxin and glomerular epithelial protein 1 (GLEPP1) (Kerjaschki *et al.* 1984; Thomas *et al.* 1994; Yang *et al.* 1996). This coat contributes to the highly negatively surface charge of podocytes and prevents foot processes from attaching to each other. Absence or chemical modification of podocalyxin causes collapse of the entire apical domain and null mice fail to form foot processes and slit diaphragms (Kurihara *et al.* 1992; Doyonnas *et al.* 2001). Therefore, it is important for the preservation of the normal podocyte architecture (Kerjaschki 1994; Doyonnas *et al.*

2001). Among the specific proteins of the apical surface membrane domain of developing and mature podocytes, the information of molecular identity is very limited. Only GLEPP1 is known as a receptor for tyrosine phosphatase and directly interacts with podocalyxin (Thomas *et al.* 1994; Yang *et al.* 1996). GLEPP1 knock-out mice only show subtle changes in the anatomy of podocytes, whereas the kidney, and glomerular structures were normal at the gross and light microscopic levels (Wharram *et al.* 2000). This means that GLEPP1 apparently can be compensated by other proteins. Whatever the precise function of GLEPP1 is, its reduced expression in glomeruli at the early course of glomerular inflammation and foot process effacement may suggest it can be a sensitive indicator of glomerular injury (Yang *et al.* 1996; Sharif *et al.* 1998)

Podocytes are structurally divided into three parts: 1) A cell body in the urinary space contains a prominent nucleus, a well-developed Golgi apparatus, mitochondria, and rough and smooth endoplasmic reticulum. 2) Major primary processes extending toward the capillaries are almost devoid of cell organelles and consist of an abundant cytoskeleton like microtubules and intermediate filaments (vimentin). This trunk is responsible for the maintenance of the complex cytoarchitecture (Drenckhahn and Franke 1988). 3) The foot processes attached to the outer aspect of the GBM. The cytoskeleton of the foot processes is composed mainly of actin-microfilaments (Fig. 2). These filaments run parallel to the longitudinal axis of the foot processes and interact with many associated proteins, such as ezrin (Hugo *et al.* 1998; Orlando *et al.* 2001), synaptopodin (Mundel P 1991), and α-actinin-4 (Kaplan *et al.* 2000).

2 *Glomerular Diseases of the renal ultrafilter*

Disruption of the glomerular filtration barrier that occurs in many acquired and inherited nephropathies, results in extensive leakage of plasma proteins and diffuse effacement of podocyte foot processes as detected by electron microscopy (EM). The nephrotic syndrome (NS) is characterized by heavy proteinuria, edemas, hypoalbuminaemia and hyperlipidemia (Glassock *et al.* 1996). Proteinuria is also a primary clinical feature in some acquired diseases that effects a huge number of individuals worldwide, including MCNS, membranous nephropathy and diabetic nephropathy. The pathomechanisms of proteinuria in these diseases are completely unknown. MCNS is the most common cause of nephrotic syndrome in childhood, and it is also quite common in adult patients. The disease usually responds to steroid therapy. In MCNS there is an effacement of podocyte foot processes of unknown cause (Niaudet

2003).

Diabetic nephropathy is one of the major complications of diabetes mellitus. It affects approximately 30% of patients with type 1 diabetes and also a large proportion of patients with type 2 diabetes. The disease, which manifests itself by persistent macroalbuminuria, ultimately leads to renal failure. Histological examination of advanced cases reveals thickening of the GBM (Parving *et al.* 2000).

Congenital nephrotic syndrome of the Finnish type (CNF), autosomal recessive steroid-resistant nephrotic syndrome (SRN) are the two genetic diseases caused by mutations in gene for proteins in podocytes of glomerular filtration barrier. Nephrin gene mutations result in CNF (Kestila *et al.* 1998), podocin gene mutations are associated with inherited and sporadic SRN (Boute *et al.* 2000; Caridi *et al.* 2001; Frishberg *et al.* 2002; Karle *et al.* 2002). Also mutations in α-actinin-4 (α-ACTN4) genes leads to focal segmental glomerulosclerosis (FSGS) (Kaplan *et al.* 2000). The molecular pathophysiology of CNF and SRN is directly connected to the formation and maintenance of the slit diaphragm, FSGS is correlated to podocyte cytoskeleton .

2.1 Congenital nephrotic syndrome of the Finnish type (CNF)

Proteinuria leading to nephrotic syndrome within the first three months of life is called congenital nephrotic syndrome (CNS) and the resulting phenotypes include MCNS, the mesangial proliferative glomerulonephritis, and FSGS (Rapola *et al.* 1992; Habib 1993). Among the CNS, CNF is the most severe, proteinuria beginning already *in utero* (Rapola *et al.* 1992; Holmberg C 1996; Khoshnoodi and Tryggvason 2001). CNF is a rare autosomal recessive inheritated disease worldwide, but in Finland, the incidence is approximately 1 in 10,000 newborns (Holmberg C 1999).

2.1.1 Clinical features

The clinical feature of CNF is chracterizecd by massive protein leakage across the glomerular capillary wall in the fetal period (Huttunen 1976). CNF children are often born prematurely with an enlarged placenta and they have retarded growth as a consequence (Holmberg C 1999). Heavy loss of proteins including albumin, immunoglobulins, plasminogen and antithrombin III directly leads to multiple abnormalities with severe hypoproteinemia, increased risk for infections, and increased risk for thrombotic complications (Holmberg C 1996; Ljungberg *et al.* 1997). In addition, vascular pathology together with hyperlipidemia contributes to the risk for

early atherosclerosis (Antikainen *et al.* 1992; Antikainen *et al.* 1994; Holmberg C 1999). CNF patients are treated with active protein and nutritional support, followed by bilateral nephrectomy, dialysis and renal transplantation (Holmberg C 1999). So far, atypical and milder forms of NS with minor *NPHS1* mutations have been described (Beltcheva *et al.* 2001; Koziell *et al.* 2002). In these cases, proteinuria can be reduced by antiproteinuric therapy (indomethacin and captopril) (Guez *et al.* 1998; Heaton *et al.* 1999; Patrakka *et al.* 2000)

2.1.2 Renal pathology

In typical cases of CNF, the kidneys are large compared to the body weight (Huttunen *et al.* 1980). At the light microscopic level, dilated proximal and distal tubuli, which might be secondary to the severe protein leakage, are seen in the renal tissue (Autio-Harmainen and J. 1981; Rapola *et al.* 1984; Rapola *et al.* 1992). The glomeruli are fibrotic and enlarged with dilatation of the Bowman´s space (Tryggvason and Kouvalainen 1975). In EM, the glomerular capillary walls shows typical changes seen in many nephrotic disorders, i.e, the fusion and effacement of the podocyte foot processes leading to a decrease in the frequency of the slit pores (Huttunen *et al.* 1980). In CNF patients carrying nonsense mutations, no nephrin is detected in the glomerular podocytes and no SD are present between the podocyte foot processes as investigated by extensive immunoelectron microscopy (Patrakka *et al.* 2000). In contrast, the GBM appears normal.

2.1.3 Genetic and molecular bases of CNF

In 1998, the gene responsible for CNF was discovered and the mystery of one of the most severe forms of NS was revealed (Kestila *et al.* 1998). Discovery of the gene which was named nephritic syndrome 1, *NPHS1,* opened new avenues to understanding the structure and mechanism of the glomerular filtration barrier. The 26-kilo base pare gene was shown to contain 29 exons. The encoded protein, nephrin, is exclusively expressed by glomerular podocytes within the kidney and predominantly located in the glomerular slit diaphragm (Ruotsalainen *et al.* 2000; Patrakka *et al.* 2001). Two common *NPHS1* mutations, Fin-major and Fin-minor, were found in >90% of the Finnish patients, but they are rare in non- Finnish patients. Fin major is a 2-bp deletion in exon 2, which causes a frameshift and the formation of a premature stop codon within the same exon, leading to a truncated 90 residue protein. Fin-minor is a nonsense

mutation in exon 26 affecting the cytoplasmic domain of nephrin and resulting in a truncated 1109-residue protein as compared with 1241-residue normal molecule (Kestila *et al*. 1998). In addition, over 60 different mutations (deletions, insertions, splicing mutations, nonsense and missense mutations) spanning the entire gene region have been reported so far from patients worldwide (Bolk *et al.* 1999; Lenkkeri *et al.* 1999; Beltcheva *et al.* 2001; Gigante *et al.* 2002; Koziell *et al.* 2002). Some mutations are enriched in different ethnic groups, e.g, in Mennonites, a 1481-delC leading to a truncated protein of 547 residues is common (Bolk *et al*. 1999) and six out of nine patients from Malta are homozygous for a nonsense mutation R1160X in exon 27 (Koziell *et al*. 2002). Detection of several sequence variants of *NPHS1* in healthy individuals indicate that this gene is quite susceptible to mutagenesis (Lenkkeri *et al.* 1999).

2.2 Autosomal recessive steroid-resistant nephrotic syndrome

Autosomal recessive steroid-resistant nephrotic syndrome (SRN) belongs to the hereditary group of idiopathic nephrotic syndrome, which is the most frequent sporadic glomerular disease in childhood. Most of the SRN patients generally respond to corticosteroid therapy. Typical SRN is characterized by an onset of proteinuria between three months and five years with edema as the dominant manifestation, as well as failure to respond to steroid therapy, rapid progression to end-stage renal disease (ESRD), absence of recurrence after renal transplantation and absence of extra-renal disorders. At least 50% of SRN patients develop ESRD (Glassock *et al*. 1996). Histologically, MCNS, FSGS and diffuse mesangial proliferation are observed. Glomeruli show a fusion of podocyte foot processes in EM (Niaudet 2003). The causative gene, *NPHS2*, encoding podocin, was mapped to chromosome 1q25-q31 and identified by positional cloning (Boute *et al.* 2000). Podocin mutations have been reported to be responsible for the main properties of SRN. More than 30 different *NPHS2* mutations, comprising nonsense, frameshift, and missense mutations, have been found to segregate with the disease. Among those, R138Q and R138X represent the two major disease–causing mutations (Boute *et al.* 2000; Frishberg *et al.* 2002). However, rapidly accumulating reports have revealed that podocin mutations also can be detected in sporadic SRN (Caridi *et al.* 2001; Karle *et al.* 2002), glucocorticoid sensitive frequent relapses (Caridi *et al*. 2003a), and sporadic FSGS in adulthood with R226Q as the most

common mutation (Tsukaguchi *et al.* 2002; Caridi *et al.* 2003b). The severity of clinical disease associated with *NPHS2* mutation alterations is highly variable.

3 *Podocyte slit diaphragm and associated proteins*

The podocyte slit diaphragm (SD) serves as a final gate in restricting the passage of molecules to the urine. In nephrotic syndromes of different types both in humans and experimental models, foot process effacement and disappearance of SD leads to proteinuria (Kanwar *et al.* 1991; Fogo 2000). Based on transmission EM findings on tannic acid-stained material, Rodewald and Karnowsky presented a model of sub-structure of the SD (Rodewald and Karnowsky 1974). It consists of rod-like units connected to a linear central filament, together forming a zipper-like pattern. The zipper was proposed to contain rectangular pores of the size of the albumin. This, together with the observed expression of ZO-1 next to SD, has led to a concept that SD is a modified tight junction (Kurihara *et al.* 1992; Fanning *et al.* 1998). However, also p-cadherin and catenin have been localized in the vicinity of SD, and it has recently been suggested that the SD could rather be a modified adherence junction (Reiser *et al.* 2000). A continuously increasing body of knowledge suggests that the SD, is actually a meshwork of multiple proteins including nephrin, Neph1, FAT1, CD2AP (CD2-associated protein), podocin, P-cadherin, zonula occludens-1 (ZO-1), and possibly some other unknown proteins.

3.1 Nephrin

Nephrin is a 180-kDa transmembrane glycoprotein which belongs to the immunoglobulin (Ig) superfamily of cell adhesion molecules. Nephrin has eight Ig-like motifs, one fibronectin type III-like domain, a short transmembrane region and a cytoplasmic tail rich in serine and tyrosine residues. In CNF patients, the podocyte food processes are effaced and the podocyte SD is missing (Ruotsalainen *et al.* 2000). In mice, inactivation of the *NPHS1* gene leads to massive proteinuria and neonatal death (Putaala *et al.* 2001). Thus the SD appears to be dependent on the expression of nephrin. A minor splice variant of nephrin mRNA lacking the sequence encoding the transmembrane domain has been reported in normal human and CNF glomeruli (Holthofer *et al.* 1999), but its significance and biological function remains to be

determined. In addition to podocyte, nephrin expression has been shown in pancreas and central neural systems in mice (Putaala *et al.* 2001). There are two mouse brain isoforms found in the mouse and rat (Beltcheva *et al.* 2003), indicating that central neural systems may contain an alternative spliced nephrin form with a unique signal peptide at amino-terminus. However, in humans, nephrin has been detected only in the glomerulus despite extensive search using immunofluorescence staining, western blotting and in situ hybridization (Kuusniemi *et al.* 2004). Recent studies in membranous nephropathy, as a common type of nephrotic syndrome in adults, have revealed decreased nephrin synthesis and partial disappearance of nephrin from the slit (Doublier *et al.* 2001). Koop and coworkers also observed a marked reduction in the protein levels, but an increase of the levels of nephrin mRNA in MCNS, FSGS, and diabetic nephropathy (Koop *et al.* 2003). There are controversial reports of low expression of nephrin mRNA in human diabetic nephropathy (Toyoda *et al.* 2004) and no changes of nephrins mRNA expression as well as its distribution pattern in MCNS, FSGS (Patrakka *et al.* 2001). Based on the structure of the SD, and the restricted localization of nephrin and recent evidence (Holthofer *et al.* 1999; Ruotsalainen *et al.* 1999; Barletta *et al.* 2003; Gerke *et al.* 2003; Khoshnoodi *et al.* 2003), it seems likely that nephrin molecules interact through their extracellular part and assemble in a head-to-head fashion to form the structural basis of the SD.

As previously reported, cell adhesion molecules (CAMs) can, in addition to mediating cell-cell junctions, also play a role in both "outside-in" and "inside-out" signaling that results in the modulation of CAMs binding characteristics (Crossin and Krushel 2000; Simmons *et al.* 2001). Nephrin is suggested to be a member of the immunoglobulin superfamily of cell adhesion molecules. Its intracellular domain contains nine tyrosine residues in human, six of these being conserved in rodents. The intracellular domain is necessary for nephrin function, since patients with the deletions of most of the intracellular domain have proteinuria and loss of foot processes (Kestila *et al.* 1998). Currently accumulating evidence has indicated that, in addition to the function as a critical adhesion protein at the slit diaphragm, nephrin can also mediate signals. In gradient centrifugation of glomerular lysates, part of nephrin can be detected in lipid rafts (Schwarz *et al.* 2001; Simons *et al.* 2001). Lipid rafts are microdomains in the cell membrane, containing sphingolipids, cholesterol, Src-family kinases, glycosyphosphatidylinostol-anchored proteins and transmembrane proteins. They directly initiate and participate in the signal transduction (Simons and Toomre 2000).

Nephrin is tyrosine phosphorylated after a injection with a podocyte-specific 9-O-acetylated GD3 ganglioside antibody and also found to bind and to be phosphorylated by Fyn kinase both in vitro and in vivo (Simons *et al.* 2001; Verma *et al.* 2003). Moreover, nephrin can trigger mitogen-activated protein kinase cascades and increase transcription factor activating protein-1 activation as well as stimulate phosphoinositide-3'-kinase/AKT–dependent signal transduction in glomerular podocyte (Huber *et al.* 2001; Huber *et al.* 2003a). These processes can be augmented by podocin. Further investigations are needed to clarify the downstream signaling pathways of nephrin and the physiological role of signaling. Phosphorylation could be involved in regulation of SD complex assembly or its dissociation by altering interactions with ligands, or phosphorylation could recruit specific signaling proteins to the complex, or it could alter endocytosis or recycling of SD complex proteins (Oved and Yarden 2002).

3.2 ZO-1

The tight junction marker ZO-1 is a 225-kDa protein of the membrane-associated guanylate kinase protein family that contains three PSD95/Dlg/ZO-1 (PDZ) domains, a src homology 3 (SH3) domain, and a guanylate kinase domain (Hung and Sheng 2002). ZO-1 is linked to the components of cell-cell junctions like occludin, catenins and the actin cytoskeleton (Kurihara *et al.* 1992; Fanning *et al.* 1998) (Fig2). Even though ZO-1 is located at the cytoplasmic face of the foot process at the site where the SD inserts (Schnabel *et al.* 1990), its expression seems to be normal in fetal CNF kidneys with Fin-major/Fin-major (Ruotsalainen *et al.* 2000). ZO-1 is tyrosine phosphorylated during foot process effacement after protamine sulfate treatment in rats (Kurihara *et al.* 1995). A recent study showed that the PDZ domain of ZO-1 binds the conserved PDZ domain binding motif present in the carboxyl terminus of the three known Neph family members, implying that ZO-1 may organize Neph1 molecules and recruit signal transduction components to the slit diaphragm (Huber *et al.* 2003b)

3.3 Neph1

Quite recently, a novel protein called Neph1 has been identified as another important component of the SD. Neph1 is a 90kDa transmembrane protein of five extracellular immunoglobulin-like domain similar to nephrin. Neph1 belongs to a family of three closely related proteins that interact with the C-terminal domain of podocin (Sellin *et al.* 2003). All three Neph proteins share a conserved podocin-binding motif. Neph1 knock-

out mice display effacement of podocyte foot processes, proteinuria and postnatal death (Donoviel *et al.* 2001). More recently, it has been shown that Neph1 forms cis-heterodimeric
interactions with nephrin (Gerke *et al.* 2003; Verma *et al.* 2003). Interestingly, disruption of the Neph1-nephrin interaction in vivo by injecting combinations of individual subnephritogenic doses of anti-Neph1 and anti-nephrin antibodie leads to proteinuria with preserved foot processes (Liu *et al.* 2003). The interaction between nephrin and Neph1 is specific and not shared with P-cadherin and appears to be an important determinant of glomerular permeability (Liu *et al.* 2003).

3.4 Podocin

Podocin is a 42 kDa integral membrane protein with a cytosolic NH_2- and COOH-terminal domain and its predicted hairpin-like structure is typical for the stomatin family. Podocin is exclusively expressed in the podocyte with the specific localization at the cytoplasmic face of the SD area. It interacts with nephrin, CD2AP, and Neph1 via its C terminus (Schwarz *et al.* 2001; Roselli *et al.* 2002) (Sellin *et al.* 2003). Podocin accumulates in an oligomeric form in lipid rafts of the SD; it can augment nephrin signaling and may serve as a scaffold protein recruiting nephrin into lipid raft microdomains which often are host for signaling proteins (Huber *et al*. 2001; Schwarz *et al*. 2001; Huber *et al*. 2003c). Podocin knockout mice die a few days to three weeks after birth from massive mesangial sclerosis which demonstrates the essential role of this protein for glomerular filtration (Roselli *et al*. 2004a). In some acquired glomerular diseases, decrease in podocin is related with the effacement of foot processes and it coincides with a rise in levels of corresponding mRNA transcripts (Koop *et al*. 2003).

3.5 CD2AP

CD2AP is an 80-kDa protein containing an actin-binding site, a proline-rich region and three SH3 domains, one of which binds to the cytoplasmic domain of CD2. CD2AP is a transmembrane protein of the Ig superfamily expressed in T cells and natural killer cells (Dustin *et al.* 1998). Within the glomerulus, CD2AP is only expressed in the podocytes, it interacts with both nephrin and podocin and it may link these two proteins to the actin-based cytoskeleton (Li *et al.* 2000; Schwarz *et al.* 2001; Saleem *et al.* 2002). Loss of CD2AP in mice generates a congenital nephrotic syndrome featured

by gradual extensive foot process effacement and death at 6 weeks of age, indicating CD2AP's role for the structural of the slit (Shih *et al.* 1999). Heterozygous mice developed glomerular changes at 9 months of age with increased susceptibility to glomerular injury by nephrotoxic antibodies or immune complexes (Kim *et al.* 2003). EM analysis of podocytes revealed defects in the formation of multivesicular bodies, suggesting an impairment in the podocyte's ability to clear plasma proteins using the intracellular degradation pathway and CD2AP is likely involved in protein trafficking and endocytosis. Two human patients with focal segmental glomerulosclerosis have been reported to have a mutation predicted to ablate expression of one CD2AP allele. This implies that CD2AP is a determinant of human susceptibility to glomerular disease, and suggests that CD2AP not only has a possible structural role in the slit diaphragm, but also targets proteins to the degradative pathway (Kim *et al.* 2003).

3.6 FAT1

FAT1 is a novel 516 kDa gigantic member of the cadherin superfamily, containing 34 tandem cadherin repeats, five EGF-like repeats, and a laminin A-G in the extracellular domain (Dunne *et al.* 1995). It is expressed in widely during development in epithelial tissues, including skin, neuroepithelium, and podocytes (Cox *et al.* 2000). Immunelectron microscopy can detect FAT1 at the base of the SD and the FAT1 cytoplasmic domain colocalizes with ZO-1 (Inoue *et al.* 2001). FAT1-deficient mice have been reported to lose the SD, develop podocyte fusion and die within 48 hr of birth. This demonstrates a necessary role for FAT1 in the formation or maintenance of SD (Ciani *et al.* 2003), but its relation to other proteins of the SD remains unclear.

3.7 P-cadherin

P-cadherin is a 120-kDa transmembrane glycoprotein that exhibits calcium-dependent homophilic cell-to-cell adhesion interactions. It consists of five cadherin domains in the extracellular part, and an α-catenin binding site in its cytoplasmic part, through which the molecule is linked to the actin cytoskeleton. During glomerular development, P-cadherin is co-located with nephrin and ZO-1 in the late S-shaped bodies. At the capillary loop stage, staining for P-cadherin has been observed at the basal margins together with nephrin and ZO-1, but not on the lateral surfaces of developing podocytes where nephrin also can be found (Ruotsalainen *et al.* 2000). P-cadherin appears not to be indispensable for maintaining the SD, since it is normally

expressed at the podocyte slit pores in CNF kidneys (Ruotsalainen *et al.* 2000), and mice lacking P-cadherin are viable (Radice *et al.* 1997). Furthermore, humans with P-cadherin gene (CDH3) mutation do not develop nephrosis, but hair and retinal defects (Sprecher *et al.* 2001)

3.8 Podocyte Cytoskeleton

The unique morphological structure of the podocyte is maintained by the cytoskeleton, a cytoplasmic system of fibers and associated proteins (See Fig1,2). Foot process effacement and detachment from the GBM are characteristic changes of most nephrotic syndromes. In some experimental kidney models and in vitro podocyte cell culture experiments, changes in the cytoskeletal and redistribution of its associated proteins have been observed (Smoyer *et al.* 1997; Hugo *et al.* 1998). Mutations in the α-ACTN4 gene, which encode an actin-filament cross linking protein, α-ACTN4, causes an autosomal dominant late onset form of FSGS (Kaplan *et al.* 2000). Kaplan et al found that mutant proteins have significantly higher co-sedimentation than the wild-type protein with F-actin and thereby proposed mutant proteins might have an increased affinity for actin filaments, and hence interference with the normal assembly/disassembly of actin filaments in glomerular podocytes (Kaplan *et al.* 2000). Mice deficient in the α- ACTN4 showed progressive proteinuria and typically death by several months of age, indicating that α- ACTN4 is required for normal glomerular function (Kos *et al.* 2003). Proteinuric α-*ACTN4*-mutant mice have reduced nephrin mRNA expression, suggesting a close relationship between the regulation of the actin cytoskeleton and the maintenance of key components of the slit diaphragm complex (Kos *et al.* 2003). Synaptopodin is another proline-rich protein intimately associated with actin microfilaments present in the foot processes of differentiated podocytes (Mundel *et al.* 1997). Several studies have shown a loss of synaptopodin expression from areas of sclerosis and weak in nonsclerotic glomeruli (Srivastava *et al.* 2001).

4 Protein quality control in endoplasmic reticulum (ER)

The ability of proteins to fold to their functional states following synthesis in the intracellular environment is a remarkable biological feature. As soon as nascent chains enter the ER, they face an environment dramatically different from that within the cytoplasm, but optimized for protein folding and maturation. Incompletely folded and

assembled proteins are, as a rule, retained and eventually degraded by an ER-associated degradation process. This unique molecular sorting phenomenon is called "quality control". The strict quality control system in the ER is essential for several reasons. By preventing the premature exit of incompletely folded or assembled proteins from the ER, it extends their exposure to the folding machinery in the ER lumen and thereby improves the chance of correct maturation. Furthermore, the ER quality control ensures that proteins are not dispatched to terminal compartments when they are still incompletely folded and potentially damaging to the cell. For example, it is essential that non-functional or partially functional ion channels, transporters and receptors do not reach the plasma membrane, where their presence could be toxic. Finally, cells use the ER quality control to regulate the transportation and activation of specific proteins posttranslationally (Hammond and Helenius 1995; Ellgaard and Helenius 2001; Ellgaard and Helenius 2003)

4.1 Protein folding and misfolding in the cell

The ER is a compartment that coordinates the synthesis, folding, export and degradation of nascent proteins. Here, newly synthesized proteins interact with chaperones that assist in protein folding. The folding and misfolding of a protein depends both on the intrinsic properties of the amino-acid sequence and on multiple contributing influences from the crowded cellular environment. Folding and unfolding are crucial steps for determining about the transport of proteins to different cellular locations. In the case of transmembrane proteins, only perfectly folded and assembled proteins can be efficiently transported to the cell membrane through the secretory pathway, while misfolded or misassembled proteins are retained and eventually are retro-translocated to the cytosol and degraded by the ubiquitin-proteasome system (Fig3) (Fewell *et al*. 2001; Helenius 2001).

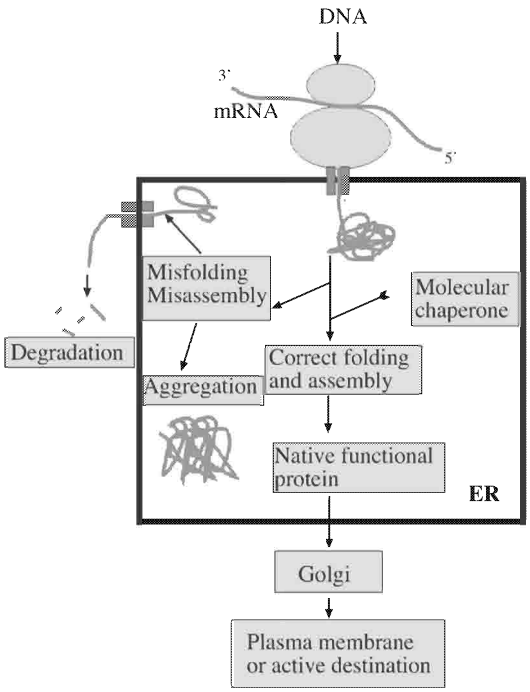

Figure 3. Regulation of protein folding in the ER. Many newly synthesized proteins are translocated into the ER, where they fold into their three-dimensional structures with the help of a series of molecular chaperones. Correctly folded proteins are then transported to the Golgi complex and then delivered via the secretory pathway to the extracellular environment. However, incorrectly folded proteins are detected by a quality-control mechanism and sent along another pathway (the unfolded protein response) in which they are ubiquitinated and then degraded in the cytoplasm by proteasomes. This retention of incompletely folded or misassembled proteins serves as a quality monitoring mechanism. The details of this process are not known, but it probably involves molecular chaperones (Ellgaard *et al.* 1999)

4.2 ER molecular chaperones

ER molecular chaperones have a dual role in promoting folding and preventing aggregation of the maturing proteins, as well as to dispatching misfolded proteins for destruction. Assuming that chaperones bind newly synthesized proteins as they fold and release them on the successful completion of the process, they could conceivably also act as monitors of the process folding. Their interactions with abnormal proteins are likely to differ from their interactions with normal proteins. For example, the proteins region binding to molecular chaperones becomes buried as it correctly folds, and the protein is released from the chaperones and transported out of the endoplasmic reticulum. In the case of misfolding proteins, the normally buried parts

continue to be exposed and bind with molecular chaperones, therefore the protein is not released but retained in the ER (Sitia and Braakman 2003). It is known that the chaperones indeed discriminate between native and non-native proteins. The correlation between prolonged association of molecular chaperones with misfolded proteins and the eventual degradation of these proteins implies that the chaperones target the misfolded proteins for degradation, wherever it may occur. When folding or assembling proteins expose hydrophobic surfaces, unpaired cysteines or immature glycans, ER-resident chaperones or oxidoreductases interact with them, and as a consequence they are retained in the ER or retrieved from the Golgi complex. The main ER molecular chaperones include Hsp70s (the 70kDa heat shock proteins), calnexin, calreticulin, the thiol-disulfide oxidoreductases and protein disulfide isomerase (PDI) (Table 1).

Table 1. **ER-resident chaperones**

Family	Main members	Functions
Hsp70s	BiP	The main protein of this family is BiP,which takes part in many aspects of ER quality control. It binds to various nascent and newly synthesized proteins and assists their folding. In addition, it is involved in the processes of ER-associated degradation and the unfolded protein response.
Hsp40s	Five ER proteins of the Hsp40 family (ERdj1–5)	They contain a luminally exposed J-domain and can stimulate BiP ATPase activity *in vitro*.
Peptidyl-prolyl isomerases (PPIases)	Cyclophilins and the FK506-binding proteins	Catalysis of *cis*/*trans* isomerization of peptidyl-prolyl bonds *in vivo* remains to be shown conclusively for these proteins.
Lectins	Calnexin and calreticulin	Interact with glycoproteins and assist their folding and assembly
Thiol-disulphide oxidoreductases	Large family of enzymes, including PDI, ERp72, ERp57, p5 etc.	Catalyses the oxidation, isomerization and reduction of disulphide bonds.

Modified from (Ellgaard and Helenius 2003)

4.3 Defective protein folding and human disease

Protein folding and unfolding are crucial ways of regulating biological activity and targeting proteins to different cellular locations. If protein folding is unsuccessful, the polypeptide is directed to the ubiquitin-regulated proteasome pathway for degradation. Ensuring accuracy in protein folding is crucial for maintaining proper cellular function. Given the complexity of the folding reactions and the need for fidelity in the process, it is not surprising that the flaws in these processes have been the molecular basis for

many hereditary diseases. In several congenital heritable disorders (such as cystic fibrosis , α1-antitrypsin (α1-AT) deficiency), the misfolded proteins altered by mutations that result in defects in intracellular transport and absent on its active sites with "loss of protein function" (Cheng *et al.* 1990; Lomas *et al.* 1992; Carrell and Lomas 1997). On the other hand, misfolded or partially folded proteins can also be produced so, such as that it exceeds the cell's degradative capacity. The proteins can form disordered intracellular and extracellular aggregates that lead to a disease with " toxic gain of function", like frontotemporal dementia with Parkinsonism and Alzheimer disease (Heutink 2000; Dobson 2001; Dobson 2003; Selkoe 2003).

5 *Potential therapeutic approaches for protein misfolding disease*

The understanding of the pathogenesis of disease caused by protein misfolds makes it possible to explore therapeutic approaches that could slow, arrest or revert disease progression. A group of low-molecular-weight compounds called "chemical chaperones" have been shown to reverse the aberrant intracellular retention of several proteins associated with human diseases. These compounds include polyols osmolytes, such as glycerol, dimethylsulfoxide (DMSO), trimethylamine N-oxide (TMAO), and 4-phenylbutyric acid (4-PBA). In addition, amino acid derivatives and membrane-permeable forms of enzyme antagonists, ligands or even substrates, can act as chemical chaperones for misfolded or mislocalized enzymes (Morello *et al.* 2000b). The mechanisms by which chemical chaperones function are not clearly understood but they are thought to stabilize improperly folded proteins, reduce aggregation, and promote the more efficient transport of affected proteins to the appropriate intracellular or extracellular destination. Chemical chaperones of the glycerol, DMSO, TMAO and 4-PBA class have general effects on multiple proteins, while antagonists, ligands and substrates are thought to affect only the specific proteins with which they interact (Table3) (Fan *et al.* 1999; Burrows *et al.* 2000; Petaja-Repo *et al.* 2002; Powell and Zeitlin 2002). The concentrations of glycerol and TMAO needed for chaperone-like effects in cell culture have been in the range of 0.5–1.2M and 50–100 mM, respectively. Studies of glycerol and TMAO given to mice by intraperitoneal or oral administration have indicated that it is possible to achieve these concentrations for TMAO but not for glycerol *in vivo* (Bai *et al.* 1999). Table 2 presents examples of the rescue of some protein folding disorders using chemical chaperones.

Table 2. Mutations of protein rescued by chemical and pharmacological chaperones

Disease	Protein	Reagents
Cystic fibrosis	CFTR	Glycerol, DMSO, TMAO, 4-PBA
Hereditary emphysema	α1-Antitrypsin (PiZ) variant	Glycerol, DMSO, TMAO, 4-PBA
Nephrogenic diabetes insipidus	Aquaporin-2	Glycerol, DMSO, TMAO
Nephrogenic diabetes insipidus	Vasoprssin V_2 receptor(V2R)	Nonpeptidic V2R antagonists. SR121463A, VPA985
Fabry	α-Galactosidase A (α-Gal A)	1-Deoxy-galatonojirimycin. A potent competitive inhibiter of α-Gal A
Gaucher's Disease	β-glucocerebrosidase (β-Glu)	β-Glu inhibitor

Adapted from (Morello *et al*. 2000a)

Aims of the Project

The discoveries of nephrin, podocin and other proteins associated with the podocyte slit diaphragm (SD) have provided a new understanding of the nature of the renal ultrafilter. This, in turn, has opened up new possibilities for studying the molecular pathomechanisms of proteinuria, a characteristic early manifestation of many kidney diseases. The goals of this thesis project were to elucidate in more detail the mechanisms of congenital nephrotic syndromes caused by different types of missense mutations in the nephrin (*NPHS1*) podocin (*NPHS2*) genes. Furthermore, nephrin-mediated intracellular signaling and possibilities for treatment of some cases of inherited congenital nephritic syndrome of the Finnish type were explored. The specific aims were to:

1. Study the subcellular localization of different nephrin missense mutants found in a subset of congenital nephrotic syndrome.
2. Examine the involvement of nephrin in intracellular signaling and identify nephrin-associated kinases.
3. Investigate the intracellular fate of some podocin mutants and their correlation with nephrin function.
4. Explore the possibility for using a chemical chaperone to rescue nephrin missense mutants from ER degradation and enable their transport to the plasma membrane.

Materials and Methods

Detailed descriptions of materials and methods used in the experiments of this thesis work can be found in the corresponding papers (number in the parenthesis) "Materials and Methods"

1. DNA constructs and site-directed mutagenesis (I, III, IV)
2. Polymerase chain reaction (PCR), and DNA sequence (I, III, IV)
3. Cell culture and transfections (I, II, III, IV)
4. Immunofluorescence staining and immunoelectron microscopy (I, III, IV)
5. Subcellular fractionation by sucrose density gradient centrifugation and Immunoprecipitation (I, II, III)
6. Anti-nephrin antibody cross-linking (II, IV)

Results and Discussion

I. Fate of nephrin missense mutants in CNF

In genetic diseases, a large number of the disease causing mutations are missense mutations that can differ between patients. These result in single amino acid substitutions that are randomly distributed along the polypeptide chain even though they cause the same disease phenotype. It is well documented that missense mutations in genes of membrane or secreted proteins often affect the transport of the mutant proteins from the ER to the plasma membrane (Cheng *et al.* 1990; Zhou *et al.* 1998). Other missense mutations may not affect trafficking of the mutant, but rather alter the protein so that it becomes non- or malfunctional (Bateman *et al.* 1996). To investigate whether missense mutations found in CNF patients lead to intracellular trafficking defects, or possible malfunction, we examined the localization of 21 disease-causing nephrin missense mutations. The different mutants had amino acid substitutions in different regions of the extracellular domain and and one in the cytosolic C-terminal part of the molecule. The expression constructs made by site-directed mutagenesis were transfected into human embryonic kidney (HEK293) cells and pools of at least five independent stable colonies were selected for further analysis. Permeabilized and non-permeabilized cells were immunostained, respectively, for intracellular and cell surface expression using a polyclonal rabbit nephrin antiserum. NPH5, a stably expressing wild type nephrin, was used as a control. Immunostaining of the different transfectans demonstrated that 16 out of the 21 mutations interfere with membrane targeting of the nephrin mutants so that they are entrapped in the ER. The cells were co-stained with caltreticulin, a known ER intracellular protein. Five mutants yielded surface staining (Table 3). Ultracentrifugation subcellular fractionations and immunoelectron microscopy further confirmed the dislocalization of the nephrin mutants, as observed by immunostaining microscopy.

Table 3. Expression of nephrin missense mutants in HEK293 cells

Exon	Location	Mutation	Cell surface location	Other Mutations found in the patient	Patient Origin (#)
2	Ig1	W64S	-	Truncation	Finland (19)
4	Ig2	I171N[a]	-	-	Turkey (2)
4	Ig2	I173N	-	S350P	France (14)
7	Ig3	G270C	-	Splicing Mutation	England (22)
9	Ig3-4	S350P	-	I173N	France (14)
9	Ig4	S366R[a]	-	-	Croatia (1)
9	Ig4	R367C	-	S724C, R1140C	France (21)
9	Ig4	P368S[a]	-	Promoter (delGA)	Netherlands (9)
9	Ig4	L376V	+	P368S	Netherlands (9)
10	Ig4	R408Q	+	C465Y, Truncation	Finland (18)
11	Ig5	C465Y	-	R408Q, Truncation	Finland (18)
12	Ig5	C528F	-	L610Q	France (15)
14	Ig6	L610Q	-	C528F	France (15)
14	Ig6	C623F	-	R408Q	North America (23)
16	SP	S724C	+	R367C, R1140C	France (21)
17	SP	R743C	+	Truncation	Finland (20)
18	Ig7	R802W	-	del172Thr	Netherland (17)
18	Ig7	R802P	-	Splicing Mutation	North America (12)
18	Ig7	A806D[a]	-	-	Morocco (3)
18	Ig7-8	R831C	-	-	North America (25)
27	Intracellular	R1140C	+	S724C, R367C	France (21)

[a]Homozygous mutations
[#] Numbers corresponding to patient numbers in (Lenkkeri *et al.* 1999)

The transport of misfolded glycoproteins in the ER is inhibited by quality control as previously explained. Calnexin and calreticulin are two ER-resident chaperone lectins that have a major role in the conformation-dependent molecular sorting of newly synthesized N-linked glycoproteins (Ellgaard *et al.* 1999). These two homologous lectins bind to almost all soluble and membrane-bound glycoproteins in the ER and ensure that misfolded proteins are retained in this compartment and eventually degraded. Our data suggested that 16 of the missense nephrin mutants studied lead to misfolding of the newly synthesized nephrin. Thus, most likely in corresponding patients the nephrin molecules are retained and degraded in the ER, instead of being transported to their site in the plasma membrane. This, in turn, may provide a possible pathomechanism for the loss of nephrin function in the SD in patients carrying those

missense mutations with defective intracellular transport to the plasma membrane. Knowledge of the crystal structure of nephrin might provide insight into how each mutation affects the protein folding and conformation process. Nevertheless, it is tempting to speculate that many of these mutations, such as W64S, I171N, S350P, R802W and R802P, result in non-conservative amino acid substitutions that lead to unfavored conformations in respective domains. Mutations leading to introduction of additional cysteine residues within Ig-domains, such as G270C, are most likely involved in the formation of incorrect disulfide bonds within each Ig-domain or such mutations may lead to new abnormal intramolecular disulfide bridges. In contrast, the mutations S724C and R743C, located in the long spacer region between Ig6 and Ig7, did not hinder the transport of the mutant proteins to the cell surface. This indicates that the spacer region is better tolerant to amino acid substitutions. Indeed, search in the protein data base revealed no homology of this spacer sequence to any known structural domain or motif, suggesting that it has a flexible structure and that missense mutations in this region may not necessarily lead to misfolding of the molecule. To this end, a nephrotic female infant with a Fin-major/R743C genotype had a kidney with a normal SD and she responded to therapy with an angiotensin-converting enzyme inhibitor and indomethacin (Patrakka *et al*. 2000). Morever, Koziell et al (Koziell *et al*. 2002) found co-existence of *NPHS1* and *NPHS2* mutations in both CNF and FSGS. Two cases of CNF like patients were detected with a homozygous R138Q mutation in *NPHS*2, but no *NPHS1* mutation. On the other hand, four FSGS patients not only have an *NPHS2* mutation, but it is also accompanied by an *NPHS1* mutation, the latter probably acting as a disease modifier representing a genetic epistasis (Koziell *et al*. 2002). This emphasizes the importance of screening both genes in CNF and FSGS, and also the joint critical role of nephrin and podocin's in the regulating glomerular filtration function.

II. Clustering-induced phosphorylation of nephrin by Src-family kinases

Based on nephrin's predicted structure, locations and function, it is widely accepted that nephrin is a key adhesion molecule of the SD, that is a podocyte specific cell-cell junction (Reiser *et al.* 2000). Clustering of receptors with multivalent ligands or antibodies can initiate signaling of CAM of the Ig superfamily, integrins and certain receptor tyrosine kinases (Heldin 1995; Juliano 2002), and we wished to examine if nephrin might also have a role in signal transduction. By adding the nephrin

monoclonal 50A9 antibody, nephrin was clustered on the surface of NPH15, cells stably expressing wild type recombinant nephrin. Cell lysates were analyzed by Western blotting with an anti-phosphotyrosine antibody. The clustering induced strong and nearly immediate increase in tyrosine phoshorylation of nephrin. By immunofluorescence microscopy nephrin was mainly visualized at the cell-cell adhesion sites before clustering. After clustering, the molecules nephrin moved from the cell-cell connections and formed the large aggregates on the apical side of the cells.

Then we studied the possible role of Src kinases in nephrin phosphorylation. Src family kinases are known to be involved in tyrosine phosphorylation of several signaling Ig superfamily proteins (Crossin and Krushel 2000; Juliano 2002). In human nephrin, Tyr_{1176} Tyr_{1193} and Tyr_{1217} are three conserved highly potent tyrosine phosphorylation sites for Src family kinases and docking sites for SH2 domain-containing adapter proteins. The Src family-selective tyrosine kinase inhibitor PP2 (Hanke *et al.* 1996), completely inhibited clustering-induced nephrin phosphorylation, suggesting that Src family kinases are major kinases phosphorylating nephrin on tyrosine residues. Decreased solubility of nephrin in Triton-containing lysis buffer and the lower recovery of clustered nephrin by immunoprecipitation were also observed. This could be due to the formation of large immunocomplexes, or to the nephrin's localization to insoluble membrane rafts. However, alteration in solubility was independent on phosphorylation, and occurred also in presence of PP2.

The Src family of kinases contains nine members in humans. Blk, Hck, Fgr, and Lck are confined to lymphoid and myeloid tissues, whereas Fyn, Src, Yes, Yrk are widely distributed throughout the organism (Korade-Mirnics and Corey 2000). Lyn is also widely distributed, although it is predominantly expressed in haematopoetic cells (Hibbs and Dunn 1997). Yrk is found in several tissues (Martins-Green *et al.* 2000), but detailed information about its expression pattern is lacking. To identify which Src family kinases are expressed in NPH15 cells and an immortalized mouse podocyte cell line, we used a pan anti-Src family specific antibody and detected several protein bands from cell lysates in Western blots analysis. The calculated molecular weights of 60 kDa, 59 kDa and 56 kDa, suggest the presence of Src, Fyn and Lyn in podocytes and NPH15 cells. Specific anti-kinase antibodies were further confirmed by Western blotting of cell lysates. In addition, we detected ubiquitous Yes kinase in both lysates, although the signal was weak in NPH15 cells.

To determine which of the Src family kinases could be involved in nephrin phosphorylation *in vivo*, HEK293 cells were cotransfected with cDNAs encoding

nephrin and the constitutively active form of either Fyn, Lyn, Src or Yes. Inactive forms of kinases were similarly transfected as negative controls. Expression of Src family kinases resulted in induction of tyrosine phosphorylation of nephrin, and particularly robust phosphorylation was observed when Src or Fyn were co-expressed with nephrin. Consistently, Fyn and nephrin coimmunoprecipited from NPH15 cells, but never from Fin-minor expressing cells. This suggests a normal association of Fyn and nephrin *in vivo*. When analyzing total cell lysates by Western blotting, we could not detect any apparent changes in phosphorylation of other proteins besides nephrin. However, when phosphoproteins were first concentrated by immunoprecipitation with an anti-phosphotyrosine antibody, and then examined by Western blotting, nephrin clustering was found to induce phosphorylation of a 46-kDa protein. Strikingly, a phosphoprotein of similar size also coimmunoprecipitated with nephrin.

More than simply mediating cellular interactions, various Ig superfamily CAMs behave as signal transducing molecules (Crossin and Krushel 2000). Nephrin has a domain and sequence structure typical for a cell-cell or cell-matrix adhesion protein. Its location is exactly and specifically in the slit diaphragm based on immunohistochemistry and immunoelctron microscopy (Patrakka *et al.* 2000; Ruotsalainen *et al.* 2000). Also in sophisticated electron tomography studies, nephrin forms an ordered two-layer structure (unpublished data). A lack of nephrin leads to absence of the SD and most foot processes (Jalanko *et al.* 2001; Putaala *et al.* 2001), which indicates that intactness of the glomerular ultrafiltration barrier is dependent on nephrin's correct presence in the SD . In other words, podocytes have to know whether nephrin is correctly positioned or not, i.e. when the nephrin molecule has reached the slit diaphragm and settled there, and when the slit diaphragm is broken and nephrin loses its normal extracellular contacts. It may be that nephrin is not only an important structural component of the filtration barrier, but it is also used as a "sensor" of the intactness of this barrier. Dislocation of nephrin from the slit diaphragm may initiate a signaling cascade that provides a response to the altered situation. It has been shown that in the anti-ganglioside antibody-induced proteinuria nephrin is dislocated to the apical pole of narrowed filtration slits, and is tyrosine phosphorylated (Simons *et al.* 2001). Earlier studies have shown that anti-nephrin antibodies are able to induce proteinuria (Orikasa *et al.* 1988) and that the perfusion with protamine sulfate leads to rapid disappearance of the slit diaphragm, foot process effacement, and induces phosphorylation of unidentified 180-kDa protein (Kurihara *et al.* 1995). Taken together, our observations strongly suggest that nephrin has, indeed, signaling functions.

Clustering likely mimics the altered interaction of nephrin with its extracellular ligands by moving nephrin on the plane of cell surface. This initiated the signal transduction by inducing rapid tyrosine phosphorylation of nephrin in one minute. The induction of phosphorylation was inhibited by PP2 indicating that Src family kinases are involved in nephrin phosphorylation. Interestingly, Fyn kinase knock-out mice exhibit podocyte foot process effacement and proteinuria (Yu *et al*. 2001). Fyn kinase immunoprecipitated with nephrin in our study suggesting that Fyn kinase either phosphorylates nephrin *in vivo* or associates with phosphorylated nephrin, or has both of these functions. Verma and his colleagues finding that Fyn binds to and phosphorylates nephrin in the lysate from isolated rat glomeruli (Verma *et al*. 2003) confirmed our assumption.

Huber and coworkers have now demonstrated that tyrosine phosphorylation of nephrin creates binding sites for phosphoinositide 3-OH kinase and then triggers serine-threonine kinase AKT -mediated downstream signal transduction inhibiting podocyte apoptosis (Huber *et al.* 2003a). Our additional observation of an unidentified 46 kDa phosphoprotein coimmunoprecipited by anti-nephrin antibodies might be one of the substrate proteins involved in the sequential nephrin-associated kinase cascade. Src, Fyn, Yes or Lyn alone, or in different combinations, participate in c-Jun's activation in signal transduction pathways (Yoshizumi *et al.* 2000; Fresno Vara *et al.* 2001). JunD expression has been connected to terminal differentiation of cells and protects cells from apoptosis (Sharma and Richards 2000; Weitzman *et al.* 2000). Protection of differentiated podocytes from apoptosis during proteinuria, i.e. when the structure of the slit diaphragm is disturbed or during temporal distributions in cell adhesion, would be of great importance. JunD is a broadly expressed partial antagonist of c-Jun. Nephrin was shown to activate c-Jun in transfected HEK293 cells (Huber *et al.* 2001). We, therefore, made an assay based on the measurement of c-Jun DNA-binding activity, that is increased when c-Jun is phosphorylated by Jun aminoterminal kinase. In clustered NPH15 cells, c-Jun activity were 2-fold higher than in clustered Fin-minor cells. Meanwhile, the DNA-binding activity of JunD was also almost 2-fold higher in clustered NHP15 cells in comparison with Fin-minor cells. In our study (II), we developed an experimental system for investigating nephrin signaling in the slit diaphragm and demonstrated that Src family kinase activity is crucial for tyrosine phosphorylation of nephrin, and that several members of Src family kinases are apparently able to catalyze nephrin phosphorylation. The most significant aim of studying nephrin signaling is to unravel how the signaling is induced *in vivo* in kidneys

and how it relates to development of various glomerulopathies. For those purposes, our study provides a cell culture model as a tool for examining the activity of Src family kinases, c-Jun and JunD as potential indicators of the signaling status of the slit diaphragm and podocyte viability.

III. Disease-causing missense mutations in the podocin gene alter normal nephrin trafficking to the plasma membrane

Co-immunoprecipitation studies have shown that podocin acts as an intracellular ligand for the cytoplasmic domain of nephrin (Schwarz *et al.* 2001). Previously, we reported that the majority of nephrin missense mutants are retained in the ER and are not transported to the plasma membrane (**I**). The aim of the this study (**III**), was to find out whether disease-causing missense mutations in the NPHS2 gene identified in patients with SRN might lead to dis- localization of the mutant podocin molecule, similarly to what we had shown for nephrin (**I**). Here, we analyzed the five subcellular locations of different disease-causing missense *NPSH2* mutations in HEK293 cells. Immunofluorescence and confocal microscopy showed that two of these missense mutations (P20L and G92C), introduced into the proximal N-terminus of the podocin molecule, had plasma membrane location similar to that of wild-type podocin. In contrast, the subcellular localization of three other missense mutants with mutations in the proximal C-terminus was drastically altered, two mutants, R138Q and R291W, being retained in the ER, while another (V180M) formed inclusion bodies in the cytoplasm. Interestingly, this abnormal subcellular location also resulted in alterations in protein trafficking of wild-type nephrin leading to co-localization of nephrin with the podocin mutants in the ER and inclusion bodies, respectively. Recently, Huber et al (Huber *et al.* 2003c) published identical data showing that disease-causing podocin R138Q and R138X interact with nephrin, but fail to recruit nephrin into rafts either because of retention in the ER or, because they get to the plasma membrane but are not targeted to the rafts (Huber *et al.* 2003c). This corresponds to our findings and underscores the synergistic role of a critical functional inter-relationship between nephrin and podocin in the pathogenesis of SRN and CNF. Our study, together with those of others (Huber *et al.* 2003c; Roselli *et al.* 2004b), suggest that some disease-causing missense mutations in the *NPHS2* gene may abolish both proper podocin and nephrin localization to the plasma membrane and lead to disruption of a normal slit diaphragm structure and function in patients with steroid-resistant nephrotic syndrome.

IV. Defective trafficking of nephrin missense mutants rescued by a chemical chaperone

In this study, we tested if some of the known disease-causing nephrin mutants retained in the ER can be relocated to the plasma membrane. Furthermore, we tested if the rescued nephrin mutants might then be functional. Sodium 4-phenylbutyrate (4-PBA) has been shown to function as a chemical chaperone that can correct the cellular trafficking of several mislocalized or misfolded mutant plasma membrane proteins, like the cystic fibrosis transmembrane conductance regulator (CFTR) and α1-antitrypsin (Rubenstein *et al.* 1997; Burrows *et al.* 2000). As discussed above, the most common consequence of missense mutations in CNF is a defect in intracellular transport and retention of the mutant proteins in the ER, possibly due to misfolding and unfavored conformation (**I**). Therefore, we explored the potential effects 4-PBA on the missense mutants found in CNF patients. The experiment was performed using HEK 293 cells stably expressing wild-type or the 21 nephrin missense mutants studied in Liu et al (**I**) (Liu *et al.* 2001). After culturing the cells with 10 mM 4-PBA in normal DMEM medium, six out of 16 missense mutants that are trapped in the ER were now transported to the cell surface based on immunofluorescence microscopy and studies with biotinylated cell surface proteins (Table 4). Subsequently, we examined if the rescued mutants interact with Neph1, as this might give us a hint if the rescued nephrin mutants were potentially functional. Nephrin - nephrin interaction, and nephrin - Neph1 association are so far the only interactions assigned for the extracellular domain of nephrin both in vitro and in vivo (Barletta *et al.* 2003; Gerke *et al.* 2003; Liu *et al.* 2003) (Khoshnoodi *et al.* 2003). A plasmid encoding the extracellular part of Neph1 tagged with the human IgG Fc-portion was transfected into normal HEK 293 cells, the wild-type NPH5 cell line, the six rescued mutant forms (W64S, S350P, S366R, R367C, R802W, R831C) and one mutant (I171N) not to rescued by 4-PBA, respectively. As expected, FITC-labeled anti-human IgG did not stain normal unpermeabilized 293 cells only expressing Neph1 and the I171N mutant, Yet, the transfection was successful, because a strong signal was detected in Triton-permeabilized cells. In contrast, when the wild-type nephrin and the rescued mutant W64S were stained for Neph1 without permeabilization, an abundant signal was detected. Repeatedly in experiments including all the six rescued mutants, they were found to be able to interact with Neph1. Furthermore, their tyrosine phosphorylation was rapidly induced by cross-linking with anti-nephrin antibodies, implying that the rescued mutants are most likely functionally

intact. These results suggest that the use of 4-PBA could become a therapeutic approach for the treatment of CNF or other similar diseases affecting renal filtration.

Table 4. Cellular location of nephrin missense mutants expressed in HEK293 cells after treatment of 4-PBA

Case	*Mutation*	*Cell surface location*	*Cell surface location after 4-PBA(10mM)*
1	*W64S*	-	+
2	*I171N*	-	-
3	*I173N*	-	-
4	*G270C*	-	-
5	*S350P*	-	+
6	*S366R*	-	+
7	*R367C*	-	+
8	*P368S*	-	-
9	*C465Y*	-	-
10	*C528F*	-	-
11	*L610Q*	-	-
12	*C623F*	-	-
13	*R802W*	-	+
14	*R802P*	-	-
15	*A806D*	-	-
16	*R831C*	-	+

During recent years, 4-PBA has been shown to effectively rescue many mutated proteins that are arrested in the ER, and allow them to be expressed on the cell surface and also be correctly functional (Rubenstein *et al.* 1997; Burrows *et al.* 2000). 4-PBA is a known orally bioavailable short chain fatty acid regularly used as an ammonia scavenger in the treatment of urea cycle disorders (Brusilow 1991). It has already been tested in clinical trials with patients suffering from cystic fibrosis. These trials are based on the observation that 4-PBA can restore maturation on the cell surface of the deltaF508 CFTR protein in vitro and in vivo (Rubenstein *et al.* 1997; Zeitlin *et al.* 2002). The mechanism of the 4-PBA effect is not yet completely clear, but it acts at least as a transcriptional regulator and modulates heat shock protein expression in the ER quality control system (Choo-Kang and Zeitlin 2001; Rubenstein and Lyons 2001). In this study, we observed that 4-PBA could prevent retention of several mutant nephrin proteins in the ER and promote their transport to the cell surface. However, 4-PBA did

not rescue the cell surface expression of all the nephrin mutants, which may reflect the degree of impairment in the folding of these proteins. Interruption of nephrin-Neph1 specific interaction is achieved by injecting combinations of individual subnephritogenic doses of anti-Neph1 and anti-nephrin, resulting in complement- and leukocyte-independent proteinuria in rats (Liu *et al.* 2003). Neph1 and nephrin's linkage in the SD appear to be an important determinant of glomerular permeability. In addition to being a structural component of the slit diaphragm, nephrin is a signaling protein that is heavily tyrosine phosphorylated when dislocated from the slit diaphragm or cross-linked with anti-nephrin antibodies (Simons *et al.* 2001) (**II**). All rescued nephrin mutants were found to interact with Neph1 on cell surface. Moreover, their tyrosine phosphorylation was rapidly induced by clustering. All this data implies that the rescued mutant nephrins are biologically functional, at least in cell culture. Consequently, this study suggests that the use of 4-PBA or corresponding chemical chaperones may be in the future a potential therapeutic approach for the treatment of mild CNF caused by the absence of nephrin on SD or in some patients with a non-familial FSGS or other similar diseases affecting on renal filtration. The generation of mice expressing nephrin missense mutations, using gene targeting in ES cells, will be necessary to test this type of drug *in vivo*.

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