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Nephrin - Intracellular trafficking and podocyte maturation

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To the ones I love

"No one can really feel at home in the modern world and judge the nature of its problems - and possible solution to those problems - unless one has some intelligent notion of what science is up to. Furthermore, initiation into the magnificent world of science brings great aesthetic satisfaction, inspiration to youth, fulfillment of the desire to know, and deeper appreciation of the wonderful potentialities and achievements of the human mind."

Issac Asimov

Summary

Congenital nephrotic syndrome of the Finnish type, CNF, is an autossomal recessive disorder caused by mutations in the *NPHS1* gene and, consequently, in its product nephrin, a 180 kDa transmembrane protein belonging to the immunoglobulin superfamily of adhesion molecules. In kidneys, nephrin is located exclusively to the podocyte slit diaphragm and it has a key role in organising and maintaining the renal ultrafiltation barrier. In Finland, 94% of the CNF phenotypes are caused by two mutations, Fin_{major} and Fin_{minor}. In contrast, the rest of the Finnish patients and patients outside Finland have unique set of mutations giving rise to the same CNF phenotype. Among those, missense mutations resulting in single amino acid substitutions in nephrin account for more that 50% of the disease-causing mutations. In this study, we investigated, *in vitro*, the fate of twenty-one-nephrin missense mutants identified in CNF patients. Our studies show that 16 out of 21 mutants are retained in the ER, and fail to reach the plasma membrane, suggesting that defective trafficking of the mutated nephrin, due to misfolding, may contribute to the CNF phenotype observed in patients carrying nephrin missense mutations.

A tightly regulated quality control prevents about one third of all proteins produced by the cells to exit the ER, and target those for degradation. Among them, there are proteins that, despite the mutations and aberrant folding, still retain part of the functional properties of the wild-type molecule. Small compounds called chemical and pharmacological chaperones have been shown to be able to rescue protein mutants from ER quality control and redirect them to their proper location. We therefore tested the potential effect of 4-PBA, one chemical chaperone, on the nephrin missense mutants retained in the ER. We demonstrate that in presence of 4-PBA many of the mutants are rescued from the ER to the cell surface. Further studies indicate that these mutants are functional, as judged by their ability to interact with an extracellular partner, NEPH-1, and to become phosphorylated upon antibody clustering. Our results suggest that 4-PBA could represent a new therapy for patients with CNF.

Nephrin has been shown to play important structural and signalling roles in podocytes, *in vitro*. In the last part of this study we investigated the impact of nephrin in late-embryonic-stage glomeuli. Our results show that the absence of nephrin, indeed, causes severe structural damage to the podocytes. However, nephrin has little impact on podocyte proliferation and apoptosis. Moreover, nephrin was able to regulate only a small set of genes, and had no impact on overall podocyte protein expression. One protein upregulated in the absence of nephrin is the tight junction component claudin 3, which is usually absent from the

glomerulus. We suggest that claudin 3 may be expressed as means for the podocyte to compensate for the loss of the proper slit diaphragm, being a component of the yet poorly understood junction-like structure observed between effaced podocyte foot processes of CNF patients. Taken together, our results suggest that nephrin has key importance in maturation of the slit diaphragm, specially the correct assembly of the filtration barrier. However, nephrin is not actively involved in podocyte development.

Original Publications

This thesis work is based on the following original publications:

I. Li Liu, **Stefania Cotta Doné**, Jamshid Koshnoodi, Alejandro Bertorello, Jorma Wartiovaara, Per-Olof Berggren and Karl Tryggvason. Defective nephrin trafficking caused by missense mutations in the NPHS1 gene: insight into the mechanisms of congenital nephrotic syndrome. Hum. Mol. Gen. 10 (23): 2637-44, 2001.

II. Li Liu, **Stefania Cotta Doné**, Kunimasa Yan, Pekka Kilpeläinen, Timo Pikkarainen and Karl Tryggvason. Defective trafficking of nephrin missense mutants rescued by a chemical chaperone. J. Am. Soc. Nephrol. 15: 1731-38, 2004.

III. **Stefania Cotta Doné**, Minoru Takemoto, Liqun He, Ying Sun, Christer Betsholtz and Karl Tryggvason. Nephrin is involved in podocyte maturation but not survival during glomerular development. Kidey Intl. *in press*, 2007.

Abbreviations

4-PBA	4-Phenylbutyrate
BrdU	Bromodeoxy uridine
CAM	Cell adhesion molecule
CASK	Calcium/calmodulin-dependent serine protein kinase
CD2AP	CD2-adapter protein
CDK	Cyclin-dependent kinases
E	Embryonic day
ECM	Embryonic extracellular matrix
ERM	Ezrin-radxin-moesin
ER	Endoplasmic reticulum
FAK	Focal adhesion kinase
F-actin	Filamentous-actin
FSGS	Focal and segmental glomerulosclerosis
GBM	Glomerular basement membrane
GLEPP1	Glomerular epithelial protein-1
GTP	Guanosine tri-phosphate
HEK	Human embryonic kidney
Hsp	Heat shock protein
IGF	Insulin-like growth factor
ILK	Integrin-linked kinase
IQGAP	IQ motif-containing GTPase-activating protein
JAM	Junctional adhesion molecule
JNK	c-Jun NH2-terminal kinase
LDL	Low density lipoprotein
MAGUK	Membrane-associated guanylate kinase
MAGI	MAGUK inverted
MAPK	Mitosis activated protein kinase
NHERF2	Na^+/H^+-exchanger regulatory factor-2
NK	Natural killer
PCNA	Proliferation cell nuclear antigen

PI3K	Phosphoinositide 3-OH kinase
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SH	src-homology domains.
TGF	Tumour growth factor
TUNEL	Transferase dUTP Nick Elongation
VEGF	Vascular endothelial growth factor
ZO-1	*Zonula occludens-1*

Table of Contents

"It is certainly impossible for any person who wishes to devote a portion of his time to chemical experiment, to read all the books and papers that are published in connection with his persuit; their number is immense, and the labour winnowing out the few experimental and theoretical truths which, in many of them, are embarrassed by a very large portion of uninteresting matter, of imagination, and of error is such, that most persons who try the experiment are quickly induced to make a selection in their reading, and thus inadvertently, at times, pass by what is really good." Michael Faraday

Review of Literature

1. The Kidney

1.1. ***Anatomical observations***

Kidneys are constituents of the excretory system and they are, as such, subject to extreme physiological conditions. All body fluids are maintained in a dynamic equilibrium with one another, hence any changes in blood composition are reflected by complementary changes in other body fluids. The kidneys are responsible for maintaining the osmotic balance of all body fluids by regulating the osmotic composition of the blood plasma and by reabsorbing 99% of the 170-180L of primary protein-free and cell-free filtrate formed each day.

Apart from osmoregulation, the kidneys also contribute to the maintenance of normal blood pressure through the renin-angiotensin-aldosterone system and to the oxygen-carrying capacity of the blood through production of the hormone erythropoietin, which stimulates erythrocyte production in the bone marrow, as well as to the maintenance of Ca^{2+} homeostasis and handling through vitamin D metabolism.

The individual functional unit of the kidney is called a nephron and it comprises the renal corpuscle, or glomerulus, the proximal tubule, the loops of Henle, the distal tubule and the connecting tubule. The number of nephrons in each kidney varies between species, with humans having approximately $1x10^6$ nephrons and rats between 30.000 and 34.000 nephrons in each kidney. Two distinct regions are observed in a kidney cross-section: an outer region, the cortex, and an inner region, the medulla. This division is believed to be important for the ability of the kidney to form concentrated urine. The cortical zone is formed by the glomerulus, the proximal tubules and part of the distal tubules, whilst the medullar zone contains both ascending and descending thin limbs and collecting ducts.

The blood flow in each mammalian kidney is provided by a single renal artery, which branches further to form the renal microcirculation network. Likewise, the blood leaves the urinary system via a single vein [30, 144, 157].

1.2. ***Developmental aspects***

The formation and maturation of the kidney is a well-orchestrated and regulated series of events involving morphogenesis, proliferation, differentiation and apoptosis. The mature mammalian kidneys develop in three stages: *pronephros*, *mesonephros* and *metanephros*, in a cranial-caudal succession and following a precise temporal and spatial sequence. In mammals, the *pronephros* and *mesonephros* are transient structures that exist only for defined periods of time. The *metanephros*, upon regression of the first two renal pairs, becomes the permanent, functional kidneys [166].

The first nephritic structures rise from the intermediate mesoderm around 22 days *post-coitum* in humans (E8 in mice). The initiating signals arise from the somites and from the surface ectoderm and induce mesenchymal cells in the intermediate mesoderm to differentiate, forming the nephric duct. The nephric duct is the first epithelial component and constitutes the central structure of the urogenital system. Its origin appears to be a result of caudal proliferation and extension of the intermediate mesoderm [56, 223]

The *pronephros* is the most primitive kidney form and is non-functional in higher vertebrates. In contrast, in lower vertebrates such as amphibians and fish they represent the embryonic kidney and are essential for survival. They comprise a filtration unit called *glomus*, an associated coiled nephritic tubule and a duct to dispose of the urine, the nephric duct (also referred to as pronephric duct and mesonephric duct or Wolffian duct in later stages). The *glomera* filter the blood and the primary filtrate accumulates in a space called the nephrocoel. Thereafter, the filtrate is directed into the nephritic tubules by the nephrostomes, ciliated funnels that connect the pronephric tubules to the nephrocoel. Within the tubules, the excess water and nutrients are reabsorbed and the waste is transported to the *cloacae* through the pronephric duct [100, 268].

In mammals the *pronephroi* cease developing. The most cranial units and the pronephric tubules degenerate completely and disappear, around *post-coitum* day 24 in humans and E9.5 in mice. The nephritic duct migrates caudally to form the mesonephric kidney [34].

The *mesonephros* develops from the mesonephric mesenchyme, a cell population within the intermediate mesoderm. Aggregates of mesenchyme cells form mesonephric tubules and renal vesicles, which give rise to structures resembling the mature nephron, with a glomerulus-like structure called the mesonephric corpuscle, associated proximal and distal tubules and a collecting tubule. The first mesonephric tubules develop around the 25th day *post-coitum* in humans (E9 in mice). The number of *mesonephroi* as well as their functional maturity and fate vary among species. Just before reaching the maximum number, the more cranial tubules start to degenerate. In females, this regression leads to a total disappearance of the organ whereas in males the most caudal parts of the tubules and the mesonephric duct will give rise to the genital organs [56, 223].

The development of the *metanephros* starts around the 28th day *post-coitum* in humans (E10.5 in mice) when a new structure called the ureteric bud sprouts from the caudal end of the nephric duct. Once the primary ureteric bud is formed, it grows towards the metanephric mesenchyme or blastema, a special population of undifferentiated cells, also derived from the intermediate mesoderm. After this first step, an intricate and tightly regulated series of reciprocal inductive signals originating from both the newly formed ureteric bud and the blastema are of uppermost importance for the forthcoming nephrogenesis. The ureteric bud itself through extensive branching gives rise to the entire urinary collecting system, including collecting ducts, renal pelvis, calyces and ureter, and the blastema undergoes extensive differentiation and originates most of the filtration unit, the nephron, from the glomerulus to the distal tube [45, 223, 268].

Branching and morphogenesis of the tubular system starts when the ureteric bud enters the blastema and the first bifid branching occurs, forming a T-shaped structure. Thereafter several other branching events take place successively until the characteristic tree-like structure of the mature collecting system is formed. The branching occurs in phases: the first phase is a fast and regular branching process that generates the primary cortex tubules, the second one is a slow phase in which the branches elongate extensively and probably will originate the long unbranched collecting tubules present in the outer part of the medulla, and the third phase generates the final cortex tubules. There is a correlation between the rounds of branching and the number of nephrons to be formed, although this relationship is not linear [45].

In coordination with ureteric bud morphogenesis the blastema also undergo profound changes in order to form the mature nephron. In a process of epithelialisation called

mesenchymal-to-epithelial transformation, the blastema cells change from non-polarised, loosely associated and fibroblast-shaped to polarised, asymmetrical, cuboidal cells, which form the nephron epithelia. The main pathways leading to the mesenchymal-to-epithelial transformation are regulated by temporal and spatial changes in the gene expression patterns of transcription factors, proto-oncogenes, growth factors and respective receptors, and are modulated by cell adhesion molecule complexes. The mature nephron starts to develop when the mesenchymal-to-epithelial transformation of the blastema is completed [27, 56, 100, 225].

In response to the invasion by the ureteric bud, cells from the blastema are induced to condense around the tips of the newly formed branches as small cell aggregates, the pretubular aggregate (Figure 1). After a burst of proliferation, these cells start to express epithelial markers and form polarised structures called renal vesicles. At this time point, the proliferation ceases and the renal vesicles develop a lumen, elongate and fold into structures called comma-shaped bodies (Figure 1). The cells start to show the first signs of differentiation. Further segmentation occurs and the comma-shape body assumes an S-shape (Figure 1). In this newly formed S-shaped body, the cell diversification is more evident. Cells closest to the ureteric bud become the distal tubule and fuse with the former, whilst the farthest portion of the S-shaped body originates the parietal epithelia, or Bowman's capsule, and the visceral epithelia composed of podocytes. The middle portions of the S-shaped body give rise to the proximal tubule and the loop of Henle. Markers for the different segments of the nephron start to be expressed and the distinct cell types start to develop their final character [34, 100, 223].

Later S-shape stage and following capillary loop stage are characterised by further differentiation of the epithelia and by the vascularisation of the glomerulus (Figure 1). During the vascularisation process, pre-existing vessels, by means of angiogenesis, as well as blastema cells by means of vasculogenesis, invade the first-formed cleft on the S-shape body and originate the capillary tuft. Podocytes assume their characteristic architecture, the slit diaphragm is formed and the endothelium develops its *fenestrae*. The maturation of the GBM accompanies the development of the glomerulus. As the podocytes and the endothelium enter their final differentiation steps, the GBM thickens and the embryonic ECM proteins are substituted with the forms present in the mature GBM, i.e. laminin-1 is substituted by laminin-11 and type IV collagen chains shift from $\alpha 1$ and $\alpha 2$ to $\alpha 3$, $\alpha 4$ and $\alpha 5$ [136, 201, 223].

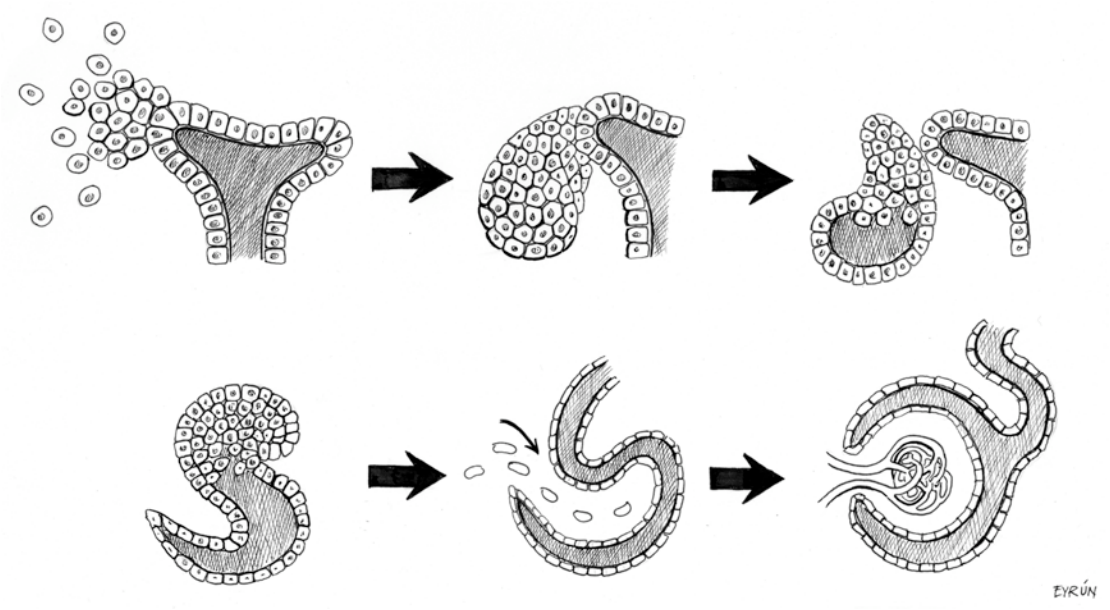

Figure 1: Schematic illustration of the development of the nephron. Adapted from ref. 223.

2. Glomerulus

The mature glomerulus consists of a tuft of capillaries and the surrounding Bowman's capsule. It has four distinct cell types: the parietal epithelial cells, the mesangial cells, the endothelial cells and the visceral epithelial cells or podocytes.

The parietal epithelial cells are the components of the Bowman's capsule and form the outer wall of the glomerulus. It consists of a single layer of cells resting on a basement membrane. The mesangial cells and mesangial matrix form the mesangium, a central region of the renal corpuscle. The mesangium plays several roles in kidney physiology, including production of vasoactive substances, growth factors and cytokines. It also provides structural support to the capillary loops and possibly regulates glomerular filtration rates. The fenestrated endothelial cells are constituents of the glomerular capillaries. The podocytes are components of the visceral epithelium of the glomerulus and are in close contact with the capillary tuft and with the GBM [157].

2.1. ***The filtration barrier***

The podocyte foot processes, together with the GBM and the fenestrated endothelium form the glomerular filtration barrier. The filtration barrier is the functional filter, which enables the production of the essentially protein- and cell-free primary urine.

The fenestrated endothelium constitutes the first barrier to the newly formed filtrate. The fenestrations are round pores, which occupy around 20% of the endothelial surface area. The pores, with diameters varying from 70-100μm, retain the blood cells inside the capillaries but do not by themselves represent a physical barrier to macromolecules. The surface of the endothelium is negatively charged, mainly due to the presence of podocalyxin, and may, therefore, contribute to the charge-selectivity of the filtration barrier [101].

The GBM is the result of a fusion, during glomerular development, between the endothelial and epithelial ECM. It is an approximately 310 nm thick highly cross-linked network composed of collagen type IV, the major component of GBM, laminins, fibronectin, entactin/nidogen and several heparan sulphate proteoglycans, such as perlecan and agrin. Although GBM was believed to be mainly a charge-selective barrier of the glomerulus, recent evidence indicates that the structural integrity of the GBM largely serves as a structural support for the capillaries [157, 258]. Transgenic mice whose GBM contains heparan-sulphate-deficient perlecan, or lacks agrin, do not develop proteinuria [91, 212]. In contrast, mutations in type IV collagen cause distortion of the GBM structure, Alport's syndrome [17], and those in the β2-laminin gene lead to a lethal form of nephrotic syndrome [174].

The podocyte foot processes are specialised structures formed at the end of the secondary processes. A gap of uniform width is present between two such adjacent foot processes. This gap is called a filtration slit and is spanned by a thin membrane, the slit diaphragm [157].

3. Podocytes

3.1. ***Structural considerations***

Podocytes, the largest cells in the glomerulus, are terminally differentiated epithelial cells with a very particular architecture. They are highly polarised and have the main cell body facing the urinary space. The cell body gives rise to long primary processes, which

thereafter branch into secondary processes and extend towards the capillaries. The foot processes envelop the capillaries in such a fashion that adjacent foot processes belong to different podocytes. The space between two adjacent foot processes is referred to as filtration slit and is bridged by the slit diaphragm [157]. The slit diaphragm defines the border between the apical and basal membrane domains in the podocyte, the apical domain comprising membrane from and above the slit diaphragm and the basal domain represented by the soles of the foot processes, which are embedded into the GBM [185].

The surface of the podocytes is covered by a glycocalix especially rich in sialoglycoproteins such as podocalyxin, podoendin and podoplanin, which confer a highly negative charge [102, 123, 124, 159]. The negatively charged podocyte surface is not only responsible for repulsing molecules, hence participating in the charge selectivity of the filtration barrier, but it also contributes to maintaining the proper structure of the podocytes by facilitating the filtration process, preventing foot processes of adjacent podocytes and the slit diaphragm from collapsing, and by preventing the podocytes from attaching to the parietal epithelia [39, 75, 249]. Podocalyxin is the major sialoglycoprotein in the glomerulus and carries more than 80% of glomerular sialic acid content. It is found on the podocyte apical membrane and in the luminal surface of the glomerular fenestrated endothelial cells [101, 124, 222] and it is associated with the podocyte actin cytoskeleton through interaction with ezrin, a member of the ERM family of actin-binding proteins, and with NHERF2 (Figure 2) [177, 250]. Other proteins present on the apical surface of the podocytes are the Heymann nephritis antigen megalin [122], an endocytic receptor from the LDL-receptor family that internalises multiple ligands, and GLEPP1, a protein phosphatase reported to contribute to the maintenance of the structure and function of the podocytes by regulating tyrosine phosphorylation of podocyte proteins [234, 255, 272, 277]. The podocytes are known for their distinct cytoskeleton, which accounts for their unique shape and for their ability to respond to the challenges imposed by the filtration process. The podocyte cytoskeleton is organised into several different levels, meaning each section of the podocyte expresses a distinct set of cytoskeletal proteins with different functions [185].

The cell body and the primary processes scaffolding are composed of intermediate filaments containing desmin and vimentin, and microtubules containing α- and β-tubulin. The microtubular structure of the primary processes hold the responsibility to connect the cell body to the foot processes, as well as to maintain the podocyte polarisation status and aid in

cell trafficking of proteins from the Golgi apparatus into the cell periphery [55, 131, 185, 243].

The podocyte foot processes are rich in microfilament bundles composed of F-actin, myosin II, vinculin, talin and actin-associated proteins such as α-actinin-4, synaptopodin and palladin. The microfilaments form a loop structure, with the base of the loop bound to the GBM though matrix receptors, whereas the top connects to the microtubules at the primary processes. This organisation confers to the podocyte the strength necessary to counterbalance the mechanical forces imposed by the capillaries [55, 64, 110, 169, 182, 185, 210].

Reorganisation of the foot process cytoskeleton due to diseases or mutations leads to podocyte foot process effacement, and hence loss of podocyte function. Podocyte effacement is a common characteristic among several congenital and acquired glomerulopathies and results in a total loss of the foot process interdigitating pattern, retraction of the processes and flattening of the podocyte cell. This process is tightly associated with cytoskeleton rearrangement and results in alteration of cell-cell and cell-matrix contacts [232]. Some of the proteins involved in podocyte effacement have been identified and their relevance in podocyte cytoskeleton regulation have been described. Mutation of the human MYH9 gene encoding non-muscular myosin heavy chain IIA found in podocytes is responsible for Fechtner's syndrome, which is characterised by nephritis, deafness and congenital cataracts among other defects [7]. α-Actinin-4 is widely expressed in podocytes and is associated with stress fibres, focal adhesion contacts and adherens junctions [141, 178]. It is essential for podocyte function since its ablation leads to severe renal failure [48, 133] and mutations in *ACTN4* gene cause FSGS [117, 160, 161]. Moreover, loss of α-actinin-4 is reported to be involved in podocyte depletion due to reduced adhesion of the podocyte to the GBM [48]. In addition to inherited diseases, puromycin-induced podocyte damage models have been found to exhibit an increase in α-actinin-4 expression prior to podocyte effacement, suggesting an involvement of cytoskeleton rearrangement in the disease process [237, 241]. Synaptopodin is a rather specific actin-associated protein expressed only in differentiated podocyte foot processes and telencephalic dendrites [167]. It interacts with α-actinin-4 and induces elongation rather than branching of actin filament bundles, which is in agreement with its pattern of expression. Short and branched actin filaments are characteristic of immature podocytes lacking foot processes whilst long and unbranched filaments are found in differentiated podocytes. This suggests that synaptopodin may be responsible for regulating

the α-actinin-4-induced actin filament bundling in podocytes, hence contributing to the complex cytoskeleton structure of the mature podocyte.

The findings that in many glomerulopathies synaptopodin expression levels are significantly reduced further support the role of synaptopodin in organising and maintaining the podocyte cytoskeleton. Indeed, synaptopodin-null mice exhibit impairment in recovering from induced proteinuria although they are, otherwise, normal. [8, 9, 16, 73, 97].

The cytoskeleton of the podocyte foot processes is also linked to the slit diaphragm. Nephrin, podocin, NEPH-1, -2, -3, P-cadherin and FAT-1 are some of the already identified components of the slit diaphragm [26, 52, 111, 125, 204, 231], and they interact with the actin filaments through adaptor and scaffolding proteins, among them CD2AP and MAGUK family members, forming a multiprotein complex responsible for maintaining the structure of the foot processes intact, as well as for signalling events driving podocyte function and survival (Figure 2) [21].

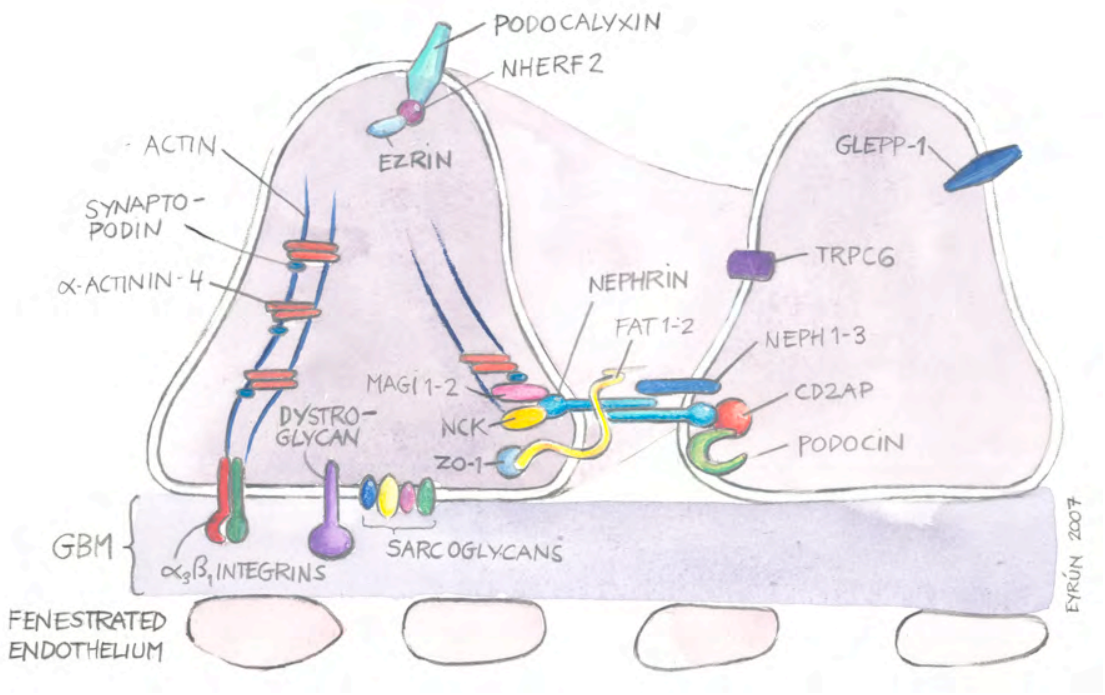

Figure 2: Illustration representing a cross-section of two podocyte foot processes, with the slit diaphragm, GBM and fenestrated endothelium.

CD2AP is a ubiquitously expressed adaptor protein first found to interact with the T- and NK-cell surface receptor CD2 and to stabilise the interaction between T-cells and antigen-presenting cells [60]. In kidneys, CD2AP is primarily expressed in podocytes and it has been shown to interact with nephrin and podocin, as well as with actin, thus serving as an adaptor molecule between the slit diaphragm and the cytoskeleton, and as a regulator of highly dynamic actin spots in podocytes [147, 230, 235, 275, 276]. CD2AP-deficient mice exhibit, in addition to a compromised immune system, severe renal failure at the age of 6-7 weeks [86, 236]. Mice heterozygous for CD2AP develop glomerular impairment at 9 months of age, suggesting that reduced levels of CD2AP caused by haploinsufficiency increase susceptibility to glomerular injury [129]. ZO-1, a peripheral membrane protein and a member of the MAGUK family of scaffolding proteins, is specifically enriched at tight junctions of epithelia and endothelia [81, 82, 244]. It is also associated with adherens junctions in some non-epithelial cells and prior to its final location in tight junctions [112, 197]. ZO-1 is one of the key regulators of tight junction assembly and it is known to associate with a number of other tight junction proteins, such as claudins, ZO-2 and –3, occluding, cingulin and JAM-1, α-catenin and F-actin, as well as with signalling proteins [228]. In podocytes, ZO-1 α, a ZO-1 isoform lacking the 80-amino acid internal α-domain, was found to be concentrated along the slit diaphragm [138, 226] and to interact with NEPH-1 [107] and nephrin [145]. CASK, another MAGUK family member, is found in synapses, tight junctions and at the podocyte foot processes, where it interacts with nephrin [146]. CASK is believed to participate in the maintenance of polarisation of epithelial cells by linking signalling molecules to the cytoskeleton [37, 92]. MAGI-1 and -2 are membrane-associated scaffolding proteins located in tight junctions as well as in the slit diaphragm. MAGI-1 was found to interact with synaptopodin, whereas both MAGI-1 and MAGI-2 interact with nephrin and α-actinin-4 [96, 146, 184]. IQGAP1 is an adherens junction-associated protein known to interact with E-cadherin and β-catenin, thereby regulating cadherin-mediated cell-cell adhesion [120, 139]. IQGAP1 also interacts with actin and regulates cytoskeleton dynamics through the small GTPases Cdc42 and Rac1 [19, 90, 248]. In podocytes, IQGAP-1 is located at the foot processes and it interacts with nephrin, which suggests a role for IQGAP1 in the regulation of slit diaphragm function by modulating cell adhesion and cytoskeleton dynamics [146, 155]. Nck is a family of ubiquitously expressed adaptor proteins consisting solely of three SH3 and one SH2 domain. In humans and mice the Nck family comprises 2 members, Nck-1 and -2. The Nck family is implicated in actin-cytoskeleton

polymerisation and organisation as well as in intracellular signalling [14, 32, 207]. Nck was recently shown to interact with nephrin [113, 266] and link this slit diaphragm protein to the podocyte actin cytoskeleton.

The soles of the podocyte foot processes are firmly attached to the GBM through protein-protein interactions (Figure 2). Moreover, properly regulated cell-matrix contacts and exchange of signals between the cell and the GBM are responsible for proliferation events during glomerulogenesis, for maintaining the mature podocyte in its terminally differentiated state, and for keeping the foot process architecture organised [22, 23, 78, 185, 193]. Podocytes attach to the GBM through two major complexes: integrin and dystroglycans. Integrins are a large family of receptors composed of an α- and a β-subunit in different combinations, conferring specificity in both substrate binding and signalling properties [78, 262]. Several different integrins are expressed along the nephron [132]. However, mainly integrin α3β1 is found at the soles of the podocyte foot processes [1, 132]. This integrin has been shown to recognise type IV collagen, laminin-521, fibronectin and nidogen, all components of the GBM. Ligand-receptor binding leads to receptor clustering and formation of focal adhesion points, and recruitment of intracellular ligands, such as cytoskeleton-associated proteins, signalling molecules and calcium-binding proteins [78, 193, 262]. Signalling events activated by integrin complexes include the FAK pathway, which is involved in the attachment of the integrins to the cytoskeleton via recruitment of adaptor proteins Crk and Nck [78, 224] and in cell survival through activation of the PI3-K/AKT pathway [40]; the ILK pathway, which is implicated in linking the integrins to the slit diaphragm [47]; and pathways which control cell cycle progression, proliferation and apoptosis [78]. Dystroglycan is the main component of the skeletal muscle dystrophin-glycoprotein complex, which also comprises dystrophin/utrophin, sarcoglycans, sarcospan, syntrophins and dysrobrevins. The dystrophin-glycoprotein complex links the cellular cytoskeleton to the basal membrane in muscle and epithelial cells [95, 279]. Dystroglycan is encoded as a single propeptide, which undergoes post-translational modification and generates two proteins, α-dystroglycan, a heavily glycosylated peripheral membrane protein, and β-dystroglycan, a transmembrane protein, which interact via their C- and N-terminus, respectively [95, 279]. Dystroglycan is ubiquitously distributed in foetal and adult muscle and non-muscle tissues [59]. In kidneys, dystroglycans are distributed in both apical and basolateral membranes [196, 269], but the intracellular binding partners utrophin, β-dystrobrevin and β2-syntrophin, responsible for linking the complex to the podocyte cytoskeleton, are found to be expressed exclusively on the

sole of the foot processes, and their extracellular ligands laminin, agrin and perlecan are in the GBM [156, 196, 203]. The exact function of the dystroglycan-complex in adult kidneys remains unclear. There are suggestions that dystroglycans may work to maintain podocyte architecture thorough its binding to the GBM, or even to maintain the integrity of the slit diaphragm, through α-dystroglycan negative charges [88, 269]. Its role in embryogenesis and kidney morphogenesis, on the other hand, is well defined. Dystroglycan-null mice fail to develop past E5.5 due to failure to form an extra-embryonic basement membrane [278]. In addition, blocking the binding of α-dystroglycan to laminin-1 causes disturbance in epithelial branching morphogenesis [58], revealing a key role of the dystrophin-glycoprotein complex in embryogenesis, epithelia development and basement membrane assembly.

3.2. ***Podocyte cell biology***

Podocytes are cells that serve several distinct functions. They contribute to the filtration barrier, regulate intracapillary hydrostatic pressure by exerting both pressure and tensile forces over the capillary loops, synthesise components of the GBM and remove macromolecules and immunocomplexes from the Bowman's space through endocytosis [15, 168].

The cell body contains a well-developed Golgi apparatus, abundant rough and smooth endoplasmic reticulum, and a great number of mitochondria and lysosomes, which agree with a high level of metabolic activity and high capacity of protein synthesis and post-translational modifications [168, 185]. A well-coordinated trafficking machinery is responsible for delivering the newly synthesised proteins from the cell body to the periphery, for secretion of active substances and for clearing the components of the ultrafiltrate and for recycling the podocyte proteins. Indeed, like neurons, podocytes feature highly regulated exocytosis/endocytosis machinery, which expresses classic exocytotic proteins including Rab3A, synapsin 1, synaptophysin, synaptotagmin1 and β-arrestin [195, 200].

Due to their terminally differentiated phenotype, mature podocytes are usually unable to proliferate. Podocytes are under tightly controlled cell cycle quiescence, due mainly to up-regulation and robust expression of CDK-inhibitors [185].

In the early stages of kidney development, the podocyte precursors are polygonal proliferative cells. In the S-shaped body stage of development, those precursors still express markers characteristics of proliferative cells, such as PCNA and cyclins A and B1 [171].

When entering the capillary loop stage, the podocytes exit from the cell cycle and loose their ability to divide. Specific markers start to be expressed, the podocytes start to develop their characteristic cell architecture and the foot processes are formed. This process is accompanied by the expression of the cell cycle inhibitors p27Kip1 and p57Kip2 [158, 171, 233].

Since there is no podocyte turn-over in healthy mature glomeruli [181], cell survival is essential. Several different mechanisms have been implicated in reduction of podocyte apoptosis and promotion of cell survival both *in vitro* and *in vivo*, among them VEGF and IGF-1 signalling pathways [28, 67, 68].

As is the case for all adherent cells, proper attachment of the podocyte foot processes to the GBM is essential for cell survival and once detached, susceptibility to apoptosis increases significantly. Detachment of podocytes from the GBM, followed by podocyte loss, is being appointed as a crucial step in the development of a series of glomerulopathies. As integrins and dystroglycans are the major matrix receptors in podocytes, it is fair to assume that podocyte apoptosis may be related to integrin/dystroglycan signalling impairment. Indeed, several studies demonstrate that integrin-ligand engagement is able to trigger the intracellular signalling cascades, which prevent cell apoptosis [69, 80]. Furthermore, abnormal integrin signalling has shown to be been involved in several different glomerulopathies [115, 137]. Nonetheless, the precise mechanisms underlying podocyte detachment, as well as events driving cell apoptosis are still under study [232]. Although detached podocytes are extremely vulnerable to apoptosis, *in vivo* studies have demonstrated that in different models for glomerulopathies, podocytes that detached from the GBM and are shed into urine are viable and can be grown *ex-vivo* [188, 189]. Recent studies have shed light has upon this question, proposing that certain slit diaphragm proteins may be able to induce signals promoting podocyte survival throughout injury [28, 67, 104].

3.3. ***The slit diaphragm***

The first observations of the slit diaphragm made by electron microscopy did not reveal its molecular organisation or composition. In 1972, Karnovsky and Ainsworth presented the first evidence of the role of the slit diaphragm as a size-selective filter [119], and in 1974, Rodewald and Karnovsky proposed a structural organisation for the slit diaphragm [208]. The model suggested a zipper-like structure where the cell membranes of adjacent foot

processes are kept approximately 35 nm apart through a central filament approximately 7 nm wide and 17 nm long [208, 227]. The molecular composition of the slit was however still obscure. In 1998, Kestilä and collaborators identified the first specific component of the slit diaphragm, nephrin [125]. Since then intense research has being performed and rapid progress has been achieved.

Today, it is clear that the slit diaphragm is a complex and highly dynamic structure formed by several different proteins that interact with each other and with the podocyte cytoskeleton. Furthermore, it has been proposed that the slit diaphragm acts not only as a physical filtration barrier but also participates in signalling events necessary to maintain the functional integrity of the filtration barrier. For this purpose, the maintenance of the polarised status of the podocytes is absolutely required. As in all cell-cell contacts, there is a constant flow of outside-in and inside-out information responsible to ensure podocyte survival and proper glomerular function, hence all the interactions inside and outside the cell membrane must be kept intact. Since the discovery of nephrin, several other proteins have been identified as components of the slit diaphragm. NEPHs 1-3 are transmembrane proteins structurally related to nephrin [231]. NEPH-1 and NEPH-2 interact with nephrin through their extracellular domains [18, 76], however, no interaction is seen between NEPH-1 and NEPH-2 [77]. Through their intracellular domain the NEPH proteins interact with ZO-1 and podocin [107, 231]. The importance of NEPH-1 in the slit diaphragm structure and function has been established through studies with mice lacking NEPH-1. These mice exhibit podocyte effacement, proteinuria and develop end-stage renal disease [52]. Podocin, the product of the *NPHS2* gene, is a 42 kDa membrane protein with similarities to the stomatin family proteins [26]. In kidneys, podocin localises exclusively to the podocyte slit diaphragm and podocin-null mice develop severe proteinuria and die a few days after birth from renal failure [211]. The *NPHS2* gene is mutated in autosomal recessive steroid-resistant nephrotic syndrome and in sporadic adult-onset nephrotic syndrome [26, 35, 71, 118, 135, 259, 274]. Podocin interacts with CD2AP [230], NEPH family members [231], TRPC6 channel [106], with the podocyte actin cytoskeleton and nephrin [221, 230]. The last association is enhanced by tyrosine phosphorylation of the nephrin intracellular domain [105] and it may be involved in maintenance of podocyte structure and function *in vivo*. FAT-1 and –2, giant protocadherins with 30+ tandem cadherin-like repeats, have also been localised to the podocyte slit diaphragm [111, 246]. FAT1 participates in cell-cell adhesion by controlling cytoskeleton dynamics [163, 252, 253]. The participation of FAT2 in podocyte biology is still under

investigation. Mice lacking FAT1 die soon after birth. They present collapse of the slit diaphragm and podocyte effacement, in addition to forebrain and ocular defects [44]. TRPC6 is a member of the transient receptor potential superfamily of cation-selective ion channels [173]. In kidneys, TRPC6 has been localised both to the glomerulus and to the tubules. In the glomerulus, expression is mainly podocyte-specific but it has also been detected in endothelial cells and, to a minor extent, in the mesangial cells. TRPC6 associates with nephrin and podocin and appears to be essential for renal function [205]. Recently, mutations in the TRPC6 gene have been associated with familial FSGS [198, 205, 280].

4. Nephrin

Nephrin, the main object of this study, is a 180 kDa transmembrane protein product of the *NPHS1* gene localised exclusively to the podocytes slit diaphragm [125, 218]. Nephrin is related to the immunoglobulin superfamily proteins and it comprises an extracellular domain containing eight immunoglobulin-like domains and a type III fibronectin-like domain, one single transmembrane-spanning helix and a short intracellular tail [125]. Nephrin actively participates in maintaining podocyte structure. Through its extracellular domain, nephrin is thought to participate in cell-cell interactions between adjacent podocytes [256]. In fact, Khoshnoodi and collaborators and Wartiovaara and collaborators demonstrated in an elegant series of experiments that nephrin promotes cell-cell contacts through homophilic interactions *in vitro* and *in vivo*, and that other slit diaphragm components contribute for this interaction [128, 273]. Indeed, the extracellular domain of nephrin has been shown to interact with the extracellular domains of NEPH-1 and NEPH-2 [18, 76, 77, 154]. Although the significance of the interaction between nephrin and NEPH-2 is still to be determined, the interaction between nephrin and NEPH-1 promotes filtration barrier stability and appears to be important for proper glomerular function [154]. The link between nephrin and the actin cytoskeleton is mediated by several foot process and cytoskeleton-associated proteins [96, 113, 145, 146, 150, 152, 184, 221, 230, 235, 266]. As in a tri-dimensional puzzle, those interactions are a key point to the structural stability of the slit diaphragm. The role of the slit diaphragm as a signalling platform has been discussed on several occasions. The first insights in this direction came from experiments describing the presence of lipid rafts in the slit diaphragm area and from the finding that podocin and nephrin are recruited into those areas

[230, 239, 242]. Lipid rafts are dynamic membrane microdomains composed of cholesterol and sphingolipids. Among the several properties of lipid rafts, it is worthy to mention that lipid rafts usually concentrate signal transduction molecules [238]. Proper recruitment of nephrin into lipid rafts has been appointed as a molecular basis of the functional podocin-nephrin complex [108]. Supporting this concept, Huber and collaborators showed that the signalling ability of nephrin is greatly enhanced by interaction with podocin [104, 105]. This complex was shown activate p38 kinase and JNK, and promote AP-1 transcription factor activation [105]. Furthermore, nephrin-podocin interaction with CD2AP was associated with the activation of PI3K and its downstream target AKT/PKB [104].

Another indication that nephrin may function as a signalling molecule comes from the fact that nine tyrosine residues in its intracellular tail, six of them conserved in rodents, are potential sites for phosphorylation and are described as docking points and initiators of a variety of signalling cascades [21, 103, 186, 187]. Indeed, nephrin can be phosphorylated by the Src-family kinase Fyn and possibly by Yes, thus providing docking sites for SH2 domain-containing proteins [267].

But how does nephrin become phosphorylated in the first place? One hypothesis is the classical model of activation of cell CAM family proteins, where homophilic and heterophilic interaction between extracellular domains trigger intracellular signalling cascades [46, 94, 114]. In agreement with this hypothesis, clustering of nephrin with monoclonal antibodies *in vitro* triggers phosphorylation of nephrin by Fyn [143]. Other evidence suggests a role of VEGF, which may, in part, be responsible for nephrin phosphorylation [67]. Different groups have addressed the physiological importance of nephrin phosphorylation. It has been demonstrated that a reduction in nephrin phosphorylation may be responsible for podocyte effacement and proteinuria [267, 288]. Li and collaborators also demonstrated that phosphorylation by Fyn enhances nephrin association with podocin and further increases downstream signalling of nephrin [151]. More recent studies have linked nephrin phosphorylation to actin cytoskeleton dynamics. Two independent groups have demonstrated that nephrin phosphorylation recruits Nck adaptor proteins, resulting in actin polymerisation and rearrangement of the actin cytoskeleton [113, 266]. In mice, podocyte-specific deletion of Nck1&2 results in defects in foot process formation, leading to congenital nephrotic syndrome [113]. Thus, the interaction between nephrin and Nck seems to be of crucial importance for the maintenance of the plasticity of the podocyte cytoskeleton and for proper podocyte function and architecture [113, 152, 266].

4.1. ***Congenital nephrotic syndrome of the Finnish type (CNF)***

CNF was originally described in Finland, where its incidence is very high [109], but today it is reported worldwide. As opposed to patients suffering from other nephrotic syndromes, CNF patients present proteinuria already *in utero* and the children are born prematurely, underweighted and featuring a large placenta, massive non-selective proteinuria and oedema. Previously, CNF children usually died within one year of birth, but now they can be submitted to kidney transplantation, the only curative treatment available [99]. Light microscopy analysis of CNF patient kidneys shows dilated tubules with massive accumulation of proteins, vacuoles filled with colloidal material, mesangial hypercellularity, expanded mesangial matrix, increased Bowman's capsule, dilated capillaries and increased size and number of glomeruli [11, 109, 199, 257].

Ultrastructural analysis of CNF glomeruli shows aberrant podocyte structure, with fusion of adjacent foot processes and absence of the slit diaphragm [10]. Despite the fact that CNF has been a known disease since 1950s, it took almost 50 years until the cause of the disease was identified. In 1994, Kestilä and collaborators found a potential candidate region in chromosome 19q13,1 and finally, in 1998, mutations were identified in a novel gene named *NPHS1*. The product of this gene was named nephrin [125, 126].

CNF is an autossomal recessive inherited disease and two mutations on the *NPHS1* gene account for 94% of all cases in Finnish population. Fin_{major}, a 2bp deletion on exon 2, causes a frameshift and generates a premature stop codon in the same exon. This mutation is found in approximately 78% of all Finnish cases. Fin_{minor}, a nonsense mutation in exon 26, leads to a truncated protein lacking almost the entire intracellular domain. Fin_{minor} phenotype is found in 16% of the Finnish chromosomes [125, 149, 183].

Non-Finnish patients present a broader spectrum of mutations [12, 20, 25, 70, 79, 125, 135, 142, 149, 183]. To date, over 80 different mutations have been described, consisting of deletions, insertions, nonsense, missense, splice-site and promoter mutations. Among those, missense mutations leading to single amino acid substitutions in nephrin represent more than half of the CNF-causing sequence variations. In addition to CNF, mutations in *NPHS1* have also been identified in patients with other types of nephrotic syndrome such as FSGS [135], diffuse mesangial sclerosis [229], minimal change nephrotic syndrome [142] and IgA nephropathy [172].

5. Adhesive junctions in mature glomeruli

In cell polarity lays the essence of all epithelia. A polarised cell is characterised by distinct distribution of proteins and lipids throughout the different plasma membrane domains (apical, lateral and basal), by the presence of junctional complexes separating the surface domains and regulating intercellular diffusion and transport, by cohesive cell-cell interactions mediated by cell adhesion complexes and by differences in the distribution of intracellular organelles within the cytoplasm. The signals leading to cell polarisation originate from cell-cell and cell-ECM contacts, the former being mediated by members of the cadherin superfamily, whereas the latter are mediated primarily by integrins [209, 254, 281].

As an organ constituted of different classes of epithelia, the acquisition and, above all, the maintenance of the epithelial phenotype is crucial for the proper kidney morphogenesis and functionality. During development the transformation of the blastema cells into epithelial cells is characterised by a gradual change in adhesive properties. In truth, mesenchymal-to epithelial transformation and further kidney maturation are characterised by a sequence of temporally distinct expression of cadherins and adherens junction-associated molecules, followed by formation of tight junctions and finally the establishment of a mature functional tissue (Figure 3) [38, 100, 193, 225, 281].

Initially, cadherin-11 is responsible for the loose adhesion seen in the blastema. Upon the first tighter cell-cell contact, it is substituted by other cadherins, which start to be expressed in different parts of the growing kidney, forming contacts called adherens junctions. The newly established cell-cell contacts are further stabilised by linkage of cadherins to the cytoskeleton through interaction with α-, β- and γ-catenins [38, 42, 61, 100, 190, 191, 193, 225].

The establishment of adherens junctions in early epithelialisation steps triggers the formation of tight junctions, and E-cadherin in particular is involved in this process. At first, cadherin complexes interact with ZO-1. Upon expression of tight junction proteins such as occludin and claudins, the interaction between ZO-1 and the adherens junction molecules subsides, and the new independent junctional complex rises. ZO-1, -2 and-3 together with other cytoplasmic proteins are responsible for linking the tight junctions to the cytoskeleton, stabilising the structure [57, 66, 74, 197, 254, 281, 286].

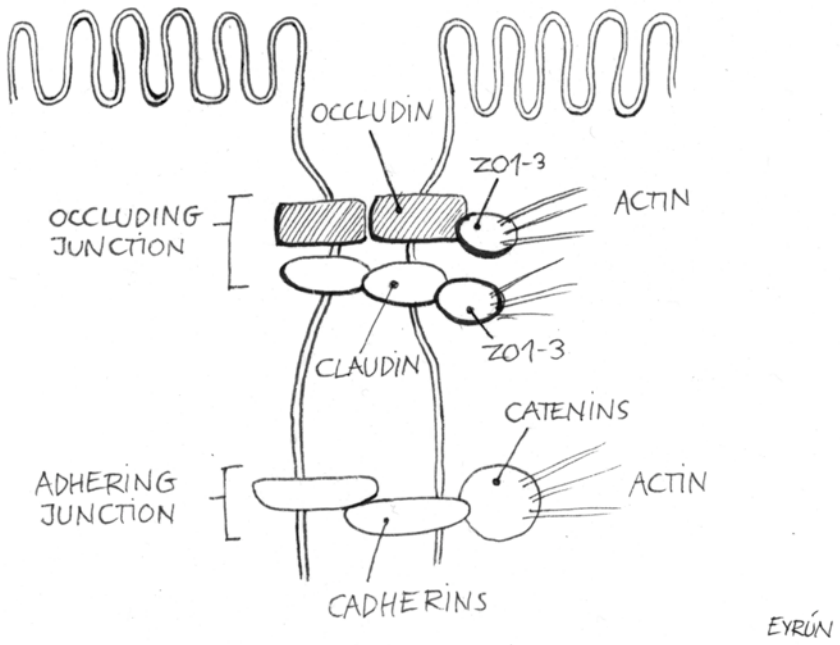

Figure 3: Schematic illustration representing adhesive junctions in epithelial cells.

In the mature kidney, the concentration and the constitution of the junctions along the nephron vary significantly and mirror the different properties inherent to each portion. Both adherens junctions and tight junctions contribute to proper kidney function [4, 5, 13, 83, 130, 193, 202, 206]. In mature glomeruli, classical junctions can be found in the endothelium, in the parietal epithelium and in the mesangial cells [65, 138, 192, 220, 285]. The podocytes, however, are devoid of classical junctions. They are linked together by the slit diaphragm, which has been considered as a modified adherens/tight junction, since it presents characteristics from both (Figure 2) [204, 226].

Along with the development of cell-cell contacts, cell-ECM contacts also contribute to kidney morphogenesis. Cell adhesion to ECM is the main factor responsible for organising the apicobasal axis of polarity, which will define the orientation of the apical and basal domains of the membrane, establishing correctly the biological compartments separated by the epithelium. The mesenchymal ECM is composed of proteins such as type I collagen and fibronectin, which will gradually be substituted by components of the mature GBM, such as type IV collagen and laminins [61, 100, 270, 286]. In the same fashion as adherens and tight

junction proteins, ECM protein expression patterns vary spatially and temporally, reflecting the differentiation pattern acquired by the cells during morphogenesis [87, 116, 148, 162, 245].

6. Protein folding and cellular quality control

The functional status of every given protein is asserted by its ability to perform the physiological role it is designated. Such tasks demand not only a correct primary amino acid sequence but also a correct and unique tri-dimensional structure. In cells, folding of one third of all newly synthesised proteins is carried out in the ER in a controlled process assisted by a variety of auxiliary proteins. Correct folding will drive the new protein into the most stable yet physiologically active state under physiological conditions and permit the mature protein to be transported to the proper location in the cell. Incorrectly folded proteins may represent a liability for the cellular function and must be avoided at any cost. ER environment is highly stressful and it greatly favours protein misfolding and aggregation, making necessary for the cell to keep a tight quality control system. Quality control mechanisms are conserved among species and it is their role to detect defective proteins and decide their fate. Faulty proteins can either be fixed or targeted for destruction [50, 51, 84, 85, 179].

6.1. ***Molecular chaperones***

Molecular chaperones are highly conserved classes of proteins, which assist protein folding and participate actively in quality control [271]. They are found in all cell compartments and take part in the majority of cellular processes, including trafficking, signal transduction and protein degradation. Molecular chaperones are sorted in different classes. Proteins from the same class share structural similarities and sequence homology, whilst members of different classes hardly share any similarities. Despite the molecular differences, however, molecular chaperones have common functional features, and many of them are rather promiscuous when clients are concerned [179].

Molecular chaperones act through a series of protein-protein interactions, by which they inhibit unproductive interactions and suppress protein aggregation. Among the mechanisms involved are recognition of exposed hydrophobic surfaces, conformational

changes in the client protein and recognition of sugar moieties on the surface of the client. If a client protein happens to be aberrant by any reason, the molecular chaperones signal to the cell quality control that this protein is not useful and should be targeted for degradation. Two main systems are responsible for degradation of aberrant proteins: ubiquitin-proteasome pathway and lysosomes. Cell quality control also plays an important role in the normal protein turnover in the cell. Proteins have a limited life span and must be replaced whenever necessary. It is responsibility of the quality control machinery to identify proteins that shall be replaced for new ones [29, 84, 85, 179, 271].

6.2. ***Protein misfolding and diseases***

When a protein fails to fold correctly, it gives rise to malfunction, which may lead to diseases. Under normal physiological conditions, the quality control mechanisms take care of the proper cleaning of the cell. However, there are times when pathological behaviour prevails and the misfolding-associated diseases become evident.

The manifestations of aberrant protein behaviour in cells are several. Loss-of-function-associated diseases are characterised by insufficient protein function, due to reduced protein production or misfolding. Two classical examples of loss-of-function pathologies are phenylketonuria, an inborn metabolic disease, and cystic fibrosis, an autosomal recessive disorder. Gain-of-function-associated diseases are characterised by accumulation/aggregation of misfolded proteins, which will interact and inhibit the native protein, in a dominant-negative fashion. Among the most studied gain-of-function pathologies are neurodegenerative disorders such as Parkinson, Alzheimer and prion-associated spongiform encephalopathies [36, 84, 85, 287].

6.3. ***Therapeutic approaches to misfolding diseases***

Eventually, the cell quality control discards proteins that could be salvaged. That occurs because even minor changes in the protein structure, due i.e. to single amino acid mutations, can lead to entrapment in the ER and targeting for degradation. Recent studies, however, show that two different classes of small molecules called chemical and pharmacological

chaperones respectively are able to reverse the intracellular retention and redirect the refolded mutants to their proper location in the cell [2, 164]

Chemical chaperones are non-specific compounds that improve the folding and trafficking without targeting any specific mutant. The most well-know chemical chaperones include dimethylsulfoxide (DMSO), glycerol, 4-PBA and trimethylamine oxine (TMAO). Pharmacological chaperones are specific compounds that work as competitive inhibitors or substrate analogues of a given protein. As chemical chaperones, these compounds aid in rescuing mutants from the cell quality control and restore proper protein function [2, 89, 164, 165].

Table 1. Mutations rescued by chemical and pharmacologocal chaperones

Disease	**Protein**	**Chaperone**
Cystic fibrosis	CFTR	Glycerol, DMSO, TMAO
Hereditary emphysema	α1-Anti-trypsin	Glycerol
Neprhogenic diabetes insipidus	Aquaporin-2	Glycerol, DMSO, TMAO
	Vasopressin V2 receptor	SR121463A, VPA985
Fabry	α-Galactosidase A	1-Deoxy-gaaxtonojirimycin
Cancer	p53	CP31398, CP257042
Gaucher's disease	β-glucocerebrosidase	β-Glu inhibitor

Adapted from reference 165

Aims

Many of the mutations identified in *NPHS1* gene after the discovery of nephrin are responsible for CNF phenotype. However, the mechanism behind this phenotype was yet to be studied. Therefore, the general aim of this study was to provide new insights into the mechanisms behind CNF.

In my opinion, there is no better way to achieve our goal than to focus our efforts on understanding nephrin, the small giant responsible for the disease in question.

Therefore the specific contribution of this study to such Herculean quest includes:

- Investigate the pathomechanisms of CNF caused by missense mutations in *NPHS1* gene.
- Explore the possibility of using chemical chaperones as a new therapy for CNF caused by missense mutations in *NHPS1*.
- Investigate the impact of nephrin gene ablation in podocyte physiology.

" It is a capital mistake to theorise before one has data. Insensibly, one begins to twist facts to suit theories, instead of theories to suit facts" Sir Arthur Connan Doyle

Methodology

Throughout this study several different methodologies have been employed. Here, I only describe briefly the methods utilised in my experiments. Detailed description on each of them can be found in the "Materials & Methods" sections of the corresponding articles.

- **DNA constructs and site-directed mutagenesis (I)**

cDNA encoding full-length or mutated nephrin was cloned into a mammalian expression vector. Site-directed mutagenesis was performed using Quick Change™ Site-Direct Mutagenesis kit using the full-length nephrin cDNA as template. The mutagenesis accuracy was verified by sequencing of the whole insert.

- **Cell culture and transfection (I, II)**

HEK-293 cells were cultured in DMEM supplemented with 10% foetal calf serum, 100U/mL Penicillin and 100mg/mL streptomycin, at 37°C, 5% CO2. Transfection was carried in 6-well plates, using Fugene™6 transfection Reagent according to the manufacturer's instructions. Clone selection was made using 1 mg/mL G-418 in the cell culture medium.

- **Immunocytochemistry (I, II)**

Cells were cultured on glass coverslips overnight. Fixation was performed using 2% buffered formaldehyde containing 0,1% glutaraldehyde at 37°C for 30 minutes. Staining with appropriate antibodies was performed at 37°C for 1 hour, followed by fluorophore-labelled secondary antibody. Results were analysed using a fluorescence microscopy.

- **Subcellular fractionation (I)**

Post-nuclear cell homogenate was layered on the top of a 0.6-2.0 M linear sucrose gradient and centrifuged at 100.000g, 4°C in a Beckman SW50.1 rotor for 18 hours. Fractions were collected using a 1mL syringe and proteins were analysed by SDS-PAGE and Western blotting.

- **Western Blot (I, II)**

Proteins were separated by SDS-PAGE and transferred to a PVDF membrane. Membranes were blocked with 5% non-fat milk for 2 hours at room temperature, followed by incubation with a primary antibody for 1 hour also at room temperature. After extensive washing, membranes were incubated with horseradish peroxidase-labelled secondary antibodies for 1 hour at room temperature, washed and immunoreactivity was detected by chemilluminescence.

- **Immunoelectron microscopy (I, III)**

Kidneys harvested from E18.5 littermate embryos were fixed with 3 % paraformaldehyde in 0.1M phosphate buffer, pH 7.4, dehydrated and embedded in Lowicryl K11M resin. Sections were blocked with 2% bovine serum albumin and 2 % gelatin in 0.1 M phosphate buffer and incubated with the primary antibody for 4 hours in a humidified chamber at room temperature, followed by thorough washes. Bound antibodies were detected with protein A coated with 10nm gold. Sections were rinsed, fixed in 2% glutaraldehyde and contrasted with 4 % uranyl acetate followed by lead citrate and examined in a Tecnai 10 microscope, at 80 kV and digital images were taken by a Megaview III camera.

- **Cell surface biotinylation (II)**

Cells stably expressing full-length or mutant nephrin were cultured on 60mm plates and treated with 4-PBA overnight. Cell surface proteins were biotinylated by incubating the cells, at 4°C, with 2mM sulfo-NHS-SS-biotin for 2 hours. Biotinylated proteins were captured by

incubating the cell lysates with streptavidin-agarose beads, overnight, at 4°C. Proteins were analysed by SDS-PAGE and Western blotting.

- **Protein co-localisation (II)**

Cells stably expressing full-length or mutant nephrin were treated overnight at 37°C with 10mM 4-PBA, followed by transfection with cDNA encoding the fusion protein ex.NEPH-1-IgG-Fc. After transfection, cells were cultured on glass coverslips and stained for nephrin and NEPH-1. Cells were analysed under fluorescence microscopy.

- **Antibody cross-linking (II)**

Near confluent cells were washed once with ice-cold DMEM containing 25 mM HEPES, followed by incubation with anti-nephrin antibody 50A9 for 30 minutes at 4°C. Medium was removed, cells were washed and incubated with goat anti-mouse IgG for 1 minute at 37°C. After gentle washing, cells were lysed and samples were analysed by SDS-PAGE and Western blot.

- **Immunohistochemistry (III)**

Eight μm Sections from both $NPHS1^{+/+}$ and $NPHS1^{-/-}$ kidneys were probed with primary antibody overnight at 4°C, followed by incubation with fluorophore-labelled secondary antibody for 2 hours, at room temperature. After extensive washing, the sections were mounted in phase-contrast mounting medium and analysed under fluorescence microscope.

- **TUNEL staining (III)**

Eight μm Sections from both $NPHS1^{-/-}$ and $NPHS1^{+/+}$ were incubated at 37°C for 1 hour with TUNEL mixture containing TdT terminal transferase and Alexa-Fluor 488-labelled dUTP. After extensive washing, the samples were mounted in anti-phase mounting medium and analysed under fluorescence microscope.

- **BrdU Staining (III)**

Pregnant female mice were treated with one single intraperitoneal injection of BrdU [50mg/kg] After 2 hours, mice were sacrificed by cervical dislocation, kidneys were dissected from the embryos and fixed with 4 % paraformaldehyde and embedded in OCT. 8µm sections of both NPHS1$^{-/-}$ and NPHS1$^{+/+}$ kidneys were stained with anti-BrdU antibody overnight at 4°C, followed by incubation with fluorophore-labelled secondary antibody. BrdU incorporation was analyzed using fluorescence microscopy.

- **PAS Staining (III)**

Sections of 8µm from both *NPHS1*$^{-/-}$ and *NPHS1*$^{+/+}$ kidneys were incubated with 0.5% periodic acid for 5 minutes at room temperature. After washing, slides were incubated with Schiff's reagent for 15 minutes, followed by 2 washes of 1 minute each with 0.55% potassium methabisulfite. Sections were washed in running water for 10 minutes, until the colour developed. Samples were counterstained with haematoxylin for 30 seconds at room temperature, dehydrated through 95% ethanol, 100% ethanol and xylene, and mounted. Analysis was performed under a light microscope.

- **Gene expression analysis (III)**

Glomeruli were isolated from E18.5 stage NPHS1$^{-/-}$ and NPHS1$^{+/+}$ embryos using a Dynabead perfusion method [251]. Total RNA was extracted, labelled and hybridized on Affymetrix Mouse Genome 430 2.0 chips. The array data were analyzed using the gcrma package [282] and affy package in the Bioconductor project [http://www.bioconductor.org].

"The best and safest method of philosophizing seems to be, first to inquire diligently into the properties of things and to establish those properties by experiences, and then proceed, more slowly, to hypothesis for the explanation of them. For hypothesis should be employed only in explaining the properties of things, but not assumed in determining them, unless so far as they may furnish experiments" Sir Isaac Newton

Results & Discussion

In Finnish population, due to founder effects, two major mutations in the *NPHS1* gene account for 94% of the CNF phenotypes. In non-Finnish populations, in contrast, the vast majority of CNF cases are caused by a broader number of mutations. Among those, missense mutations represent approximately half of the disease-causing genotypes. They result in single amino acid substitutions along nephrin and most of them lead, curiously, to a similar phenotype as seen in Fin_{major} and Fin_{minor} patients. It is well described that amino acid substitutions in polypeptide chains may cause disturbances in the mature proteins, leading to defective folding, impaired trafficking and, ultimately, loss-of function. We therefore speculated that this would be the mechanism responsible for the CNF phenotype observed in patients. Through site-directed mutagenesis we generated nephrin mutants representing 21 amino acid substitutions in nephrin. The constructs were transfected into HEK-293 cells and stable colonies were selected for further analysis. We observed that, despite being translated by the cells, nephrin fails to reach the plasma membrane in 75% of the mutants (Table 2 and Article I). This was demonstrated by immunoflourescence staining of the stable transfectants with or without prior Triton X-100 permeabilisation. Thus, our results indicated that the mutant nephrin protein might be disposed of by the cellular quality control.

In eukaryotes, transmembrane proteins and proteins destined for secretion are translated and modified in the rough ER. Calreticulin and calnexin are two ER-resident chaperones, which aid the folding and sorting of almost all N-glycosylated proteins, through recognition and binding to specific sugar moieties attached to the newly synthesised polypeptide [29, 62, 63].

Nephrin is a transmembrane protein, glycosylated at nine asparagine residues within its extracellular domain [127]. Therefore we speculated that our mutants might be retained in the ER because of mutant protein misfolding. Indeed, double immunofluorescence staining showed that nephrin mutants failing to reach the plasma membrane co-localise with calreticulin. Further studies showed that, on a linear sucrose gradient, wild-type nephrin migrated together with Na^+K^+ATPase, a plasma membrane protein, whilst the mutant

S366R, a representative of the ER-retained mutants, migrated together with calreticulin, thus confirming the results.

Table 2: Expression of nephrin missense mutants in HEK293 cells

Exon	Location	Mutation	Cell surface location	Other mutations found at the same patient	[b]Patient origin
2	Ig1	W64S	-	Truncation	Finland (19)
4	Ig2	I171N[a]	-	-	Turkey (2)
4	Ig2Ig3	I173N	-	S350P	France (14)
7	Ig3-4	G270C		Splicing mutation	England (22)
9	Ig4	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	N. America (23)
16	SP	S724C	+	R367C, R1140C	France (21)
17	SP	R743C	+	Truncation	Finland (20)
18	Ig7	R802W	-	del1172Thr	Netherlands (17)
18	Ig7	R802P	-	Splicing mutation	N. America (12)
18	Ig7	A806D[a]	-	-	Marocco (3)
18	Ig7-8	R831C	-	-	N. America (25)
27	Intracellular	R1140C	+	S724C, R367C	France (21)

[a]Homozygous mutations

[b]Numbers correspond to patient numbers in ref. 149

Aberrant folding often give rise to diseases, and the impairment of the ability to fold can originate from single amino acid substitutions in the polypeptide chain, as seen in cystic fibrosis [41] and QT syndrome [291]. Our results strongly suggest that the same is true for the disease-causing nephrin missense mutants. Notably, all the nephrin mutants retained in the ER contain a non-conservative mutation within the Ig domain. It is also noteworthy that disease-causing mutations often involve replacement of a cysteine residue or addition of a new one, which most likely have severe effects on the formation of correct disulfide bonds and

result in retention of the mutant protein inside the ER. In contrast, mutations in the spacer region do not result in impaired transport, indicating that the spacer region is more tolerant to mutations. Equally, conservative mutations such as L376V do not affect the protein trafficking, nor does the intracellular mutation R1140C. The mutants transported to the plasma membrane, however, are very likely functionally defective. It is also worth noting that some of the CNF patients presented more than one heterozygous mutation in different alleles (Table 2). In this context, it is relevant to mention that the mutation R367C found in an affected child was also found in the healthy mother [149], indicating that unknown mutations inherited from the father or a *de novo* mutation in the affected patient may be responsible for the final phenotype. Since the publication of our results, several other missense mutations had been found in *NPHS1* [70, 79, 135]. This information may be helpful in explaining why individuals heterozygous for mutations R831C and C623F are affected. In addition, Koziell and collaborators found co-existence of *NPHS1* and *NPHS2* mutations in both CNF and FSGS patients [135], emphasising the complexity of CNF phenotypes. In summary, our data suggest that misfolding and impaired trafficking of nephrin missense mutants may account for a large number of CNF phenotypes.

Thus far, the only curative treatment for CNF is kidney transplantation, which may incur many difficulties for the patients. For those whose mutations result in complete absence of nephrin, such as homozygous Fin_{major} mutation, no other alternative is available. However, alternative treatments might become possible for patients carrying some nephrin missense mutations. In fact, a nephrotic infant with Fin_{major}/R743C genotype responded to treatment with angiotensin-converting enzyme inhibitor and indomethacin [183], corroborating the idea that pharmacological approaches might represent a useful alternative for treating particular CNF cases.

In recent years, a series of chemical and pharmacological chaperones have been used to rescue mutated proteins from the ER quality control and redirect them to the plasma membrane (Table 1). Therefore we set out to study whether the ER-retained nephrin missense mutants could be redirected to the cell surface. Thereafter, if this were the case, whether we wanted to test whether the proteins are functionally active.

To this end, we treated the stable transfected cells expressing the nephrin missense mutants with 4-PBA, a chemical chaperone, and examined whether the mutants were able to bypass the ER quality control and move to the plasma membrane. After incubation with 4-

PBA, six out of sixteen ER-retained mutants could be found at the plasma membrane (Table 3 & Article II).

Table 2: Cellular location of nephrin missense mutants expresses in HEK-293 cells

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	-	+

4-PBA is an orally administrated short-chain fatty acid used regularly in treatment of urea cycle disorders [31]. 4-PBA has been shown, both *in vitro* and *in vivo*, to promote trafficking of mutant proteins [33, 43, 93, 175, 213, 215, 216, 240, 261, 283, 284], as well as to prevent protein aggregation [49], induce differentiation and maturation of malignant cells [6, 134, 247], influence lipid metabolism [121] and promote protein synthesis [3, 53, 54], all with great impact on different types of diseases. The most well studied effect of 4-PBA is in restoring the cystic fibrosis conductance channel to the plasma membrane [213, 290] and it has already been tested with some degree of success in human patients with cystic fibrosis [240, 289]. The mechanism of action of 4-PBA seems to be related to the reduction of ER stress and regulation of transcription of HSP70 class of molecular chaperones [43, 49, 180,

214, 217, 283]. 4-PBA was not able to rescue all the nephrin mutants, which may reflect the different degrees of damage caused by improper polypeptide folding.

As a test for the functional activity of the rescued nephrin mutants, we examined whether they were able to interact with NEPH-1, the only known protein to interact with nephrin through its extracellular domain [18, 76]. Furthermore, nephrin association with NEPH-1 has been shown to be essential for maintenance of the SD integrity, since disruption of this interaction leads to proteinuria [154]. A plasmid encoding the extracellular domain of NEPH-1 tagged with human IgG Fc-portion was transfected into wild-type HEK-293 cells, HEK-293 expressing wild-type nephrin and cells expressing the six rescued mutants and one non-rescued mutant (I171N). As expected, non-permeabilised wild-type HEK-293 cells and cells expressing the non-rescued mutant did not stain with flourophore-conjugated anti-human Ig antibody. The success of the transfection was verified by staining of the cells after Triton X-100 permeabilisation. In contrast, non-permeabilised cells expressing both wild-type nephrin and the rescued mutants showed a strong plasma membrane staining, indicating the presence of interaction between nephrin and NEPH-1. This interaction is a strong indication that nephrin mutants savaged from ER quality control might be able to function normally.

Nephrin is regarded as a signalling molecule, with tyrosine phosphorylation indicated as the key event for its function [21, 103]. Thus, we investigated the ability of the rescued mutants to become phosphorylated upon antibody clustering [143]. Cells expressing wild-type nephrin and the six rescued mutants were incubated with an antibody against extracellular domain of nephrin, followed by a brief incubation with secondary antibody. Phosphorylation status was analysed by Western blotting. All six mutants rescued by 4-PBA presented robust tyrosine phosphorylation after antibody clustering, providing further evidence that the rescued nephrin mutants may, thereby, be as active as wild type nephrin.

Based on the results from other disease models and the results obtained in this study, 4-PBA could represent a future therapeutic approach for CNF. A natural future step would be to investigate the effect of 4-PBA in transgenic animal models or even in clinical trials.

The importance of nephrin as a constituent of the podocyte slit diaphragm and glomerular filtration barrier has already been discussed above. However, its importance for podocyte development has not yet been addressed. It is tempting to speculate that nephrin, through protein-protein interactions or signalling events involving tyrosine phosphorylation, would play an active role in development and maturation of the glomerulus. The damages observed in the glomerulus of both CNF patients [10, 11, 109, 199] and nephrin-null mice

[194] would speak in favour of such an assumption. Therefore, we decided to investigate the involvement of nephrin in glomerular development, and in the assembly and organisation of the podocyte foot processes and the slit diaphragm, using nephrin null-mice (*NPHS1*$^{-/-}$) as a model (Article III).

Histological analysis of E18.5 *NPHS1*$^{-/-}$ kidneys showed the characteristic defects observed in CNF, such as dilated Bowman's space and tubules dilated with massive protein accumulation. Transmission electron microscopy demonstrated the presence of abnormally broad foot processes and complete collapse of the slit diaphragm. The foot processes seemed to be attached to each other through a junction-like structure. In contrast, the *NPHS1*$^{+/+}$ kidneys were normal, as expected (Article III). Since nephrin is such an essential component of the slit diaphragm, one would expect its absence to cause major alterations in glomerular biology. To explore this hypothesis, we decided to study cell proliferation and rate of apoptosis in nephrin-null mice and their wild-type littermates.

For the analysis of cell proliferation rate we took advantage of BrdU incorporation. Our results showed no differences in proliferation rates between *NPHS1*$^{-/-}$ and *NPHS1*$^{+/+}$ kidneys at E18.5. Further analyses with antibodies against cell-cycle inhibitors p27 and p57 showed no difference in expression of these markers when comparing *NPHS1*$^{-/-}$ and *NPHS1*$^{+/+}$ glomeruli. In early stages of kidney development, podocyte precursors are cells with high proliferation rate. When entering the capillary loop stage, the podocytes stop dividing, acquire their characteristic architecture and start to express mature podocyte markers. Nephrin expression is first detected in mouse at the early capillary loop stage and in humans at the late S-shape body stage, coinciding with ceasing of cell proliferation [185].

In order to study the apoptosis rate in the kidneys, we performed TUNEL staining, and counted the number of WT-1 positive nuclei, which represents the number of podocytes. These studies showed that the absence of nephrin does not increase apoptosis rate in *NPHS1*$^{-/-}$ glomeruli when compared with *NPHS1*$^{+/+}$. Podocyte loss has been observed in several acquired glomerulopathies [185, 232]. However, that does not seem to be the case in the mouse model for CNF. In agreement, Kuusniemi and collaborators showed similar results for human glomeruli [140]. Instead, we found increased apoptosis events in the inner part of the kidney, corresponding to the medullar tubules. In fact, reports in the literature appoint albumin as being responsible for apoptosis in renal proximal tubular cells, mainly due to ER stress [176]. Consequently, it is possible that the increase in apoptosis observed in the medullar tubules of *NPHS1*$^{-/-}$ kidneys is due to highly increased albumin excretion.

Based on our results we suggest that the absence of nephrin does not interfere with podocyte proliferation or apoptosis in E18.5 glomeruli. However, our model does not allow us to speculate on nephrin participation in such events during adulthood. Thus, further experiments are necessary to elucidate the role of nephrin in podocyte survival in steady-state glomeruli.

Nephrin is part of the slit diaphragm multiprotein complex and interacts with several other proteins, stabilising the filtration barrier structure. Moreover, it is known that malfunction of many other slit diaphragm proteins leads to renal failure [26, 44, 52, 236, 280]. Therefore, we decided to investigate the impact of the lack of nephrin on glomerulus protein expression and localisation, as well as on glomerular gene expression.

Immunofluorescence staining showed no difference in the expression of the other podocyte-specific proteins between *NPHS1*$^{-/-}$ and *NPHS1*$^{+/+}$ glomeruli. Similarly, no changes were observed in the staining pattern of the GBM components collagen type IV chains, laminin chains, perlecan, nidogen and fibronectin between *NPHS1*$^{-/-}$ and *NPHS1*$^{+/+}$ glomeruli. These findings indicate that the podocyte foot processes keep some degree of organisation, even in the absence of nephrin.

Our microarray analyses using RNA extracted from E18.5 *NPHS1*$^{-/-}$and *NPHS1*$^{+/+}$ glomeruli showed that, surprisingly, lack of nephrin has very little impact on overall glomerular gene expression profile (Table 4 & Article III). Only 7 genes were downregulated more than 2-fold and 11 genes were upregulated more than 2-fold in *NPHS1*$^{-/-}$.

Despite evidence showing that tyrosine phosphorylation of the nephrin intracellular domain is involved in several essential signalling events in podocytes [104, 105, 113, 143, 266, 267], our results clearly indicate that such signalling events are second to the severe structural changes imposed by the destabilisation of the protein-protein interactions in the foot processes/slit diaphragm area. We and others [219] have shown that, when nephrin is absent from the slit diaphragm, the adjacent podocytes are linked together through a junction-like structure. Interestingly, one of the proteins upregulated in *NPHS1*$^{-/-}$ was claudin 3, a tight junction protein (21.6, p=0.0267). It is known that the mature slit diaphragm originates from classical adhesion junctions, adherens and tight junctions, and in its mature state it conserves characteristics of both [204, 226] being, therefore, considered a modified adherens/tight junction.

Table 4. List of genes up- or down-regulated at least 2-fold in Nphs1$^{-/-}$ glomeruli when compared with Nphs1$^{+/+}$mice.

	Probe Set ID	Gene Title	Gene Symbol	KO/WT	P-value
	down-regulated genes				
1	1422142_at	nephrosis 1 homolog, nephrin (human)	Nphs1	-37.30	0.00215
2	1452135_at	glutathione peroxidase 6	Gpx6	-5.70	0.03097
3	1449883_at	FXYD domain-containing ion transport regulator 2	Fxyd2	-4.30	0.02962
4	1450344_a_at	prostaglandin E receptor 3 (subtype EP3)	Ptger3	-3.60	0.03946
5	1421286_a_at	ATPase, H+/K+ transporting, alpha polypeptide	Atp4a	-3.20	0.03974
6	1425138_at	guanylate cyclase activator 1B	Guca1b	-2.50	0.04590
7	1423407_a_at	fibulin 2	Fbln2	-2.50	0.01354
	up-regulated genes				
1	1415935_at	SPARC related modular calcium binding 2	Smoc2	3.80	0.01271
2	1445626_at	Lectin, galactose binding, soluble 3 (Lgals3), mRNA	Lgals3	3.10	0.03965
3	1434651_a_at	claudin 3	Cldn3	3.30	0.02670
4	1449195_s_at	chemokine (C-X-C motif) ligand 16	Cxcl16	2.90	0.02731
6	1428664_at	vasoactive intestinal polypeptide	Vip	2.50	0.00531
7	1417156_at	keratin complex 1, acidic, gene 19	Krt1-19	2.40	0.00892
8	1418203_at	phorbol-12-myristate-13-acetate-induced protein 1	Pmaip1	2.30	0.01715
10	1437213_at	nudix (nucleoside diphosphate linked moiety X)-type motif 21	Nudt21	2.20	0.04729
11	1451021_a_at	Kruppel-like factor 5	Klf5	2.20	0.01668

Probe ID is listed according to Affymetrix annotation.
The analysis was carried on using gcrma normalisation. Nphs1$^{-/-}$ n=5 and Nphs1$^{+/+}$n=4, $p \leq 0.05$.

Claudins are main components of tight junctions. The claudin family comprises 24 members, each of them being expressed in a tissue-specific manner, such that every tissue has its own set of claudins [72, 260, 263, 264]. In the nephron nine different claudins have been

identified [4, 130, 153, 206, 264, 265] and mutations in some of them are responsible for kidney impairment [98]. However, only claudins 1 and 2 have been identified in the glomerulus, in the Bowman's capsule. Since microarray analysis revealed strong upregulation of claudin 3, we investigated the expression of this protein in *NPHS1*$^{-/-}$ kidneys. Immunofluorescence staining showed a significant difference in claudin 3 expression between *NPHS1*$^{-/-}$ and *NPHS1*$^{+/+}$ kidneys. So far, the presence of claudin 3 has been reported only in tubules, more specifically, in thin ascending limb, thick ascending limb, distal tubule and collecting duct [130, 153]. We found expression of claudin 3 in the Bowman's capsule of *NPHS1*$^{-/-}$ glomeruli and, to a lesser extent, in *NPHS1*$^{+/+}$. More importantly, the expression of claudin 3 in *NPHS1*$^{-/-}$ glomeruli extended to the podocytes. Tight junctions are responsible for sealing cell-cell contacts, and play an important role in orchestrating cell polarity, proliferation and differentiation [24]. Our findings suggest that nephrin participates in those processes through regulation of tight junction proteins such as claudin 3. In agreement with our hypothesis, Nagai and collaborators had shown that the coxsackievirus and adenovirus receptor CAR is expressed in glomerulus and its expression is increased in PAN-induced nephrosis [170]. Taken together, these data strongly support a model where in the absence of nephrin, downregulation of tight junction proteins is delayed as means of maintaining proper kidney function. Moreover, the presence of tight junction proteins at the presumed slit diaphragm provides the first insights into the nature of the junction-like structures found between the effaced foot processes. The precise mechanism involved in regulation of claudin 3 within the glomerulus and the exact nature of these contacts is still to be elucidated.

Conclusions

The discovery of nephrin, the product of the *NPHS1* gene, represented a hallmark for understanding not only CNF but also the functioning of the kidney filter. Since then large number of new molecules involved in glomerular function have been identified and the mechanism behind several other kidney diseases have been clarified.

Fin_{major} and Fin_{minor}, the two main mutations found in Finnish CNF patients, result in complete absence of nephrin. However, the mechanisms behind the CNF phenotype in patients carrying missense mutations in *NPHS1* have been poorly understood. In this study we provide evidence that in many of these cases, CNF is, at least partially, due to retention of the mutant nephrin inside the ER, which leads to absence of the protein in the slit diaphragm.

Missense mutations have been shown to be the cause of several other well-known diseases, such as cystic fibrosis and phenylketonuria. For some of these diseases, the use of chemical and pharmacological chaperones represents an alternative treatment. Here we show that this can also be the case for CNF. 4-PBA, a chemical chaperone already used in patients, was able to rescue some of the nephrin mutants from the ER, resulting in cell surface expression. Two different functional assays indicated that the rescued mutants behave indistinguishably from the wild-type nephrin. The results thus propose that 4-PBA could be a candidate for the treatment of CNF patients carrying missense mutations.

Nephrin has been described as a scaffolding component of the filtration barrier as well as a signalling protein involved in podocyte biology. This study shows that nephrin is essential for podocyte maturation and slit diaphragm formation. However, our findings indicate that nephrin has only a modest role in podocyte and glomerulus development.

The data presented here are important contributions for the understanding of the glomerulus and filtration barrier function both in healthy and disease conditions. Nevertheless, they represent only a part of what is yet to be done and indeed further studies are needed. My hope is that, in the not-too-far future, the data acquired here can be used as tools in the search for deeper understanding and more detailed information on kidney filtration process. Perhaps it would be too bold to wish even for CNF cure…

"On ne connaît que les choses que l'on apprivoise, dit le renard. Les hommes n'ont plus le temp de rien connaître. Ils achètent des choses toutes faites chez les marchands. Mais comme il n'exite point des marchands d'amis, les hommes n'ont plus d'amis. Si'tu veux un ami, apprivoise-moi!" Antoine de Saint-Exupéry

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To me this whole experience has been like a roller coaster ride, with all the ups, downs, loops and turns. The same excitement, alternating moments of apprehension, euphoria, happiness and sadness.

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Everything in life has a beginning and mine was in a not-so-small city in Brazil. There I have my family: My parents - Gladston and Maria Imaculada, to whom I owe the greatest gifts I was ever given: my education, my values, my persistence, my perseverance, my "stubbornness"... They are and they will always be my model. This work is for them as, in face of all the sacrifices they made throughout their lives, they deserve more than me. And my beloved sisters Julliana and Ana Paula, their partners, and our little Benjamin, so small and already fill our lives with joy; they mean the world to me.

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I am not a native English speaker, so I am bound to commit absolute heinous crimes against the English language. Mark prevented me from burrying myself in such felonies. He, however, did not read this section. Therefore, he may be excused from any mistakes found after page 51.

Sometimes I believe we researchers are creative thinkers. And to be able to make our magic happen we need visionary men and women to support our ideas. People with a foward mind, people who

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So the journey continues...

Stefania Cotta Dore'

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