

THESIS FOR DOCTOR OF PHILOSOPHY IN MEDICAL SCIENCES

Characterization of Novel Genes of Importance for Renal Glomerular Function and Disease

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KAROLINSKA INSTITUTET

Stockholm, Sweden 2013

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Cover:

A glomerulus depicted with the letters of the genetic code. The X symbolises a mutation.

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ABSTRACT

Glomerular kidney diseases are a major health care burden. The glomerular filtration barrier consists of three layers: the slit diaphragm that bridges the interlocking foot processes of the podocytes, the glomerular basement membrane and fenestrated endothelial cells. The filtration barrier is permselective to plasma macromolecules based on size, shape, and charge. The molecular makeup of the filtration barrier determines its permselectivity. Knowledge about the molecular mechanisms of the glomerular filtration barrier has been gained with the study of genes mutated in humans and animal models of glomerular kidney disease.

In the thesis work, we performed a proteome analysis of healthy glomeruli in mice using two-dimensional gel electrophoresis coupled to mass spectrometry. A total of 232 unique proteins were identified from 414 gel spots. This study provided a snapshot of the glomerular proteome that can serve as reference for future glomerular protein biomarker studies.

We describe the expression and physiological function of the gene *Glcc1* in zebrafish. Histological analysis of *Glcc1* showed expression in podocytes and mesangial cells. *In vivo* and *in vitro* studies demonstrated that *Glcc1* expression is induced by glucocorticoids. Depletion of *Glcc1* by morpholino knockdown resulted in the development of pericardial edema and defects in glomerular filtration. Our results suggest a role for *Glcc1* in glomerular injury and proteinuria.

Knockdown experiments of the paralogs *Plekhh1* and *Plekhh2* in zebrafish resulted in gross morphological changes in the glomerulus, including thickening of the glomerular basement membrane and disorganization of the podocyte foot processes associated with a defective filtration barrier. These results suggest a role for *Plekhh1* and *Plekhh2* in regulating podocyte foot process morphology in zebrafish. We further characterized *Plekhh1* and *Plekhh2* in knockout mouse models. Single knockouts of *Plekhh1* and *Plekhh2* do not develop any apparent phenotype. *Plekhh1* and *Plekhh2* deficient mice were intercrossed to produce mice lacking both genes. This yielded fewer than expected number of double knockout offspring, suggesting functional redundancy. Ultrastructural analysis of surviving double knockout mice did not reveal changes in glomerular morphology suggesting that *Plekhh1* and *Plekhh2* are largely redundant for kidney function in mice.

These results give insight into glomerular biology and pathomechanisms of kidney disease that might provide a basis for translational research in the future.

Keywords: Kidney glomerulus, podocytes, slit diaphragm, proteinuria, zebrafish, knockout mouse.

to my dear Grandparents.

Utan þessa dags bak
við árin og fjall- lvegina streyma
fram lindir mínar. Ef ég legg aftur au-
gun ef ég hlusta, ef ég bið heyri ég þær
koma eftir leyningunum grænu langt
innan úr tímanum hingað, hingað úr
fjarska. Þær hljóma við eyru
mér þær renna gegnum
lófa mína ef ég legg
aftur augun



— Hannes Pétursson, Farvegir

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*In the long history of humankind
(and animal kind, too) those who
learned to collaborate and improvise
most effectively have prevailed*

— Charles Darwin

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ABBREVIATIONS

2-DE	Two-dimensional gel electrophoresis
μm	micrometer
ATG	translation initiation site
DBP	Vitamin-D binding protein
DNA	Deoxyribonucleic acid
cDNA	complementary DNA
dpf	days post fertilization
EGFP	Enhanced green fluorescent protein
FSGS	Focal segmental glomerulosclerosis
GBM	Glomerular basement membrane
Glci1	Glucocorticoid induced transcript 1
HSPG	Heparan sulfate proteoglycan
hpf	hours post fertilization
H&E	Hematoxylin and Eosin
iEM	Immunoelectron microscopy
KOMP	Knockout Mouse project
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
MALDI-TOF	Matrix-assisted laser desorption/ionization
NC1	Non-collagenous domain 1
nm	nanometer
PAS	Periodic acid-Schiff
Plekhh1	Pleckstrin homology domain containing, member 1
Plekhh2	Pleckstrin homology domain containing, member 2
QTL	Quantitative trait locus
RNA	Ribonucleic acid
mRNA	messenger RNA
RT-PCR	Real time polymerase chain reaction
SD	Slit diaphragm
TEM	Transmission electron microscopy
qPCR	quantitative polymerase chain reaction

PUBLICATIONS

This thesis consists of an extended summary and the following appended papers:

- Paper I** Sam Tryggvason, Masatoshi Nukui, **Asmundur Oddsson**, Karl Tryggvason, and Hans Jörnvall. Glomerulus proteome analysis with two-dimensional gel electrophoresis and mass spectrometry. *Cell Mol Life Sci* **64**:24 (Dec. 2007), 3317–3335
- Paper II** Yukino Nishibori, Kan Katayama, Matalena Parikka, **Asmundur Oddsson**, Masatoshi Nukui, Kjell Hulténby, Annika Wernerson, Bing He, Lwaki Ebarasi, Elisabeth Raschperger, Jenny Norlin, Mathias Uhlén, Jaakko Patrakka, Christer Betsholtz, and Karl Tryggvason. Glcc1 deficiency leads to proteinuria. *Journal of the American Society of Nephrology : JASN* **22**:11 (Nov. 2011), 2037–2046
- Paper III** **Asmundur Oddsson**, Matalena Parikka, Lwaki Ebarasi, Patricia Rodriguez, Yukino Nishibori, Kjell Hulténby, Annika Wernerson, Christer Betsholtz, Jaakko Patrakka, and Karl Tryggvason. “Zebrafish Plekhh1 and Plekhh2 are involved in organization of foot processes and normal kidney function”. manuscript. May 2013
- Paper IV** **Asmundur Oddsson**, Mark Lal, Kjell Hulténby, Annika Wernerson Annika, Jaakko Patrakka, and Karl Tryggvason. “Analysis of Plekhh1 and Plekhh2 knockout mice reveal redundancy of the paralogs in kidney function”. manuscript. May 2013
- Other publications not included in the thesis
- Paper A** Jaakko Patrakka, Zhijie Xiao, Masatoshi Nukui, Minoru Takemoto, Liqun He, **Asmundur Oddsson**, Ljubica Perisic, Anne Kaukinen, Cristina Al-Khalili Szigarto, Mathias Uhlén, Hannu Jalanko, Christer Betsholtz, and Karl Tryggvason. Expression and subcellular distribution of novel glomerulus-associated proteins dendrin, ehd3, sh2d4a, plekhh2, and 2310066E14Rik. *Journal of the American Society of Nephrology : JASN* **18**:3 (Mar. 2007), 689–697
- Paper B** Lwaki Ebarasi, **Asmundur Oddsson**, Kjell Hulténby, Christer Betsholtz, and Karl Tryggvason. Zebrafish: a model system for the study of vertebrate renal development, function, and pathophysiology. *Curr Opin Nephrol Hypertens* **20**:4 (July 2011), 416–24

Paper C

Unnur Styrkarsdottir, Gudmar Thorleifsson, Patrick Sulem, Daniel F. Gudbjartsson, Asgeir Sigurdsson, Aslaug Jonasdottir, Adalbjorg Jonasdottir, **Asmundur Oddsson**, Agnar Helgason, Olafur T. Magnusson, Bragi Walters, Michael L. Frigge, Kristin Bergsteinsdottir, Margret H. Ogmundsdottir, Jacqueline R. Center, Tuan V. Nguyen, John A. Eisman, Claus Christiansen, Erikur Steingrimsson, Jon G. Jonasson, Laufey Tryggvadottir, Gudmundur I. Eyjolfsson, Asgeir Theodors, Thorvaldur Jonsson, Thorvaldur Ingvarsson, Isleifur Olafsson, Thorunn Rafnar, Augustine Kong, Gunnar Sigurdsson, Gisli Masson, Unnur Thorsteinsdottir, Kari Stefansson. Nonsense mutation in the LGR4 gene associates with several human diseases and other traits. *Nature* (in Press)

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Part I

Extended Summary

1

Introduction

1.1 Glomerular Filtration Barrier

The kidney glomerulus comprises the Bowman's capsule and a tuft of intertwined capillaries, which constitute the actual kidney blood filter. The main function of the kidney is to sieve small molecular weight plasma waste products into the Bowman's space to produce primary urine. The primary urine is further modified in the tubular system to generate the final urine waste (Figure 1.1a and 1.2a). In one day, the two kidneys produce approximately 180 liter of primary urine, but the final excreted volume is about 1.5 liter per day. In contrast to plasma, the urine is practically devoid of proteins of the size of albumin or larger. Glomerular ultrafiltration occurs in a capillary wall made up of fenestrated endothelial cells, glomerular basement membrane (GBM), and the slit diaphragm of podocytes (Figure 1.1b and Figure 1.2b). The filtration barrier is permselective to plasma macromolecules based on size, shape, and charge. The permselective properties of the glomerular filtration barrier are dictated by pore sizes of the GBM and slit diaphragm and to some extent charge selectivity. Defects and damage of components of the filtration barrier can cause excessive leakage of protein into urine, a condition called proteinuria (Table 1 and Table 2). Proteinuria is a major factor promoting the progression of kidney disease and a common feature in most renal disorders.

1.2 Podocytes

Podocytes are highly differentiated epithelial cells that enwrap glomerular capillaries [7]. Podocytes constitute a cell body and major cytoplasmic projections (primary processes) that divide into secondary processes and subsequent foot processes. The foot processes are ordered in such a way that processes of neighboring cells interdigitate like the fingers of a folded hand (Figure 1.1b). A specialized cell-cell junction termed slit diaphragm, bridges the interdigitating foot processes. Like other epithelial cells, the podocytes are polarized and feature distinct apical and basal domains. The slit diaphragm is the site of contact between neighboring foot processes and forms the border dividing the podocyte foot process plasma membrane into an apical domain facing the urinary space and a basal domain resting on the GBM (Figure 1.1c and 1.1d). The different plasma membrane domains are interconnected through the cytoskeleton via a network of protein-protein interactions, actin-filament and microtubules that maintain the distinctive cell shape of the podocyte (Figure 1.3). In proteinuric diseases, a flattening of foot processes is often

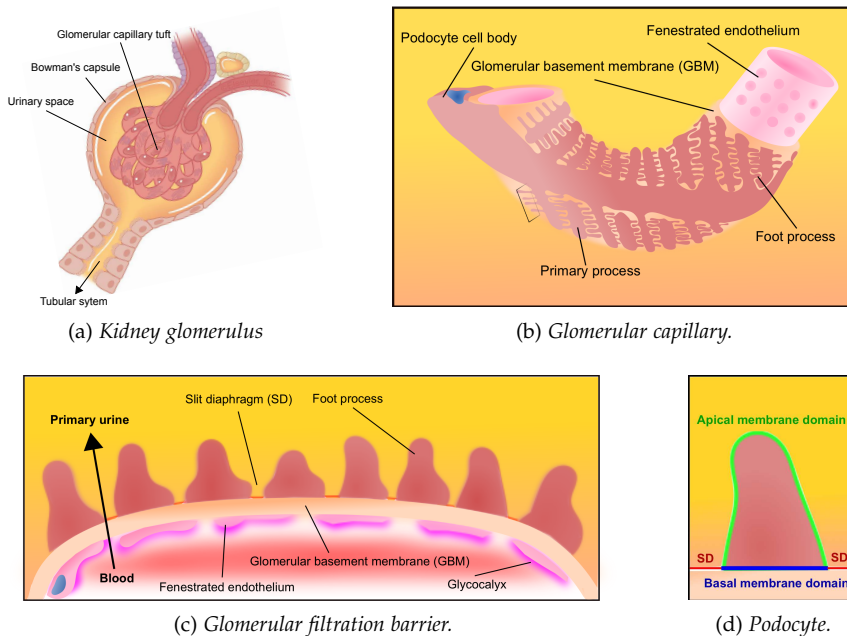


Figure 1.1: Glomerular filtration barrier. (a) The kidney glomerulus is a tuft of capillaries within the Bowman's capsule. (b) The Glomerular filtration barrier is made up of three layers. Fenestrated endothelial cells, glomerular basement membrane and podocytes with a cell body, primary processes and interdigitating foot processes. (c) Glomerular filtration from blood to primary urine occurs through a layer of fenestrated endothelium covered with a glycocalyx, the glomerular basement membrane (GBM) and the slit diaphragm bridging podocyte foot processes. (d). Podocytes are polarized cells. The slit diaphragm (SD) divides the podocyte foot processes into an apical membrane domain facing the urinary space and basal membrane domain resting on the GBM.

observed, a phenomenon referred to as effacement [7, 8]. The whole podocyte flattens as a result of retraction, a widening and shortening of the foot processes. This gives the appearance of a continuous cytoplasmic sheet casing the glomerular capillary. Currently the detailed pathomechanism of foot process effacement and its involvement in the development of proteinuria are not well understood.

1.2.1 Slit Diaphragm

The slit diaphragm is a specialized cell-cell junction located immediately above the GBM (Figure 1.1c). Apart from proteins like cadherins and catenins, that are constituents of the most common types of cell junctions, the slit diaphragm contains proteins that are specialized for carrying out the ultrafiltration process. [9, 10]. Today, the slit diaphragm is considered to be the most critical part of the glomerular filtration barrier [11]. A number of components of the slit diaphragm are mutated in patients with nephrotic syndrome (Table 1) that is characterized by massive

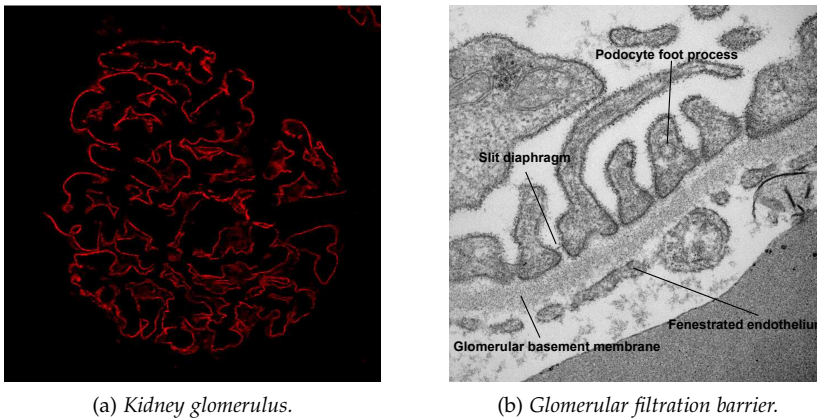


Figure 1.2: Immunohistochemical staining of Nephrin in the kidney glomerulus. (a). A continuous strong staining on the outside of glomerular capillary loops is observed. (b). Transmission electron micrograph showing the three layers of the glomerular filtration barrier.

proteinuria.

Nephrin forms the backbone of the slit diaphragm and it was the first protein located in this unique structure (Figure 1.2a and 1.3) [12]. Deleterious mutations in nephrin cause collapse of the slit diaphragm and massive proteinuria already in utero in humans, and mice lacking nephrin exhibit a similar severe phenotype (Table 1) [13, 14, 15, 11]. Nephrin is a transmembrane protein with a short intracellular domain, an extracellular domain containing eight immunoglobulin-like motifs and a fibronectin motif. The extracellular domains of Nephrin from neighboring foot processes likely form homodimers spanning the uniformly wide slit diaphragm (Figure 1.3) [16]. Based on electron tomography results and Nephrin interaction studies, the Nephrin molecules form a zipper-like structure that can act as a filter [17]. This kind of zipper-like structure has been proposed earlier based on transmission electron microscopic studies [18].

NEPH1 possesses five immunoglobulin-like domains and is structurally related to Nephrin. NEPH1 localizes to the slit diaphragm where it forms homodimers with other NEPH1 molecules and heterodimers with Nephrin [19]. Mice lacking NEPH1 develop massive proteinuria much like Nephrin knockout mice [14]. Furthermore, disruption of the glomerular filtration barrier has been recapitulated by morpholino knockdown of NEPH homologs in zebrafish [20]. Podocin is an intramembrane protein with a hairpin like shape where both N- and C- termini face the cytoplasm (Figure 1.3).

Podocin interacts directly with the CD2-associated protein, NEPH1 and Nephrin at the slit diaphragm where it also forms homo-oligomers with other podocin molecules [21, 22, 23]. Mutations in podocin cause steroid resistant congenital nephrotic syndrome (Table 1) [24]. Mutated Podocin fails to localize to the slit diaphragm where it might have a scaffolding function as the location of Nephrin is also affected [25]. Inactivation of podocin in mice mirrors the human phenotype with heavy proteinuria, hypercholesterolemia and progressive renal failure [24, 26, 25, 27].

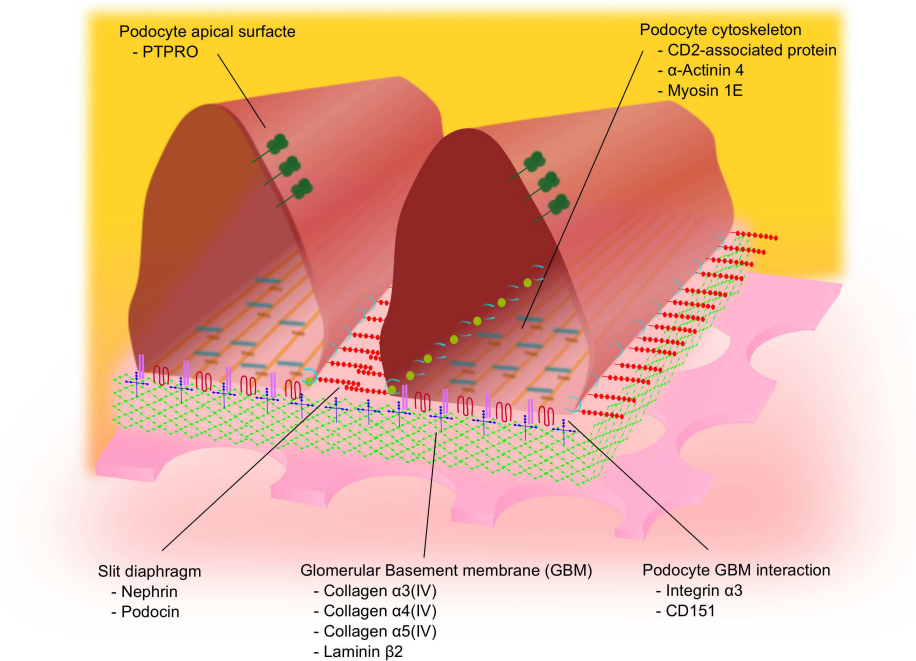


Figure 1.3: Illustration of podocyte foot processes showing key components of the glomerular filtration barrier. Mutations in the genes encoding these proteins have been linked to human disease and a renal phenotype in animal models (Table 1). In the glomerular basement membrane mutations in all the components of type IV Collagen have been linked to Alport Syndrome and mutations in Laminin β 2 are linked to nephrotic syndrome. Foot processes are basally anchored to the glomerular basement membrane via Integrin α 3 and CD151 both are linked to nephropathy with extra renal manifestations. Mutations in the slit diaphragm proteins Nephrin and Podocin cause congenital nephrotic syndrome, like PTPRO that is located at the apical surface of podocytes. Mutations in the actin cytoskeleton proteins CD2-associated protein, β -actinin-4 and Myosin1E are associated with focal segmental glomerulosclerosis.

Cadherins are transmembrane proteins that play an important role in cell adhesion. Two cadherin proteins have been located to the slit diaphragm, the large protocadherin FAT1 and vascular endothelial cadherin (CDH5) [28]. FAT1 is needed for the filtration barrier as FAT1 knockout mice and zebrafish develop severe proteinuria and pronephric cyst, respectively (Table 2) [29, 30, 31]. Renal abnormalities in CDH5 knockout mice have not been determined, as they show complete embryonic lethality during organogenesis.

Cell polarity is fundamental for the proper function of most cell types. A complex of proteins involved in cell polarity co-localize with Nephrin on the cytoplasmic side of the slit diaphragm. This polarity complex is composed of Partitioning defective 3 (PARD3), Partitioning defective 6 (PARD6G) and Protein kinase C ι (PRKC ι) [32, 33]. PARD3 interacts with nephrin and NEPH1, and recruits the PARD6G and PRKC ι to the slit diaphragm. The absence of PRKC ι in podocytes causes proteinuria in mice indicating that this cell polarity complex is important in regulating podocyte cell polarity and a functional glomerular filtration barrier [32, 33]. Crb2b is a polarity protein of the crumbs family that has been shown to affect localization of nephrin in podocytes. Crb2b depleted zebrafish larvae show disrupted glomerular filtration associated with gross changes in podocyte foot processes morphology mis-localization of nephrin to the podocyte apical surface [34]. Crb2 knockout mice show complete embryonic lethality before kidney organogenesis [35].

TRPC6 is a transient receptor potential channel involved in the regulation of intracellular calcium concentration. In podocytes, TRPC6 is located to the slit diaphragm region and mutations in TRPC6 have been linked to familial focal segmental glomerulosclerosis (Table 1) [36, 37]. Despite this TRPC6 knockout mice have an intact glomerular filtration barrier [38, 39]. However, overexpression of TRPC6 in transgenic mice results in a phenotype resembling focal segmental glomerulosclerosis [40].

1.2.2 The Podocyte Apical Surface

Apically the podocytes face the urinary space of the Bowman's capsule and the glomerular filtrate (Figure 1.1c). The apical plasma membrane is covered with a glycocalyx made up from negatively charged molecules. The negative charge of the apical surface is believed to be important for repelling the plasma membrane of a neighboring foot process which in turn maintains a space between foot processes, and podocytes and the Bowman's capsule. Podocalyxin is an O-glycosylated and sialylated transmembrane protein that is the major component of the negatively charged glycocalyx of podocyte foot processes [41]. Mice lacking podocalyxin have severe podocyte malformations that hinder normal glomerular filtration and formation of primary urine resulting in renal failure [42]. The negative charge of podocytes is neutralized in inducible models of acquired podocyte disease in rodents such as those caused by puromycin and protamine sulfate, resulting in a collapse of the slit and disruption of the highly ordered foot process architecture with proteinuria as a result [41].

The transmembrane receptor tyrosine phosphatase PTPRO is located at the apical plasma membrane of podocytes (Figure 1.3). PTPRO has a large extracellular domain containing multiple fibronectin type III repeats and a single cytoplasmic phosphatase active site. Mutations in PTPRO have been shown to be associated with nephrotic syndrome in humans (Table 1) [43]. Furthermore, PTPRO deficient mice exhibit abnormal podocyte morphology, reduced glomerular filtration rate, and are susceptible to high blood pressure (Table 1) [44]. Thus, PTPRO is necessary for

the maintenance of normal podocyte structure and plays a role in the regulation of glomerular filtration.

1.2.3 Podocyte Cytoskeleton

The cytoskeleton renders podocytes its distinctive shape and coherence and is essential for a functional filtration barrier. The cytoskeleton of the podocyte cell body and primary processes mostly contains intermediate filaments and microtubules, while actin filaments are predominant in the foot processes that run lengthwise along their axis (Figure 1.3). Components of the cytoskeleton receive and mediate signaling through cues from the extracellular environment that can result in changes in cell shape. For example, during foot process development, Nephtrin molecules are phosphorylated which results in their binding to intracellular NCK proteins (NCK1 and NCK2) that then interact with actin monomers which assemble into oligomers [45]. Also, defects and injuries of podocytes are known to cause remodeling of the actin cytoskeleton which can lead to foot process effacement that generally is accompanied by proteinuria [7, 8]. During repair of foot process and slit diaphragm damage and effacement, Nephtrin molecules are phosphorylated similarly as during development, which leads to reconstruction of the actin cytoskeleton [46]. The NCK proteins bind to the cytoplasmic tail of Nephtrin and recruit other proteins involved in the regulation of actin dynamics. NCK protein mediated actin polymerization and cytoskeletal reorganization through Nephtrin is essential for the normal filtration barrier as mice lacking both Nck proteins in podocytes develop massive proteinuria [45]. The formation of foot processes and effacement are dynamic processes that occur very fast after initial damage. Loss of foot processes in effacement can be repaired quickly such as in many cases of minimal change nephrosis, the most common cause of nephrosis in children.

Nephtrin and the slit diaphragm are connected to the actin cytoskeleton via CD2-associated protein (CD2AP) and NCK proteins [47, 48, 49]. CD2AP binds both nephtrin and actin establishing a link between the slit diaphragm and the actin cytoskeleton. This link is crucial for the filtration barrier as CD2AP deficient mice exhibit massive proteinuria and foot process effacement. Like other key constituents of the podocytes cytoskeleton mutations in CD2AP have been associated with segmental scarring of the kidney glomerulus in humans, a condition referred to as focal segmental glomerulosclerosis (FSGS) (Table 1) [49, 50].

Further insight into the regulation of actin dynamics has been gained through studies of the actin modulating proteins Cofilin1 (CFL1) and Inverted formin FH2 WH2 domain containing (INF2). Cofilin1 is part of the ubiquitous cofilin family of proteins that are essential for the remodeling and disassembly of actin filaments [51]. Podocyte specific inactivation of Cofilin1 in mice results in a loss of organized actin cytoskeletal architecture, susceptibility to podocyte injury, proteinuria and gradually results in foot process effacement [52]. This implies that Cofilin1 is an essential regulator of actin dynamics in podocytes. Furthermore, it has been demonstrated *in vitro* that nephtrin may play a role in the regulation of Cofilin1. INF2 is highly expressed in podocytes. (Figure 1.3) INF2 is a member of the heterogeneous diaphanous-related formin family and it has been shown to control actin polymerization as well as depolymerization. Mutations in INF2 have been identified as a common cause of familial FSGS underlining the importance of dynamic rearrangement of the podocyte cytoskeleton in maintaining a functional glomerular filtration barrier [53, 54].

Another actin binding protein linked to familial FSGS is α -actinin-4 (Figure 1.3) [55]. Interestingly a podocyte specific expression of the most common disease causing α -actinin-4 mutation in a mouse model mirrors the human disease phenotype [56, 57]. A complete disruption of α -actinin-4 in a mice leads to detachment of podocytes from the GBM and proteinuria indicating that α -actinin-4 is an essential part of the podocyte cytoskeleton [58, 56]. The actin-associated protein Synaptopodin has been identified as a regulator of α -actinin-4 activity. Synaptopodin deficient mice have normal kidney function and podocyte morphology. However, the Synaptopodin knockout mice demonstrate impaired recovery after induction of podocyte injury [59].

The nonmuscle class I myosin MYO1E is highly enriched in podocytes. It is made up of a motor domain; a regulatory domain and a long tail domain involved in cross-linking actin filaments (Figure 1.3). MYO1E has been linked to a recessive form of FSGS [60]. Inactivation of MYO1E in mice leads to proteinuria, podocyte foot process effacement, thickened GBM and glomerulosclerosis [61]. A podocyte specific inactivation of MYO1E in mice mirrors this phenotype emphasizing the role of MYO1E in podocyte actin cytoskeleton organization [62].

Another protein, worth mentioning here is Phospholipase c ϵ 1 (PLCE1). PLCE1 has been associated with congenital nephrotic syndrome in humans and morpholino mediated gene silencing in zebrafish results in disruption of the glomerular filtration barrier and disorganization of foot processes (Table 1). Unlike other genes linked to nephrotic syndrome, PLCE1 is not a structural protein. In contrast, it is an enzyme, a member of the phospholipase C superfamily [63, 64]. Mice with targeted mutations in PLCE1 do not however show a renal phenotype. The exact role of PLCE1 in podocyte biology remains obscure.

1.3 Glomerular Basement Membrane

Basement membranes are a specialized form of extracellular matrix that underlies all epithelial, and endothelial cells, and surrounds peripheral nerves as well as muscle and fat cells throughout the body. The glomerular basement membrane (GBM) is a sheet of extracellular matrix that separates the fenestrated endothelial cells from the podocytes (Figure 1.1c and 1.2b). Like other basement membranes, the GBM contains laminin, type IV collagen, nidogen, and heparan sulfate proteoglycans (HSPGs). However, the GBM is a structurally unique type of basement membrane. It is thicker (240-370 nm) compared to most other basement membranes. This is due to the fact that the GBM forms during glomerulogenesis by fusion of endothelial and pre-podocyte basement membranes. In addition, the specific isoforms of laminins and collagen type IV present in the GBM are different from those found in most other basement membranes. The GBM has an important role in the formation and maintenance of the glomerular filtration barrier, and mutations in components of the GBM are associated with hereditary human glomerular diseases.

The podocytes are anchored to the GBM through transmembrane receptors such as integrins and tetraspanins (Figure 1.3). Integrins are heterodimeric proteins with alpha and beta subunits that show differential expression between cell types and disease states. In podocytes, $\alpha3\beta1$ integrin is the major isoform and is necessary for the development of the glomerulus. Mutations in the integrin $\beta3$ chain, have been associated with interstitial lung disease with nephrotic syndrome and epidermolysis bullosa in humans (Table 1). Mice deficient for the integrin $\beta3$ chain in podocytes develop massive proteinuria postnatally and show complete foot process effacement with severe

defects of the GBM ultrastructurally (Table 1) [65, 66]. A similar phenotype is seen in mice lacking the integrin β_1 chain in podocytes [67]. Interestingly, Integrin-linked kinase (ILK) directly interacts with integrin β_1 and mediates integrin signal transduction. ILK has also been found to interact with Nephrin linking integrin and slit diaphragm signaling [68]. Podocyte specific inactivation of ILK causes mislocalization of Nephrin, severe proteinuria and kidney failure in mice (Table 2)[68, 69]. This clearly underlines the importance of the $\alpha_3\beta_1$ integrin isoform for the development and maintenance of the glomerular filtration barrier. Tetraspanins are a multigene family encoding four-transmembrane domain proteins that organize other membrane proteins such as $\alpha_3\beta_1$ integrin. The tetraspanin CD151 co-localizes with integrin α_3 at the basal plasma membrane of foot processes (Figure 1.3). CD151 knockout mice develop proteinuria and exhibit severe thickening and splitting of the GBM that precede podocyte abnormalities mimicking the phenotype of integrin α_3 knockout mice (Table 1). This indicates a critical role for this protein in the maintenance of the filtration barrier [66, 70].

Type IV collagen forms the structural framework of the GBM. The extensively cross-linked GBM type IV collagen meshwork provides tensile strength to the glomerular capillary wall (Figure 1.3). During development the triple-helical type IV collagen molecules of the GBM are composed of α_1 and α_2 chains. However, after birth, this isoform is replaced by $\alpha_3\alpha_4\alpha_5$ trimers that contain more disulfide crosslinks. Mutations in any of adult GBMs collagen chains cause Alport's syndrome (Table 1) [71, 72], a disease characterized by the distortion of the GBM structure and a progressive renal hematuria disease that usually leads to renal failure [73]. A similar phenotype is observed in corresponding mouse models lacking the $\alpha_3\alpha_4\alpha_5$ trimers and in mice lacking the non-collagenous domain (NC1) of the α_3 chain that specifically disrupts the assembly of Type IV collagen [74, 75, 76, 77, 78]. The collagen network binds directly to laminin in the GBM and is connected to podocytes via plasma membrane linker proteins such as integrin.

The heterotrimeric laminins are permanent features of all basement membranes where they form a cross-linked meshwork with type IV collagen (Figure 1.3). During development, the GBM laminin is laminin-521 containing the $\alpha_5\beta_2\gamma_1$ chains [79]. Mutations in the Laminin β_2 chain are associated with perinatal disease termed Pierson's syndrome, which is characterized by massive proteinuria and ocular abnormalities [80]. Mice lacking the Laminin β_2 chain exhibit disorganized GBM and massive proteinuria, which further underlines the importance of this laminin in the glomerular filtration barrier (Table 1). Importantly, massive protein leakage through the filtration barrier is detected in these mice before any abnormalities in podocytes and loss of Laminin β_2 results in compensatory upregulation of Laminin β_1 resulting in a structurally intact but defective GBM that likely is the cause of proteinuria [81, 82, 83]. Laminin α_5 (Lama5) knockout mice die late during development as a cause of defects in basement membranes in various tissues including the GBM [82, 84, 85]. A mouse model with a hypomorphic mutation in Lama5 die as a result of polycystic kidney disease and renal failure by four weeks of age [84]. Podocyte specific expression of wildtype Lama5 in the hypomorphic mutant mice prevents proteinuria and development of kidney cysts. On the other hand, a podocyte specific inactivation of Lama5 results in a thickened and damaged GBM and podocyte effacement [85]. Nidogen1 is thought to be involved in the assembly of the Collagen IV and Laminin network of the GBM. Inactivation of Nidogen1 does not result in an obvious phenotype in mice. However, a deletion of the Nidogen interacting domain of Laminin γ_1 (Lamac1) results in kidney agenesis emphasizing the important role of laminins in the formation of the GBM [86]. Interestingly the $\alpha_3\beta_1$ integrin isoform is a receptor of laminin-521 and it is likely that disruption of the integrin-laminin interaction results in detachment and loss of podocytes. Heparan sulfate proteoglycans are made up of a core protein with one or more

covalently attached glycosaminoglycans.

Three HSPGs have been identified within the GBM, agrin, perlecan, and collagen XVIII [87, 88]. HSPGs are believed to contribute to charge-selectivity of the ultrafiltration process since removal of their negative charge after intravenous administration of heparanases is reported to cause increased permeability of the filtration barrier in animal models [89]. However, the role of HSPGs in glomerular filtration has been challenged, as mice lacking podocyte-derived agrin and perlecan lacking heparin-sulfate side chains do not show exhibit proteinuria [90, 88]. On the other hand, mice lacking collagen XVIII $\alpha 1$, the gene product of COL18A1, show mesangial expansion and podocyte foot process abnormalities [87, 91, 92].

1.4 Fenestrated Glomerular Endothelium

Endothelial cells line the inner surface of the vascular tree throughout the body and form a barrier between blood and tissues. Endothelial cells in various vascular beds have quite different structural and functional properties. The glomerular endothelium is fenestrated and discontinuous with large pores. Whereas some discontinuous endothelia are permeable to water and small solutes, others, such as the brain capillary endothelium, are highly impermeable. In the glomerulus, endothelial cells are an important part of the glomerular filtration barrier, although their direct role in restricting the passage of macromolecules through the capillary wall is still debated. Morphologically, there are two types of glomerular endothelial cells. Cells located near the hilus of the capillary loop that do not have fenestrae and endothelial cells located in the peripheral regions of the glomerular tuft that are flat and highly fenestrated. These fenestrae of the plasma membrane range from 70 to 100 nm in diameter, vastly bigger than the diameter of albumin. The fenestrae constitute 30–50% of the glomerular capillary wall surface area. Unlike many other fenestrae in endothelial cells, those present in the glomerulus lack diaphragms [93].

Intact glomerular endothelial cell fenestrae are essential for the normal hydraulic conductivity of the glomerular capillary wall. In many human disorders, such as in pre-eclampsia and in the hemolytic uremic syndrome, the integrity of glomerular endothelial cell fenestrae is lost. These diseases are associated with reduced glomerular filtration rate. Similar findings have been observed in animal models in which glomerular endothelial fenestrations are affected. Of note is that VEGF-A has been shown to be a key factor in the formation and maintenance of glomerular endothelial cell fenestrations [94, 95]. The glomerular endothelial cells are enveloped in an extracellular surface layer, glycocalyx, mostly made up from negatively charged glycoproteins, glycosaminoglycans and membrane associated proteoglycans (Figure 1.1c). The surface layer might hinder the passage of albumin and other plasma proteins. This idea is supported by studies in various experimental models where the permeability of the glomerular capillary wall is increased after endothelial cell surface layer disruption.

Table 1.1: Genes linked to glomerular kidney disease in humans.

Location	Gene Sumbol	Disease (OMIM ID)*	Mouse Phenotype†	Zebrafish Phenotype‡	Ref.
Slit diaphragm	<i>NPHS1</i>	Nephrotic syndrome, type 1 (256300)	Proteinuria, podocyte foot process effacement, absent podocyte slit diaphragm	Glomerular filtration disrupted	[15, 11, 96, 2]
	<i>NPHS2</i>	Nephrotic syndrome, type 2 (609995)	Proteinuria, glomerulosclerosis, podocyte foot process effacement, absent podocyte slit diaphragm, abnormal renal glomerulus basement membrane morphology	Glomerular filtration disrupted	[26, 25, 27, 97]
	<i>PLCE1</i>	Nephrotic syndrome, type 3 (610725)		Glomerular filtration disrupted, foot process effacement, disorganization of slit diaphragms	[63]
	<i>TRPC6</i>	Glomerulosclerosis, focal segmental, 2 (603965)			
GBM	<i>LAMB2</i>	Nephrotic syndrome, type 5 (614199)	Proteinuria, fused podocyte foot processes, increased renal glomerulus basement membrane thickness, abnormal glomerular filtration barrier function		[81]
	<i>COL4A3</i>	Alport syndrome, autosomal recessive (104200, 203780)	Proteinuria, hematuria, increased renal glomerulus basement membrane thickness, podocyte foot process effacement, expanded mesangial matrix, renal glomerulus fibrosis		[75, 74, 77]
	<i>COL4A4</i>	Alport syndrome (203780)	Proteinuria, hematuria, expanded mesangial matrix, glomerulosclerosis		[98]
	<i>COL4A5</i>	Alport syndrome, autosomal dominant, autosomal recessive (301050)	Proteinuria, hematuria, dilated glomerular capillary, podocyte foot process effacement, abnormal renal glomerulus basement membrane morphology, glomerulosclerosis, mesangial cell hyperplasia		[76]
Basal surface	<i>CD151</i>	Nephropathy with pretibial epidermolysis bullosa and deafness (609057)	Proteinuria, podocyte foot process effacement, absent podocyte slit diaphragm, decreased podocyte number, increased renal glomerulus basement membrane thickness, glomerulosclerosis		[66, 70]
	<i>ITGA3</i>	Interstitial lung disease, nephrotic syndrome, and epidermolysis bullosa, congenital(614748)	Proteinuria, podocyte foot process effacement, absent podocyte foot process, increased renal glomerulus basement membrane thickness, glomerulosclerosis		[65, 66]
Apical surface	<i>PTPRO</i>	Nephrotic syndrome, type 6 (614196)	abnormal podocyte foot process morphology, decreased renal glomerular filtration rate		[44]
Cytoskeleton	<i>ACTN4</i>	Glomerulosclerosis, focal segmental, 1 (603278)	Proteinuria, glomerulosclerosis		[58]
	<i>INF2</i>	Glomerulosclerosis, focal segmental, 5 (613237)			
	<i>MYO1E</i>	Glomerulosclerosis, focal segmental, 6 (614131)	Proteinuria, hematuria, podocyte foot process effacement, increased renal glomerulus basement membrane thickness glomerulosclerosis, expanded mesangial matrix		[61]
	<i>CD2AP</i>	Glomerulosclerosis, focal segmental, 3 (607832)	Proteinuria, abnormal podocyte morphology, glomerulosclerosis, expanded mesangial matrix	Glomerular filtration disrupted	[49, 50, 97]

*Online Mendelian Inheritance in Man (OMIM).

†Mouse genome informatics (MGI).

‡Zebrafish Model Organism Database (ZFIN).

Table 1.2: Genes linked to glomerular kidney disease in mouse and zebrafish.

Location	Gene Symbol	Mouse Phenotype [§]	Zebrafish Phenotype [¶]	Ref.
Slit diaphragm	<i>Neph1</i>	Proteinuria, abnormal renal glomerulus morphology, expanded mesangial matrix	Glomerular filtration disrupted	[14, 20]
	<i>Fat1</i>	Fused podocyte foot processes, abnormal podocyte slit junction morphology	Pronephric cysts	[29, 31]
	<i>Prkci</i>	Glomerulosclerosis, fused podocyte foot processes, abnormal podocyte polarity, abnormal podocyte slit diaphragm morphology		[33, 99]
	<i>Crb2</i>		Glomerular filtration disrupted, abnormal podocyte slit diaphragm morphology	[34]
Basal surface	<i>Itgb1</i>	Proteinuria, increased podocyte apoptosis, podocyte foot process effacement, abnormal renal glomerulus basement membrane morphology, dilated glomerular capillary		[67]
GBM	<i>lmac5</i>	Proteinuria, hematuria, abnormal glomerular capillary endothelium morphology, podocyte foot process effacement, increased renal glomerulus basement membrane thickness, glomerulosclerosis,		[82, 84, 85]
	<i>lamc1</i>	Abnormal renal glomerulus morphology, decreased renal glomerulus number, abnormal kidney development		[86]
	<i>Agrn</i>	Increased renal glomerulus basement membrane thickness		[90]
	<i>Col18a1</i>	Podocyte foot process effacement, expanded mesangial matrix		[91, 92]
Cytoskeleton	<i>Nck1, Nck2</i>	Proteinuria, glomerulosclerosis, fused podocyte foot processes, abnormal glomerular filtration barrier function		[47]
	<i>Cfl1</i>	Proteinuria, abnormal podocyte foot process morphology, podocyte microvillus transformation	Glomerular filtration disrupted,	[52, 100]
	<i>Ilk</i>	Proteinuria, detached podocyte, decreased podocyte number, podocyte foot process effacement, abnormal podocyte slit diaphragm morphology, increased renal glomerulus basement membrane thickness		[68, 69]
Apical surface	<i>Podxl</i>	Anuria, absent podocyte foot process, absent podocyte slit diaphragm		
Fenestrated endothelium	<i>Vegfa</i>	Proteinuria, abnormal glomerular capillary endothelium morphology, absent glomerular endothelium fenestra, abnormal renal glomerulus basement membrane thickness, glomerulosclerosis, decreased renal glomerulus number		[95, 94]

[§]Mouse genome informatics (MGI).[¶]Zebrafish Model Organism Database (ZFIN).

Aims of the Thesis

The objectives of this thesis work are part of a large systems biology approach to increase the understanding of how glomerulus specific proteins are involved in biological and pathological processes of kidney glomeruli. An initial and central part of the study has included isolation of glomeruli from healthy mouse kidneys and characterization of their transcriptomes and proteomes. The specific objectives of this thesis were to characterize the proteome of normal mouse glomeruli, study the role of some proteins, *Glcc1*, *Plekhh1* and *Plekhh2*, highly upregulated in glomeruli using zebrafish and mouse as a model system.

Specific Aims

Paper I

To identify proteins in normal mouse kidney glomeruli by two-dimensional gel electrophoresis and MALDI-TOF mass spectrometry.

Paper II

To determine the expression pattern, sub-cellular location and function of *Glcc1* in the kidney.

Paper III

To elucidate the *in vivo* function of the paralogous genes *Plekhh1* and *Plekhh2* in zebrafish.

Paper IV

To determine the effect of double knockout of *Plekhh1* and *Plekhh2* on kidney function in mice.

3

Methodology

The methods applied in the thesis are described in further detail in the materials and methods section of the appended papers (I-IV).

Glomerular isolation (Paper I-IV)

Mice were anesthetized and perfused through the heart with magnetic beads. The kidneys were removed, minced and digested with DNaseI. The digested tissue was pressed through a cell strainer and washed several times. Finally, the glomeruli were collected with a magnet and washed. The glomeruli were counted and the purity was controlled under a light microscope. In paper II and III, glomeruli were micro-dissected from transgenic zebrafish expressing enhanced green fluorescent protein under a podocin promoter.

Two-dimensional gel electrophoresis (Paper I)

A method of electrophoresis in which the first dimension is Isoelectric focusing according to the electrochemical charge of the protein and the second dimension is separation by gel electrophoresis according to molecular weight.

In-gel digestion (Paper I)

Gel pieces from two-dimensional gel electrophoresis were destained, reduced, alkylated and digested with trypsin. Peptides were extracted, desalted and concentrated prior to mass spectrometric analysis.

Mass spectrometry (Paper I)

The type of a mass spectrometry used was matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF). MALDI-TOF uses an electric field to accelerate peptides and then measures the time they takes them to reach the detector. If the particles all have the same charge they will have the same kinetic energy and the velocity will depend on their mass. Lighter peptides reach the detector first. Identification of proteins was performed using the ProteinProspector MS-Fit and Mascot search engines.

Western blotting (Paper II)

A method of detecting a specific protein in a tissue extract. Proteins are separated according to molecular weight using one-dimensional gel electrophoresis. Proteins are then transferred by blotting from gel to membrane. Specific proteins can be localized on the membrane using antibodies.

Northern blotting (Paper II)

A method to detect specific mRNAs. RNA is separated on a gel and transferred to a membrane by blotting. Specific mRNAs are detected using a radio-labeled anti-sense probe.

Southern blotting (Paper III)

A method of detecting a specific genomic DNA fragment. Restriction enzyme digested DNA is separated on a gel and transferred to a membrane by blotting. Specific DNA fragments are detected with anti-sense radio-labeled probes.

Zebrafish filtration assay (Paper II-III)

In Paper II, a novel method was used to assess proteinuria in zebrafish larvae. Briefly, morphant zebrafish larvae were collected at four dpf and kept in five ml of medium. After 24 hours, four ml of medium were collected and proteins were precipitated from the solution and concentrated in a smaller volume. Finally, proteins were separated using one-dimensional gel electrophoresis and stained for the presence of protein. In Paper III, a different method to assess proteinuria in zebrafish larvae was applied. Transgenic zebrafish expressing vitamin-D binding protein fused to enhanced green fluorescent protein (DBP-EGFP) were used to assess proteinuria. Morphant zebrafish were collected at four days post fertilization and fixed in paraformaldehyde. Fixed embryos were embedded in plastic and sectioned through the glomerulus. Sections were examined under a fluorescent microscope for the presence of fluorescent protein in tubuli as a sign of proteins having traversed the glomerular filtration barrier.

Quantitative and Real-Time RCR (Paper II-IV)

Quantitative Real-Time PCR (qPCR) was used to detect gene transcripts in total RNA isolated from mouse glomeruli wild-type and knockout mice. Real-Time PCR (RT-PCR) was used to characterize expression of genes using cDNA libraries from various adult mouse tissues, isolated mouse and zebrafish glomeruli as template for RT-PCR.

Immuno-histochemistry (Paper (Paper II-IV)

Immuno-histochemistry staining was used to detect proteins in frozen- or paraffin-sections of kidney glomeruli.

Histology (Paper II-IV)

Mouse kidneys and embryos were fixed, dehydrated and embedded in paraffin. Zebrafish larvae were fixed, dehydrated and embedded in plastic. Thin sections were stained with Periodic acid-Schiff's staining (PAS) alternatively Hematoxylin and Eosin (H&E) staining.

Immunoelectron and transmission electron microscopy (Paper II-IV)

Kidney biopsies from mice and zebrafish larvae were prepared for ultrastructural analysis by fixation, dehydration and embedding. For transmission electron microscopy, ultra-thin sections were made, treated to enhance contrast and examined under a transmission electron microscope. For immunoelectron microscopy, ultra-thin sections of kidneys were incubated with antibodies and examined under a electron microscopy. Random images of glomerular capillaries were taken for semi-quantification of protein expression.

Morpholino antisense oligonucleotide and mRNA injecton in Zebrafish (Paper II-IV)

Zebrafish (*D. rerio*) embryos used in this thesis were derived from the AB strain. Zebrafish embryos were microinjected with antisense morpholino oligonucleotides at the one to four cell stage and monitored for a phenotype at two to four days post fertilization. Morpholino oligonucleotides were designed to Morpholino to block or alter mRNA splicing of *Plekhh1* and *Plekhh2*. Rescue experiments were done by co-injecting *in vitro* transcribed mRNA with morpholino oligonucleotides.

Knockout mouse models (Paper III-IV)

A *Plekhh2* gene knockout construct was designed. The construct was electroporated

into a 129sv derived embryonic stem cells that were used to generate a chimeric *Plekhh2* knockout mouse line. The mice were backcrossed onto a C57BL6/NTac background for nine generations. *Plekhh1* knockout mice line was generated by order from the NIH knockout Mouse project (KOMP). The *Plekhh1* knockout mouse line used in this thesis was created from B57BL6 derived ES cell line (JM8A3), obtained from the NCRR-NIH supported KOMP Repository and generated by the Mouse Biology Program at the University of California-Davis. *Plekhh1* and *Plekhh2* mice were intercrossed to generate double knockout mice.

Results and Discussion

The results of the papers included in this thesis are briefly summarized and discussed in the following sections. For more details see appended papers I-IV.

4.1 Paper I

Glomerulus proteome analysis with two-dimensional gel electrophoresis and mass spectrometry

In this initial proteome analysis study, we applied two-dimensional gel electrophoresis and MALDI-TOF mass spectrometry to identify proteins from murine kidney glomeruli to create a snapshot of the glomerular proteome that could serve as a reference to identify biomarkers of glomerular disease by proteome analyses. Kidney glomeruli were isolated from healthy wild-type mice using a method developed by Takemoto *et al.* [101]. Quality and number of glomeruli obtained was assessed by light microscopy showing glomeruli largely free from Bowman's capsule and tubuli contaminants. From a single mouse, about 20,000 glomeruli are normally obtained, yielding $\approx 100 \mu\text{g}$ of protein. For the first dimension of protein separation, glomerular protein extracts were subjected to iso-electric focusing (IEF) using a range of immobilized *pH* gradients. For the second dimension, proteins were separated in an 8–16 % linear gradient polyacrylamide gel. After separation in two dimensions, proteins were visualized on gels with either silver or coomassie brilliant blue (CBB) staining. Excised spots were digested with trypsin and analyzed by MALDI-TOF mass spectrometry. Protein identification was performed by peptide-mass fingerprinting using a minimum of four matching peptides and coverage of 12% of the length of the query. Minimal expectation for valid identification was $P < 0.05$. Candidate biomarkers were validated by *de novo* peptide sequencing using liquid chromatography-tandem mass spectrometry (LC-MS/MS). A total of 232 proteins were identified from 414 excised spots. Comparison to similar proteomic studies of the kidney glomerulus revealed a surprisingly small overlap, only 53 out of the 232 identified proteins in this study [102, 103]. Different mass spectrometric methods and quality of tissue preparations could be a plausible explanation for this observation. In this context, it is noteworthy that previous proteomic studies of the kidney glomerulus have not successfully identified proteins specific for the podocyte slit diaphragm. We were able to identify α -actinin-4, integrin $\alpha 3$, CLIC5 and nephrin, all of which have been linked to glomerular disease in humans [13, 55, 104, 105]. Furthermore, Cofilin1 was identified, shown to be important for modulating cytoskeletal dynamics of podocytes in animal models [51]. However, the majority of the proteins identified in this study were housekeeping and structural proteins and a comparison

with proteins identified in proteome studies of endothelial and mesangial cells revealed a large overlap [103, 106]. Furthermore, comparison between proteins identified in our study and a cDNA library prepared from kidney glomeruli revealed that several proteins were not represented by their corresponding cDNA [101]. This highlights the importance of using different approaches to characterize gene expression as mRNA and protein stability differ significantly from each other.

Two-dimensional gel electrophoresis is a very powerful technique and has been applied with great success in resolving less complicated protein solutions like urine, blood and whole proteomes of bacteria that harbor a genome encoding for $\approx 3,000$ proteins [107, 108, 109]. However, when it comes to more complex proteins solutions with $>10,000$ proteins the technique starts to reach its limits, the chance of proteins overlapping increases and poor resolution of hydrophobic, very basic, large, and small molecular weight proteins becomes more evident. Improved and more sensitive alternatives to classic two-dimensional electrophoresis are emerging and have to a large extent replaced the method. As an example of a improved version of the technique is a study by Miyamoto *et al.* where they analyzed proteins extracted from human kidney glomeruli applying a novel approach [110]. First, proteins were pre-fractionated using one-dimensional gel electrophoresis. This was followed by a two-dimensional separation using solution-phase iso-electric focusing in the first dimension and gel electrophoresis in the second prior to *de novo* peptide sequencing with LC-MS/MS. Applying this methodology Miyamoto *et al.* were able to successfully identify $\approx 3,000$ proteins demonstrating that sample pre-fractionation prior to mass spectrometry substantially increases peptide identifications. Despite its limitations two-dimensional gel electrophoresis is a useful technique that continues to be used and is constantly being improved and optimized [111, 112, 113].

4.2 Paper II

Glcc1 deficiency leads to proteinuria

This study describes the expression and physiological function of the gene *Glcc1* (glucocorticoid induced transcript 1) that previously was shown to be upregulated in kidney glomeruli in a transcriptome study [101]. Immuno-histochemistry and expression analysis using RT-PCR and northern blotting further confirmed the expression of *Glcc1* in kidney glomeruli. To determine the expression of *Glcc1* within glomeruli in more detail, we carried out immunogold electron microscopy that revealed localization of gold particles mainly in podocytes and mesangial cells. Expression studies of *Glcc1* using RT-PCR showed expression in a wide range of tissues in accordance with our northern blot results. In a previous study it was shown that expression of *Glcc1* is induced by glucocorticoids in mouse thymocytes [114, 115]. This observation leads us to the hypothesis that the glucocorticoid dexamethasone might induce expression of *Glcc1* in podocytes. Using immortalized mouse podocytes in culture we demonstrated increased expression of *Glcc1* in podocytes treated with dexamethasone. Furthermore, we showed that this induction of gene expression might be mediated through the glucocorticoid receptor. To confirm these results *in vivo*, we treated mice with dexamethasone. Glomeruli isolated from these mice indeed showed an increase in *Glcc1* expression as compared with control mice. These results prompted us to carry out functional studies in zebrafish larvae. To confirm the expression of *Glcc1*, we carried out immuno-staining with an anti-*Glcc1* antibody showing specific immuno-

reactivity in glomeruli of five day old zebrafish larvae. Expression of *Glcc1* was further analyzed in glomeruli micro-dissected from transgenic zebrafish expressing green fluorescent protein under the podocin promoter confirming our previous observation. Next, we applied morpholino mediated gene inactivation in zebrafish larvae to get insight into the physiological function of *Glcc1*. Morpholino oligonucleotides were designed to block the translation initiation site of the *Glcc1* transcript or to interfere with RNA splicing. Gene specific targeting of the splice blocking morpholinos, designed to interfere with splicing of the acceptor and donor sites of exon 2, was demonstrated with RT-PCR and sequencing. Depletion of *Glcc1* by specific morpholino injection resulted in development of a pericardial edema, curvature of the dorsal body axis and short stature at four days post fertilization. This was not observed in embryos injected with standard control morpholinos at the same concentration. To further establish specificity of the gene inactivation, we co-injected full-length zebrafish *Glcc1* mRNA along with *Glcc1* splice blocking morpholinos. This greatly reduced the penetrance of the phenotype from 79% to 15%, indicating that the effects of the morpholino mediated gene inactivation are specific to *Glcc1*. Histological analysis of *Glcc1* morphant zebrafish larvae showed dilated glomerular capillary loops and expanded Bowman's space. Transmission electron microscopy revealed partial effacement of podocyte foot processes. To determine the integrity of the glomerular filtration barrier, we applied a novel glomerular filtration assay. Briefly, three day old wild-type and *Glcc1* morphant zebrafish larvae were incubated in equal volumes in cell culture plates. After 24 hours, the medium was collected and proteins were precipitated from the solution and analyzed by gel electrophoresis. Medium from *Glcc1* morphant embryos showed two bands of approximately 70 and 150 kilodaltons. Bands of the same size were also observed in medium from nephrin morphant larvae that served as positive controls. Both bands were excised and were subjected to *de novo* sequencing by mass spectrometry. This revealed that both bands originated from zebrafish vitellogenin that is a transport protein highly abundant in the yolk and blood of zebrafish larvae. Furthermore, we observed decreased glomerular *Glcc1* expression in mouse models of adriamycin and lipopolysaccharide of acute kidney injury, as well as in a diabetic mouse model (db/db) indicating that *Glcc1* expression might be generally down-regulated following podocyte injury.

In summary, our results suggest that *Glcc1* plays an important role in the maintenance to the glomerular filtration apparatus. Interestingly, shortly before our study was published, *Tantisira et al.* showed in a genome-wide association study that variants in the promoter region of human *GLCCI1* could be linked to glucocorticoid responsiveness in patients with asthma [116]. These findings combined with the results of our study spurred *Cheong et al.* to examine whether variants in *GLCCI1* could predict glucocorticoid responsiveness in patients with nephrotic syndrome. No statistically significant differences were noted in the rather small cohorts of glucocorticoid responsive and non-responsive patients [117]. This needs to be studied in a larger cohort of patients to determine if genotyping variants in *GLCCI1* has a value as a diagnostic parameter for patients with nephrotic disease. Independently, *Glcc1* was identified as a candidate within a quantitative trait locus (QTL) on Chromosome 6 associated with proteinuria based on expression differences between a proteinuria resistant and proteinuria prone inbred mouse strains [118]. This QTL in mice is concordant with a proteinuria QTL on chromosome 7 in humans detected in European Americans as part of the *Family Investigation of Nephropathy and Diabetes* (FIND) study [119]. These studies further suggest a possible link between *Glcc1* and glomerular disease. On this note, it is tempting to speculate that *Glcc1* might play a role in the modulation of glucocorticoid response in patients with nephrotic disease. To date, *Glcc1* has not been studied in the context of knockout mice. Generation of a *Glcc1* knockout mice line could give insight

into the role of *Glcc1* in the pathogenesis of glomerular disease. Generation of a *Glcc1* knockout mice line could give insight into the role of *Glcc1* in the pathogenesis of glomerular disease.

4.3 Paper III

Zebrafish Plekhh1 and Plekhh2 are involved in organization of foot processes and normal kidney function

The primary aim of this study was to characterize the physiological role of *Plekhh2* which we previously identified as a highly upregulated protein in podocytes of normal mice [101]. Furthermore, it was demonstrated using immunogold electron microscopy that *Plekhh2* has reduced expression in podocytes in a mouse model of adriamycin induced nephrosis, as well as in patients with focal segmental glomerulosclerosis (FSGS) and minimal change nephrotic syndrome [120, 121]. To further investigate the role of *Plekhh2* in the kidney and disease, we generated a knockout mouse line deficient for *Plekhh2* that was backcrossed for nine generations onto a C57BL6/NTac background. However, analysis of *Plekhh2* deficient mice revealed that all *Plekhh2*^{-/-} knockout mice were born at expected Mendelian ratios and were apparently normal. *Plekhh2*^{-/-} knockout mice did not develop proteinuria for up to eight months of age, nor did they show abnormalities in kidney morphology. The mutation was also backcrossed for four generations onto 129/SvOla and DBA/2J background to exclude potential sub strain differences. The lack of phenotype of *Plekhh2* deficient mice led us to hypothesize that the paralog of *Plekhh2*, *Plekhh1*, might compensate for the loss of *Plekhh2* function. Gene expression analysis using qPCR showed that *Plekhh1* was indeed two-fold up-regulated in glomeruli of *Plekhh2*^{-/-} mice, suggesting that up-regulated *Plekhh1* might redundantly compensate for *Plekhh2*. Both *Plekhh1* and *Plekhh2* show expression in kidney glomeruli. Immuno-histochemistry with anti-*Plekhh1* and *Plekhh2* antibodies shows linear staining following the capillary loops of the glomerulus in human kidneys. Furthermore, we were able to determine the cellular location of *Plekhh1* and *Plekhh2* within the glomerulus to podocyte foot processes using immunogold electron microscopy. To further study the *in vivo* function of the paralogs *Plekhh1* and *Plekhh2* in the glomerulus, we applied antisense morpholinos to inactivate gene expression in zebrafish larvae. RT-PCR was used to confirm expression of *Plekhh1* and *Plekhh2* in micro-dissected zebrafish glomeruli. Using an anti-*Plekhh1* antibody, we were also able to localize immunoreactivity to the glomerulus on cross-sections of four days post fertilization zebrafish larvae. No immunoreactivity was observed using an anti-*Plekhh2* antibody in zebrafish. We designed non-overlapping translation initiation site (ATG) and splice blocking antisense morpholino oligonucleotides against each gene. As a specificity control mutated versions of the splice blocking morpholinos containing five random mutations were designed. All morpholinos were injected at the one to four cell stage and the phenotype was scored at four days post fertilization. Both ATG and splice blocking morpholinos against *Plekhh1* and *Plekhh2* resulted in pericardial edema, a typical sign of kidney failure in zebrafish embryos. Larvae injected with mismatched splice morpholinos showed no significant penetrance of the phenotype. Histological analysis of the morphant embryos showed gross changes in glomerulus morphology. The Bowman's space was enlarged and the number of podocytes was reduced, exposing large, bare capillary loops. Transmission electron microscopy of the pronephric glomerulus revealed disorganized podocyte foot processes and thickening of the glomerular basement membrane (GBM) in both *Plekhh1* and *Plekhh2* knockdown zebrafish larvae. The effect of *Plekhh1* and *Plekhh2* knockdown on the integrity of the glomerular filtration

barrier was tested using transgenic zebrafish expressing a fusion-protein made up from vitamin-D binding protein (DBP) fused with enhanced green fluorescent protein (EGFP) in the blood circulation. The DBP-EGFP fusion-protein should not traverse the filtration barrier in healthy zebrafish. Passage of DBP-EGFP fusion-protein through the kidney filter was monitored from cross sections of pronephric filtration apparatus. In *Plekhh1* and *Plekhh2* morphant larvae the passage of DBP-EGFP was seen as endocytic vesicles reabsorbed into the tubular epithelia, this was not observed in larvae injected with standard control morpholino.

Taken together, although the *Plekhh2* knockout mice exhibits no phenotype depletion of *Plekhh1* or *Plekhh2* gene expression by morpholino injection in zebrafish results in disorganization of podocyte foot processes, thickening of the GBM and proteinuria. This suggests a role in the organization of podocyte foot process architecture and the formation of a functional renal filter in zebrafish. Future studies examining double knockout mice will likely be needed to fully elucidate the contribution of *Plekhh1* and *Plekhh2* for the integrity of the filtration barrier.

4.4 Paper IV

Analysis of Plekhh1 and Plekhh2 knockout mice reveal redundancy of the paralogs in kidney function

To determine the compensatory roles of *Plekhh1* and *Plekhh2* in kidney function we decided to generate a knockout of *Plekhh1*. *Plekhh1* knockout mice were generated by replacing exons 11-14, which encode for tandem PH domains, with a lacZ expression cassette. In heterozygous *Plekhh1*^{+/-} mice β -galactosidase activity was detected in several tissues including brain, spinal cord, lung and kidney's. Heterozygous *Plekhh1*^{+/-} mice were intercrossed to generate *Plekhh1*^{-/-} knockout mice. *Plekhh1*^{-/-} knockout mice are born at expected Mendelian ratios, are both viable and fertile, and have no morphological kidney abnormalities as observed by transmission electron microscopy. Likewise, the observation that *Plekhh2* knockout mice do not develop a kidney phenotype suggests that the lack of phenotype of single knockouts of *Plekhh1* and *Plekhh2* is caused by functional compensation. We were, therefore, interested in determining the impact of deleting both *Plekhh1* and *Plekhh2* on kidney function. To address this question, we intercrossed *Plekhh1* deficient mice with previously described *Plekhh2* mutant mice to produce mice lacking both genes. This yielded fewer than expected number of double knockout offspring, as well as *Plekhh1*^{-/-}/*Plekhh2*^{+/-} mice. These results suggest a redundant function for *Plekhh1* and *Plekhh2*. Surprisingly, the surviving double knockout mice did not show abnormalities of the kidney on histological and ultrastructural examination. However, at the ultrastructural level, changes were observed in *Plekhh1*^{-/-}/*Plekhh2*^{+/-} mice. Despite this, surviving double knockout and *Plekhh1*^{-/-}/*Plekhh2*^{+/-} mice did not develop proteinuria for up to six months of age.

This study demonstrates a genetic interaction between *Plekhh1* and *Plekhh2*. Both *Plekhh1*^{-/-}/*Plekhh2*^{-/-} double knockouts and *Plekhh1*^{-/-}/*Plekhh2*^{+/-} mutant mice show reduced perinatal survival, indicating functional redundancy between *Plekhh1* and *Plekhh2*. Previous observations indicate that *Plekhh2* localises to the actin-rich lamellipodia of cultured human podocytes and interacts with β -actin and the cytoskeletal protein Hic-5 [121]. This is interesting in light of the fact that cytoskeletal changes in podocytes are associated with foot process effacement [122]. This is consistent with the finding that podocytes of *Plekhh1*^{-/-}/*Plekhh2*^{+/-} mice show changes in ultrastructural organization of the podocyte foot process morphology, suggesting a possible

role of Plekhh1 and Plekhh2 in the maintenance of the podocyte cytoskeleton. Surprisingly, β -galactosidase activity was not detected in glomeruli of adult mice. In the kidney β -galactosidase activity was restricted to tubular cells. This is in contrast to our previous data that demonstrates expression of Plekhh1 in both mouse and zebrafish glomeruli using RT-PCR and in human glomeruli with immuno-histochemistry. The cause of this discrepancy is somewhat unclear. One explanation can be missing regulatory information in the sequence deleted in Plekhh1 knockout mice or decreased stability of the LacZ transcript as an alternative explanation.

The generation of Plekhh1/Plekhh2 deficient mice is the initial step in the understanding of the *in vivo* role of Plekhh proteins in mammals. For future studies, these mice will have to be analyzed under physiological stress conditions in order to determine the definitive *in vivo* function of Plekhh1 and Plekhh2.

Conclusions

Techniques allowing for the isolation of pure kidney glomeruli have facilitated the study of the molecular makeup of the kidney glomerulus using different “omics” approaches [123]. Others and we have been involved in characterizing both the transcriptome and proteome of the kidney glomerulus with the aim of gaining knowledge about the patho-mechanism of glomerular disease [101, 110, 124, 113, 125].

In paper I of this thesis, we applied two-dimensional gel electrophoresis and mass spectrometry to identify proteins in glomeruli isolated from healthy mice. The aim was to gather data to get a snapshot of the healthy glomerular proteome to serve as a reference for future work on disease models. The technique chosen for this work was two-dimensional gel electrophoresis, which is a widely used technique capable of resolving a complex mixture of thousands of proteins. However, the technique has limitations that are reflected in the rather limited number of proteins identified in our study. A conclusion that might be drawn from our work is that two-dimensional gel electrophoresis is not sufficient to characterize the whole proteome of a complex mini-organ such as the kidney glomerulus. However, the glomerular proteome will not be defined by a single method. Our study is one of the first attempts to catalog the protein components of the kidney glomerulus and it can be seen as a small step towards defining the glomerular proteome. Proteomic techniques are still developing rapidly and future studies will give us a deeper understanding of the glomerular proteome.

The main objective of this thesis is to validate the functional importance of putative candidate genes emerging from our transcriptome studies with the aim to reveal novel gene function with generation and analysis of animal models. The conservation of genes and genetic networks across species is one of the biggest conceptual advances of the genomic revolution. This has provided credence for using diverse animal models to study disease processes. We have chosen to use both zebrafish and mice as animal models. The optical clarity and rapid development of zebrafish embryos combined with morpholino mediated gene inactivation allows swift assessment of candidate genes and the mouse model provides a good approximation of human biology [126, 127]. The combination of these powerful models provides a way to functionally validating putative candidate genes. Using this strategy we have followed up a number of genes through functional studies. Two of them are presented in this thesis, *Glcc1* and *Plekhh2*.

In paper II, we describe the functional characterization of the glucocorticoid-induced transcript 1 (*Glcc1*) in zebrafish. This work demonstrates the usefulness of zebrafish as a model for kidney disease. In this study, we were able to demonstrate expression of *Glcc1* in podocytes and mesangial cells in glomeruli. Furthermore, morpholinos targeting *Glcc1* induced morphological

changes in foot processes associated with a defective filtration barrier. These results and those of others have spurred some interest in *Glcc1* [116, 117, 118]. Further studies of knockout mice and human patients will likely yield interesting discoveries regarding the function of *Glcc1*. Furthermore, in this study we have introduced new techniques to support phenotyping glomerular disease in zebrafish that will be valuable for researchers in this field. Measuring proteinuria is an important tool in kidney research to directly measure the permeability of the glomerular filtration barrier. Since zebrafish live in water, a trivial task like measuring proteinuria becomes difficult. Developing a robust reproducible method to easily screen for proteinuria in zebrafish would facilitate large-scale mutagenesis screens in zebrafish to identify genes essential for glomerular filtration.

In the work described in paper III of this thesis, we study the *in vivo* function of *Plekhh2* and its paralog *Plekhh1* in zebrafish. Knocking out *Plekhh1* and *Plekhh2* in zebrafish caused a penetrant phenotype which was not the case in knockout mice of the same genes. This could reflect physiological differences between the mouse and zebrafish models. Physiological differences should be taken into consideration when studying the same gene in different animal models. Furthermore, the method of gene inactivation should be kept in mind when interpreting phenotypic outcomes. When using morpholinos to inactivate gene expression off-target effects are a cause of concern. These are effects caused by morpholinos influencing things other than the target sequence. This has to be addressed by putting in place proper controls, the morphant phenotype should be recapitulated with a second non-overlapping morpholino against the same transcript, morpholinos containing random mutations can be designed and the observed phenotype should be rescued by co-injecting a rescue mRNA (morpholino controls paper).

In study IV of this thesis, we demonstrate functional redundancy between *Plekhh1* and *Plekhh2* in mice. The classic knockout/knockdown approach to evaluate gene function gives insight into necessity but can be complicated by functional redundancy. Genes are likely to gain redundant copies for backup purposes by duplication events during the course of evolution. In the selection of candidates for functional validation the presence and similarity of homologous genes should be taken into account. However, as often is the case in biology, compensatory effects of homologous genes is difficult to predict.

The knowledge produced by our work on validating potential candidate genes of glomerular disease will provide a basis for translational studies. The limits of our strategy are that with a powerful technique like transcriptomic profiling the number of candidate genes accumulates faster than what can be functionally validated in detail. In the future, humans will increasingly be used as models to study human disease. With the advent of population-scale genome sequencing in combination with extensive phenotype information, the direct characterization of deleterious mutations in humans by association mapping is possible [128]. This will complement the use of animal model by identifying genes linked with disease and quantitative traits, leading to more targeted use of animal models. Importantly, this approach will also help exclude functionally redundant genes and thus will reduce the number of animal experiments needed.

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