PUTTING THE PIECES TOGETHER: ELUCIDATION OF PODOCYTE BIOLOGY IN THE HOMEOSTASIS OF THE KIDNEY FILTRATION BARRIER

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Putting the pieces together: Elucidation of podocyte biology in the homeostasis of the kidney filtration barrier

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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SUMMARY OF THE THESIS

End-stage renal disease (ESRD) is a devastating condition that can only be treated with chronic dialysis or kidney transplantation. ESRD treatment costs account for up to 10% of healthcare budgets in the Western world. The 5-year survival for patients in dialysis is only about 40%. Glomerular disease processes are the main cause of ESRD. Despite this, our basic knowledge on the biology and disease processes of the glomerulus is poor. As a result, we still lack effective therapy options to stop the progression of glomerular diseases.

In this thesis we have identified a number of candidate genes and proteins that could have an essential role in the glomerular homeostasis. In the first project, we identified a group of neural proteins, Hip1, Nfasc and Olfm12, which are enriched in podocytes. We used these markers to provide further evidence that podocytes are present in glomerular crescent lesions that occur in inflammatory diseases of the glomerulus.

In project 2, we studied the functional role of another neural protein, dendrin, in the kidney by generating and characterizing a knockout (KO) mouse line. Previously, we and others have shown that dendrin is very specific to podocytes and interacts with cd2ap and nephrin, two podocyte proteins imperative for the maintenance of the kidney barrier. The KO mouse model showed that dendrin is not needed for the development or maintenance of the glomerulus filtration barrier. Furthermore, the outcome of glomerular disease in two injury models was unaffected by the absence of dendrin. This suggests that dendrin does not have a role in the development of glomerular damage in these two models.

In project 3, we identified Tmem234, Slfn5, Lrrc49 and Znf185 as highly podocyte-enriched molecules. Morpholino knockdown experiments in zebrafish showed that the silencing of Tmem234 results in podocyte foot effacement and proteinuria in pronephros. Thus, Tmem234 seems to have an important role in the formation of functional filtration barrier in zebrafish pronephros, and therefore it is reasonable to speculate that it can have also an important role in the mammalian kidney.

Lastly, in project 4 we identified a mediator protein subunit, Med22, to be essential for the kidney filtration barrier. In zebrafish pronephros, Med22 morphants exhibit defective capillary loop formation and leak large proteins to tubuli. In mice, full KO mice die during embryonic development. In podocyte-specific KO animals kidney development proceeds normally. However, these mice exhibit proteinuria starting from 8 weeks of age that progresses to ESRD by 16 weeks of age. Histological analysis shows the accumulation of caveolin-positive vesicles in the cytoplasm of podocytes. As these vesicles became larger, we detected the loss of podocytes that leads to glomerulosclerosis and ESRD. Thus, Med22 seems to regulate vesicular trafficking in podocytes and be essential for podocyte survival.

This thesis provides novel insights into podocyte biology and obviously opens up new possibilities to study these candidate genes in glomerular function and pathology.
POPULÄRVETENSKAPLIG SAMMANFATTNING


I den första artikeln identifierade vi proteiner som har beskrivits förut så som specifika för nervsystemet, Hip1, Olmf2 och Nfasc - men som också uttrycks i glomeruli. Vi kunde konstatera att i en sjukdomsprocess i glomerulus, och när andra särskilda podocyt proteiner försvinner, finns det fortfarande podocyter i glomeruli. Vår artikel visar att podocyter i glomeruli är kvar längre än tidigare beräknat.


RESUMEN DE LA TESIS (CIENCIA POPULAR)

La enfermedad renal en estado terminal se describe como el fallo renal sin posibilidad de mejora. Las principales causas de enfermedad renal son la hipertensión y la diabetes, ambas en alza a nivel mundial. Menos de la mitad de los pacientes en diálisis sobreviven después de 5 años en tratamiento, todos a la espera de un trasplante de riñón. Por tanto, es importante encontrar tratamientos que ayuden a re-establecer la función renal y a protegerla, para poder evitar la enfermedad renal terminal.

El riñón se compone de dos partes, la médula y la corteza. En la corteza se encuentra la unidad de filtración del riñón. La sangre entra por los capilares, pasa por la barrera de filtración (llamada glomérulo) donde se filtran toxinas, sales y otros elementos hacia el espacio urinario para producir la orina primaria. El glomérulo está compuesto por tres capas, un epitelio con poros, una membrana basal y los podocitos. Los podocitos son células especializadas que no se dividen, y en más del 70% de los casos de fallo renal, la pérdida de podocitos es la responsable de la insuficiencia renal. En esta tesis buscamos entender cómo funcionan los podocitos, qué proteínas expresan y cuáles son importantes para que el podocito, el glomérulo y el riñón funcionen adecuadamente.

En el primer artículo identificamos proteínas que se habían descrito anteriormente como específicas para el sistema nervioso (Hip1, Olmf2 y Nfasc) y que también se expresan en el glomérulo renal. Hemos podido determinar que cuando hay un proceso de enfermedad en el glomérulo, y se pierden proteínas específicas para podocito renal, quedan podocitos en el glomérulo, ya que encontramos Hip1, Olmf2 y Nfasc en glomérulos con daño. Nuestro trabajo demuestra que los podocitos permanecen en el glomérulo más tiempo de lo que antes se pensaba.

En el segundo artículo hicimos un modelo en ratón de una proteína identificada por nuestro grupo anteriormente, Dendrin, que también se consideraba específica del sistema nervioso y que igualmente se expresa en el glomérulo renal. En éste artículo vimos que los ratones sin Dendrin no tienen ningún problema de salud. También vimos que los marcadores específicos de podocitos permanecen intactos y aunque Dendrin interactúa con otras proteínas importantes para el riñón, Dendrin, per se, no es indispensable para el glomérulo renal.


LIST OF PUBLICATIONS


II. Xiao, Z., Rodríguez, PQ., He L., Betsholtz C., Tryggvason T., Patrakka J. Wntp- and Gadd45a-interacting protein dendrin is not crucial for the development or maintenance of the glomerular filtration barrier. PLOS ONE. 2013; 8(12): e83133.


IV. Rodríguez PQ, Jahnukainen T., Guo J., Ebarasi L., Tryggvason K., Patrakka, J. Mediator complex protein 22 regulates vesicular trafficking in podocytes and is essential for the maintenance of the glomerular filtration barrier. Manuscript

Other Publications not included in the thesis


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1 AIMS OF THE THESIS

I. Characterize the expression of neural proteins Nfasc, Hip1 and Olfm2a in the kidney.

II. Develop a KO mouse model for podocyte-enriched protein dendrin and analyze its function in the kidney glomerulus in vivo. Challenge KO mice to determine the role of dendrin in disease models.

III. Characterize the expression and function of Tmem234, Znf185, Lrrc49 and Slfn5 in the kidney.

IV. Develop and analyze a KO mouse line for mediator complex protein Med22 in order to determine its role in the glomerular filtration barrier.
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<td>ACR</td>
<td>Albumin Creatinine Ratio</td>
</tr>
<tr>
<td>BUN</td>
<td>Blood Urea Nitrogen</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>Cas9</td>
<td>CRISPR Associated protein 9</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic Kidney Disease</td>
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<tr>
<td>CNF</td>
<td>Congenital Nephrotic Syndrome of the Finnish Type</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered Regularly Interspaced Short Palindromic Repeats</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>dpf</td>
<td>Days Post-Fertilization</td>
</tr>
<tr>
<td>ES</td>
<td>Embryonic Stem Cell</td>
</tr>
<tr>
<td>ESRD</td>
<td>End-Stage Renal Disease</td>
</tr>
<tr>
<td>FSGS</td>
<td>Focal Segmental Glomerulosclerosis</td>
</tr>
<tr>
<td>Gadd45a</td>
<td>Growth Arrest And DNA Damage Inducible Alpha</td>
</tr>
<tr>
<td>GBM</td>
<td>Glomerular Basement Membrane</td>
</tr>
<tr>
<td>GLOM</td>
<td>Glomerulus</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human Embryonic Kidney Cells 293</td>
</tr>
<tr>
<td>hpf</td>
<td>Hours Post-Fertilization</td>
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<td>IP</td>
<td>Immuno Precipitation</td>
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<td>KD</td>
<td>Knockdown</td>
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<td>KO</td>
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<td>Med22</td>
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<td>NEO</td>
<td>Neomycin</td>
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<tr>
<td>Nfasc</td>
<td>Neurofascin</td>
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<tr>
<td>NZW</td>
<td>New Zealand White (Rabbits)</td>
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<td>Olfml2a</td>
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<td>qPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>ROK</td>
<td>Rest of Kidney: Kidney fraction without glomerulus</td>
</tr>
<tr>
<td>ROF</td>
<td>Rest of Fish without pronephros</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcription Polymerase Chain Reaction</td>
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<tr>
<td>SD</td>
<td>Slit Diaphragm</td>
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<tr>
<td>Slfn5</td>
<td>Schlafen Family Member 5</td>
</tr>
<tr>
<td>T2D</td>
<td>Type 2 Diabetes</td>
</tr>
<tr>
<td>Tmem234</td>
<td>Transmembrane protein 234</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>WT-1</td>
<td>Wilmer’s Tumor 1</td>
</tr>
<tr>
<td>Wtip</td>
<td>Wilms Tumor 1 Interacting Protein</td>
</tr>
<tr>
<td>Znf185</td>
<td>Zinc Finger Protein 185</td>
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2 INTRODUCTION

2.1 THE KIDNEY AND ITS FILTRATION BARRIER

The kidney is our body’s filtration machine. Through the kidney passes approximately 20% of our heart blood volume per minute to be cleaned from toxins and restored with electrolytes. The ultrafiltration occurs through the glomerular capillary wall. This barrier works in a size and charge selective manner. Glomeruli generate about 180 liters of primary urine a day. Most of this filtrate is reabsorbed back into the systemic circulation in the tubular system and we ultimately produce 0.5-2.5L of urine a day.

![Diagram of the kidney](image_url)

Fig. 1. Renal glomeruli are located in the kidney cortex. The three-layered glomerular capillary wall, with endothelial cells, the glomerular basement membrane and podocyte foot processes interconnected by slit diaphragms, forms the ultrafiltration barrier.

The glomerulus filtration barrier is formed of three layers (Fig. 1). The innermost layer is formed of fenestrated glomerular endothelial cells. These cells have fenestrations of 70-100nm in size and their health is dependent on the vascular endothelial growth factor (VEGF). The glomerular endothelium is in charge of synthesizing growth factors for the normal renal function and for this it is in constant interaction with podocytes. The middle layer of the filtration barrier is the glomerular basement membrane (GBM), which is an acellular matrix whose main components are type IV collagens, proteoglycans, laminins and nidogens. (Hudson et al. 1993; Hudson et al. 2003) Mutations in the GBM components are associated with human kidney diseases, such as Alport and Pierson syndrome, highlighting the importance of this structure.

Lastly, the podocyte layer envelops the glomerular capillary loops and forms the final filtration barrier in the glomerulus. Podocytes are specialized epithelial cells that are terminally differentiated. Morphologically, podocytes have prominent cell bodies that lie in the urinary space. From there they send large cytoplasmic extension called...
major processes. These extensions further divide to smaller processes, termed foot processes, closer to the capillary wall. Foot processes attach podocytes to the GBM. Interdigitating foot processes are connected by highly specialized cell-cell junctions called slit diaphragms just above the GBM. (Tryggvason & Wartiovaara 2005)

2.2 PODOCYTE FOOT PROCESSES AND PROTEINURIA

Podocytes are very dynamic cells with a prominent cytoskeleton that can effectively respond to changes in their environment. The cytoskeleton of foot processes is composed mainly of actin filaments. This actin-based machinery is vital for a sustained kidney filtration, as defects in actin-regulating proteins of podocyte foot processes, such as alpha-actinin-4, result in proteinuria. Importantly, the foot process cytoskeleton is reorganized in almost all proteinuric states, the phenomenon known as foot process effacement. The effacement is believed to be a key event in the pathogenesis of proteinuria.

Basally foot processes are attached to the GBM through a number of adhesion molecules, including integrins. In podocytes, heterodimer α3β1 is the most highly expressed integrin. The proper adhesion of podocytes to the GBM is essential for the kidney filter as mutations in α3β1 integrins result in proteinuria and progressive glomerular disease in both man and mouse.

![Fig. 2 Key molecular components of the podocyte: Two interdigitating foot processes are shown emphasizing structural and functional regions: cytoskeletal components, adhesion complexes and the slit diaphragm. Additional gene products include, PGr (prostaglandin receptor) subtypes and podocalyxin, along with its interacting partners NHERF-2 and ezrin. V, vinculin; T, talin; P, paxillin. (Michaud et al. 2007)](image)

2.3 THE SLIT DIAPHRAGM

The slit diaphragm is the highly specialized cell-cell junction of podocytes. It plays a central role in the maintenance of the filtration barrier function. This is highlighted by the fact that mutations in molecular components of the slit diaphragm, such as nephrin and podocin, result in massive proteinuria in both man and mouse. The structure is connected to the actin cytoskeleton of foot processes via a number of
linker molecules, including nck proteins and cd2ap. This link is also essential for the filtration barrier function as KO animals for nck and cd2ap proteins develop massive proteinuria. Dendrin is a cytoplasmic component of the slit diaphragm that interacts directly with nephrin and cd2ap. However, its functional significance in the glomerulus is unknown.

### 2.4 PODOCYTE MAJOR PROCESSES

Major processes are cytoplasmic extensions between podocyte cell body and foot processes. Bundles of microtubules and intermediate filaments support major processes. However, besides microtubules and the intermediate filament protein vimentin, the components that form these cellular projections are largely unknown.

### 2.5 MOUSE MODEL FOR KIDNEY DISEASES

Mice are commonly used as an animal model to study kidney biology and diseases. The physiology of the kidney seems to be very similar between man and mouse. Manipulation of genome is well established in mouse and this makes it often the most obvious choice as an animal model. In addition, there are several well-established disease models in mouse that can be used in studies on kidney disease. However, there are also several drawbacks in using mice as a model. The reproductive cycle in mice is rather long which slows the research down. Additionally, the maintenance of mice is reasonably expensive. The relevance of many disease models in mouse to human disease processes can also be questioned. Another problem is that the genetic background of mice affects kidney disease progression and sensitivity; making the interpretation and standardization of experiments difficult. (Gurley et al. 2006) (Wang et al. 2000).

### 2.6 ZEBRAFISH AS A MODEL ORGANISM

The zebrafish (*Danio rerio*) is a tropical sweet water fish that is very popular as an aquarium fish but also widely used as a vertebrate model organism in research. There are several reasons why zebrafish has become so useful in scientific research:

1) The zebrafish genome has 71.4% gene orthology with the human genome. The orthologs are even higher if we look at disease-causing genes (82%, Mendelian Disease Database).

2) Zebrafish has the same major organs as humans and physiology of these tissues seems to be very similar to that of humans. This conservation is sometimes even better than in mouse (see Figure 3 - representation of electrocardiogram)

3) Zebrafish eggs are fertilized outside the mother’s body. This facilitates studies in early development.

4) Zebrafish embryos are almost transparent that helps researchers to visualize internal structures.

5) Zebrafish are much cheaper to maintain than mice.
6) Zebrafish produce hundreds of offspring at short intervals providing quickly plenty of material for studies.
7) Zebrafish grow and develop fast, speeding up analyses.
8) A large number of drug metabolism pathways are well conserved making it a suitable model for pharmacological studies.
9) Manipulation of gene expression is relatively easy through use of morpholinos.
10) The zebrafish genome has been fully sequenced. This facilitates the genetic manipulation of zebrafish genome.

![Conserved targets](image1.png)
![Conserved drug metabolism](image2.png)
![Conserved physiology](image3.png)
![Conserved pharmacology](image4.png)

*Fig.3 Zebrafish has very similar genome, physiology, drug metabolism and pharmacology compared to humans.* (Barros et al. 2008)

### 2.7 ZEBRAFISH AS A MODEL TO STUDY PODOCYTES

Zebrafish embryos develop quickly with most organs being fully developed at 48 hours post fertilization (hpf). (Drummond et al. 1998) In the case of the kidney, the zebrafish larvae have a pronephros, a single glomerulus formed of two glomeruli that fuse at the midline (Fig. 4).
The pronephros is responsible for the blood filtration and osmoregulation in the zebrafish larvae. It has been estimated that filtration capabilities of pronephros start from about 72 hpf (Drummond 2003). Morphologically, the zebrafish pronephros is very similar to that of mammalian glomerulus. Endothelial cells are fenestrated in the pronephros; podocyte foot processes with slit diaphragms envelope the GBM, identical to that seen in humans. (Fig. 5). Moreover, podocytes in pronephros seem to express the same critical molecules as those in mammalian kidney, including nephrin and podocin. Gene silencing experiments using morpholinos have shown that even their function is conserved between the species as knockdowns of nephrin and podocin in zebrafish larvae results in protein leakage.

2.8 MANIPULATION OF GENE EXPRESSION IN ZEBRAFISH

The use of morpholinos has been the most popular way to manipulate gene expression in zebrafish. Morpholinos are chemically modified oligos with properties similar to those of genetic material. Morpholinos bind and block translation of mRNA \textit{in vitro} and \textit{in vivo}. (Nasevicius & Ekker 2000) When designed properly they are thought to have a limited amount of non-specific effects in silencing other genes. However, the major drawback of them is that one can get only a transient effect on gene expression lasting only up to 2-3 days.

The other popular way to alter gene expression in zebrafish has been the use of genetic mutants. In the past, these mutants were generated for instance by high
throughput N-ethyl-N-nitrosourea (ENU) mutagenesis. More recently, the targeted genetic manipulation has become easier in zebrafish with the introduction of CRISPR-technology (“clustered regularly interspaced short palindromic repeats”). The CRISPR/Cas9 is a type of prokaryotic immune system that confers resistance to foreign genetic material by cutting it. This system is now being exploited by delivering Cas9 nuclease and an appropriate guide RNA into a cell to cut the gene at a desired location. With the methodology one can for instance induce deletions or add genes. The advantage of mutants in comparison to morpholinos is that it is more specific and that one generates a permanent mutant line. This allows more sophisticated mechanistic studies in zebrafish. The major drawback of this methodology is that it is much slower than the use of morpholinos.
3 RESEARCH APPROACH

Following is a summary of the techniques used for the thesis project; *techniques used in subsequent papers are not repeated*. More details about specific procedures, primers and antibodies are available on the published papers and the manuscripts at the end of this thesis.

3.1 NEURONAL PROTEINS IN PODOCYTE MAJOR PROCESSES AND THEIR EXPRESSION IN GLOMERULAR CRESCENTS

Reverse Transcriptase-PCR
The expression of candidate genes in the mouse kidney was studied by RT-PCR using cDNA isolated from glomerular and the kidney fraction devoid of glomeruli. The glomerular isolation was performed as previously described. (Takemoto et al. 2002)

Antibody Generation
To generate polyclonal antibodies against the human Hip1 and Olfm12a, NZW rabbits were immunized with corresponding purified recombinant proteins.

Western Blotting
Lysates of glomerular tufts and the kidneys fractions lacking glomeruli were obtained from human cadaver kidneys unsuitable for transplantation due to vascular abnormalities (from the fourth Department of Surgery of Helsinki, Finland). Glomeruli were isolated using a standard sieving method.

Immunofluorescence
Normal human kidney samples were collected from human cadaver kidneys (as described above). Human fetal kidney samples were from a 20-week old fetus, non-viable due to neural tube defects and hydrocephalus (University Hospital of Helsinki, Finland). Fresh frozen sections of human kidney were fixated with acetone at -20C, and blocked with 5% normal goat or donkey serum prior to staining with the corresponding antibodies.

Immunoelectron microscopy
Tissue sample preparations and immunolabeling experiments were carried out as described previously (Wernerson et al. 2003).

Immunoperoxidase staining
Paraffin-embedded renal biopsies were processed using standard procedures.

3.2 DENDRIN IS NOT CRUCIAL FOR THE GLOMERULAR FILTRATION BARRIER
**Generation of dendrin-deficient mouse line**

Dendrin KO mice were generated using Velocigene technology. Correctly targeted ES line was used to generate chimeric male mice that were then bred to C57BL/6 female to generate N1 mice. The mice used in this study were backcrossed over 5 generations onto the C57BL/6 background.

**Immunohistochemistry and histological analysis**

For histological analysis, mouse kidney samples were fixed in 4 % paraformaldehyde followed by dehydration and embedding in paraffin. Samples for immunohistochemistry were collected by snap-freezing kidneys on dry ice. The dendrin antibody and immunohistochemistry procedure has been described previously (Patrakka et al., 2007).

**Urine analysis**

Urine samples were collected from dendrin KO and control mice for up to 1 year of age. The presence of albuminuria was analyzed by running 2 µl of urine on SDS-PAGE gel stained with Coomassie blue or PAGE-Blue stain.

**Mouse disease models**

We induced glomerular injury and proteinuria in dendrin KO mice by LPS-injection and albumin overload as previously described (Xiao et al., 2011). Each mouse was injected with 13-µg/g of body weight of LPS intraperitoneally. For the albumin overload experiment, we injected 200 µg of BSA in 400µl intraperitoneally on four consecutive days.

Adriamycin induced proteinuria model was established in BALB/c mouse that were injected via the tail vein at a dosage of 10.5 mg/kg body weight as previously described (Wang et al. 2000)

**Yeast two-hybrid assay**

The coding sequence of mouse dendrin was cloned to a bait plasmid pGBKT7, and used to screen a custom-generated mouse kidney glomerulus cDNA library. The yeast two-hybrid (y2h) screening was performed according to the manufacturer’s instructions. The positive clones were sequenced and analyzed with NCBI’s BLAST database.

**Co-immunoprecipitations**

For coimmunoprecipitations, HEK293 cells were co-transfected with full length myc-tagged Gadd45a and flag-tagged dendrin constructs, or full length myc-tagged Wtip and HA-tagged dendrin constructs, respectively. Experiments were performed according to standard procedures.

**Microarray analysis**

Three dendrin-null and three littermate mice at the age of 13 months were used to profile glomerular transcriptomes. Glomeruli were isolated as described previously
and total glomerular RNA was extracted. Microarrays were performed using GeneChip® Mouse Gene 1.0 ST Array.

3.3 GENE SCREEN IDENTIFIES TMEM234 AS AN IMPORTANT COMPONENT OF THE FILTRATION BARRIER

Expression in mouse glomerulus
qPCR was performed using standard procedures using Fast SYBR® Green (Life Technologies).

Pronephric glomerulus isolation in zebrafish
The expression of candidate genes in the zebrafish was studied by RT-PCR using cDNA isolated from pronephric glomerulus and the zebrafish larvae devoid of pronephric glomerulus. Larvae were sacrificed at 96 hpf and the GFP positive glomeruli were microdissected.

Knockdown of genes in zebrafish using morpholino
Zebrafish were housed following European and Swedish animal husbandry and ethical guidelines. Morpholino injections were performed using standard procedures on AB wildtype or podocin-GFP transgenic larvae.

Histological analysis of zebrafish larvae
The histological analysis of zebrafish pronephric glomerulus was performed on JB-4 embedded larvae using PAS staining. Samples were collected at 72hpf and 96hpf and processed according to standard procedures. Transmission electron microscopy was performed on 96hpf larvae fixed with glutaraldehyde following standard procedures.

Pronephric glomerulus quantification
Ten to twelve 96hpf larvae from control and morpholino-injected larvae were histologically processed, stained and imaged. Subsequently the nuclei of each pronephric glomerulus were quantified. Statistical significance was determined using Student’s T-test.

Proteinuria Assay
A total of 35 control and 40 morphant 72hpf larvae were successfully injected into the common cardinal vein with 10kDa rhodamine-labeled dextran and 500kDa FITC-labeled dextran in 0.2M KCl as described before. (Drummond et al. 1998)(Ebarasi et al. 2009) Larvae were taken 16hrs post-injection and fixed as described before. (Ebarasi et al. 2009)

3.4 MED22 IS NEEDED FOR THE MAINTENANCE OF THE GLOMERULUS FILTRATION BARRIER

Med22 full KO and conditional KO analysis
Med22 mice were acquired from EUCOMM in a C57b/6 129Sv background mix, where they were heterozygous for a Laz/Neo cassette. Heterozygous mice for the cassette were bred with Th-IRES (a global cre deleter as described by Lindeberg et al. 2004) mice to generate the full KO allele. In parallel, mice heterozygous for the cassette were bred with a FLP-deleter line to remove the NEO and LacZ portion of the cassette to generate a floxed mouse line. This line was then crossed with podocin-cre to inactivate the gene exclusively in glomerular podocytes.

**Urine analysis**

Albuminuria was analyzed using Exocell’s Albuwell kit and creatinine using Bioassays kit.

**Blood analysis**

Blood urea and albumin values were determined using Abcams Urea and Albumin ELISA kits.

### 3.5 LIMITATIONS AND ADVANTAGES OF TECHNIQUES USED

**Using Protein Atlas antibodies to screen for proteins: Specificity**

We have had the fortune to collaborate with the Human Protein Atlas project (Uhlén et al. 2005) where they generate antibodies for all human proteins. This has been a great advantage when dealing with orphan proteins, since previously (like in project 2) our own antibodies had to be generated. However, the high throughput generation of antibodies has its limitations, as the confirmation of the specificity of antibodies is perhaps not always optimal. In some cases it may be that the antibody binds unspecifically or the antibody exhibits poor versatility to be confirmed in different techniques. We have tried to avoid these problems by confirming results in both mouse and human tissue whenever possible. Also, we have used both immunofluorescence and Western blotting experiments to detect proteins, as well as validated the results at the mRNA level.

**Using morpholinos to silence genes in zebrafish**

Morpholino technology in zebrafish was at the front of gene expression manipulation for years, making it an easy and affordable way to screen for gene candidates in an appropriate *in vivo* model. Recently, the poor correlation between morphant and mutant phenotypes in zebrafish was reported (Kok et al. 2014), raising concerns about off-target effects of morpholinos. However, this study was only limited to the context of early vascular phenotypes. For renal studies, our basic requirement is intact cardiac and vascular function (so that the pronephros can be formed), which means that we discard larvae with defective circulation from our studies in the first two days post-fertilization. Therefore, it is possible that the co-relation between morphants and mutants could be greater in renal morphants than in vascular morphants.
Morpholino-mediated downregulation of genetic transcripts in zebrafish have been of great use in different fields for many years. However, it is obvious that CRISPR technology (“Clustered regularly interspaced short palindromic repeats”) is now getting more popular also in zebrafish studies. This system is now being exploited by delivering Cas9 nuclease and an appropriate guide RNA into a cell to cut the gene at a desired location, this to induce deletions or add genes. When compared to morpholinos, CRISPR technology allows a permanent and more specific inactivation of genes. This comes with a time-price tag, since the larvae with the desired modification have to be screened to see that the mutation went germ-line. For this, zebrafish have to reach reproductive age, and then their offspring have to be screened. So even though fewer off-targets effects are expected, the speed and convenience of the zebrafish model for analyzing renal mutations could now be comparable to that of mice in a timelne. In the scope of this thesis we do not use CRISPR/Cas9 for our studies in zebrafish, although zebrafish mutants for Tmem234 (project 3) and Med22 (project 4) have been generated and in the near future we will start analyzing knockout larvae.
4 RESULTS

Following is a summarized version of the results presented in the projects included in the thesis. For more details, please refer to the respective articles.

4.1 NEURONAL PROTEINS IN PODOCYTE MAJOR PROCESSES AND THEIR EXPRESSION IN GLOMERULAR CRESCENTS

Expression of Hip1, Nfas and Olfml2a in mouse and human kidney

First, we analyzed the expression of Hip1, Nfas and Olfml2a in the mouse kidney by comparing the glomerular fraction to the rest of the kidney. The PCR product for Hip1, Nfas and Olfml2a was only amplified in the mouse glomeruli.

The expression of Hip1, Nfas and Olfml2a was then studied in the human kidney using Western Blotting. In this approach, we detected the corresponding proteins only in glomerular fractions and no signal was seen in kidney fractions lacking glomeruli.

In immunofluorescence, Hip1, Nfas and Olfml2a were detected in the glomeruli and located at the side of the urinary space of nephrin. Double labeling with vimentin showed overlapping immunoreactivity in the major processes.

Immunoelectron microscopy was used to confirm the subcellular localization. Gold label for Hip1 and Olfml2a was observed in major processes of podocytes. Taken together, Nfas, Hip1 and Olfml2a are localized specifically to podocyte major processes.

Expression of Hip1, Nfas and Olfml2a in human kidney development

The two earliest stages of glomerular development, vesicle and S-shaped stages, showed no Hip1 or Nfas expression. In the capillary stage glomerulus, a later stage in where the formation of major and foot processes begins, immunoreactivity was observed for Hip1 and Nfas. At this stage, the staining for these two proteins co-localized with that of vimentin. This subcellular localization suggests an association to developing major processes and supports our findings in the adult kidney.

The expression of Olfml2a during the vesicle stage glomerulus did not show any immunoreactivity. Surprisingly, at the S-shaped stage, Olfml2a presented positive signal in the invading endothelial cells located in the glomerular cleft. In the later stages, more mature endothelial cells did not present any positivity for Olfml2a. Instead, at the capillary stage, a clear signal for Olfml2a was observed in podocytes and this co-localized with that of vimentin. This supports our findings in the adult kidney.

Expression of novel podocyte markers in glomerular crescents

We used our novel podocyte markers to investigate the cellular content of glomerular crescents. Immunoreactivity for Hip1 was observed in 14 of the 26 crescents that were identified in the biopsy materials analyzed. Olfml2a staining was clearly detected in 21 of the 31 glomerular crescents identified. The positive signal for these podocyte markers was detected in less than half of the cells in cellular crescents, and in
these cells, the staining was seen diffusely in the cytoplasm. Anti-Nfasc antibodies did not give any reliable signal.

In order to validate our results, we analyzed the expression of Pdlim2 in glomerular crescents. Pdlim2 is a molecular component of podocyte foot processes, and importantly, Pdlim2 is in the kidney, podocyte-specific (Sistani et al. 2011). We chose Pdlim2 as a marker for podocytes because during podocyte maturation it is expressed earlier than foot process markers nephrin and synaptopodin. In patients with crescentic glomerulonephritis, Pdlim2 presented positive signal in 30 of the 43 glomerular crescents identified. Thus, cells in glomerular crescents showed positivity for three podocyte markers, which strongly supports the idea that podocytes are present in these lesions.

4.2 DENDRIN IS NOT CRUCIAL FOR THE GLOMERULAR FILTRATION BARRIER

Establishment of dendrin-deficient mouse line

Two coding exons of dendrin and the intervening intron were replaced with an eGFP cassette and the elimination of dendrin in homozygous mice was confirmed by immunohistochemistry. No signal for dendrin was observed in +/- mice whereas littermate control mice showed strong dendrin expression in the glomerulus.

Lack of dendrin does not affect glomerular development or barrier function

Previously, others and we have located dendrin specifically to the cytoplasmic side of the podocyte slit diaphragm (Asanuma et al., 2007; Patrakka et al., 2007). Light and electron microscopic examination showed that the glomerulogenesis proceeded normally in dendrin KO mice and mature glomeruli showed normal morphology. Urine analysis of dendrin knockout mice did not show significant albuminuria.

Furthermore, immunostaining patterns for slit diaphragm proteins nephrin and podocin, as well as foot process protein synaptopodin and major process marker vimentin were not altered in dendrin-deficient glomeruli in comparison to controls.

Expressional profiling of dendrin-deficient glomeruli

We performed microarray profiling of glomeruli from dendrin KO mice. As expected, the dendrin expression was downregulated in KO glomeruli. Besides the downregulation of dendrin, the expression profiles showed little changes. No significant expressional changes were detected in well-known podocyte genes, including slit diaphragm genes that have been shown to bind to dendrin.

LPS or BSA-overload induced kidney injury

To study if dendrin has a role in compensatory mechanisms in the glomerulus, we challenged dendrin KO mice with LPS-injection and BSA overload. Both LPS-injection and BSA overload resulted in significant albuminuria but no difference was observed between the dendrin-deficient and control mice.

Wtip and Gadd45a bind to dendrin
To analyze protein-protein interactions of dendrin, we performed a yeast two-hybrid screen. To identify meaningful interactions occurring in the podocyte, we used our own glomerular cDNA library (Sistani et al., 2011) as a prey-library. Two of the identified candidates caught our attention, Wtip and Gadd45a. Wtip has been shown to localize to the slit diaphragm and shuttle to nucleus in injured podocytes, similarly to the previous report for dendrin (Kim et al., 2010). Gadd45a was, on the other hand, significantly upregulated in the dendrin-deficient glomeruli.

The association of dendrin with Gadd45a was confirmed by transfecting HEK293 cells with expression constructs followed by co-immunoprecipitation. Immunoprecipitation of double transfected cells with anti-flag antibody brought down myc-tagged Gadd45a together with flag-tagged dendrin. The other way around, immunoprecipitation with anti-myc antibody co-immunoprecipitated flag-dendrin, whereas the control IP with a non-related expression construct does not bring down flag-dendrin. To get support these results, we analyzed the localization of dendrin and Gadd45a in the glomerulus. In the normal glomerulus, strong immunoreactivity for dendrin was observed as a linear line around capillary loops indicating localization to foot processes. In addition, a weak reactivity in nuclei of podocytes was observed. No dendrin was detected in nuclei of other glomerular cells. Staining for Gadd45a was detected in nuclei of all glomerular cells, including podocytes. Importantly, the staining for dendrin in podocyte nuclei overlapped with that of Gadd45a. In adriamycin-induced nephropathy, immunoreactivity for dendrin in nucleus was increased as 95% (124/133 in 10 glomeruli) of podocyte nuclei showed reactivity for dendrin.

We confirmed the interaction between dendrin and Wtip using co-immunoprecipitation experiments. We transfected HEK 293 cells with HA-tagged dendrin and myc-tagged Wtip. Dendrin co-immunoprecipitated with Wtip in double-transfected cells, whereas Wtip was not detected in the control immunoprecipitation. To get support for these results, we tried to perform immunostaining experiments with various Wtip antibodies. However, in our hands, no reliable signal for Wtip was detected in immunohistochemical experiments and thus no colocalization studies could be made with dendrin.

4.3 GENE SCREEN IDENTIFIES TMEM234 AS AN IMPORTANT COMPONENT OF THE FILTRATION BARRIER

RT-PCR and immunofluorescence analysis identifies Tmem234, Znf185, Lrrc49 and Slfn5 as new molecular components of the podocyte

RT-PCR and qPCR experiments showed that all four transcripts were enriched in the glomerulus in comparison to the kidney fraction lacking glomeruli. The amplification of the Tmem234 transcripts gave significant signal also in the kidney fraction lacking glomeruli, whereas other transcripts gave only minimal or no signal in the non-glomerular fraction.

Staining of adult human kidney sections with antibodies directed against Tmem234, Znf185, Lrrc49, and Slfn5 gave strong glomerular immunoreactivity, and only weak signals were detected in rest of the kidney. Double labeling with nephrin, a foot process marker showed overlapping reactivity for Tmem234, Znf185, Lrrc49 and
Slfn5. Staining with major process marker vimentin showed colocalization only with Znf185.

Expression during glomerular development

To validate the association of these proteins with podocytes, we analyzed their expression pattern during glomerulogenesis. Staining of human fetal kidney sections gave strong immunoreactivity in developing podocytes. The expression of all four proteins was first detected at the capillary stage glomerulus. At this stage the formation of foot and major processes begins and the earliest expression of podocyte markers, such as synaptotopin and podocin, is observed. (Nagata et al. 1998; Mundel et al. 1997)

At the capillary stage, Tmem234, Znf185, Lrrc49 and Slfn5 were colocalizing with nephrin at the basal aspects of pre-podocytes. Znf185, on the other hand, colocalized also with vimentin during podocyte development. To summarize, studies in glomerulogenesis confirmed the association of Tmem234, Lrrc49 and Slfn5 to foot processes, as well as the association of Znf185 to both foot and major processes.

Zebrafish orthologs and their expression

Zebrafish was chosen as a model to study the functional role of new proteins in the glomerular filter. For Tmem234 and Znf185 orthologs were identified, whereas no zebrafish orthologs were found for Slfn5 and Lrrc49. Therefore, no further studies were performed with Slfn5 and Lrrc49 in zebrafish.

The pronephric glomerulus expression of Tmem234 and Znf185 was confirmed using RT-PCR and qPCR. Strong expression for both genes was detected in the pronephric glomerulus, whereas weaker or no signal was observed in the zebrafish fraction lacking glomerulus.

Knockdown of zebrafish orthologues

Morpholinos were designed to knockdown the expression of Tmem234 and Znf185 orthologs in zebrafish. To assess whether knockdown affected the pronephric glomerulus the presence of GFP expression in transgenic podocin-GFP larvae was analyzed. A decreased GFP expression in a living fish was considered as a sign of podocyte and pronephric glomerulus injury.

The experiments showed successful downregulation of gene expression for Tmem234 and Znf185. Macroscopically, Tmem234 morphants exhibited mild pericardial edema at 72 hpf that progressed slightly by 96 hpf in comparison with control larvae. GFP-positivity in pronephric podocytes was often lost in the morphants. Light microscopy examination of morphants confirmed the pronephric glomerulus abnormalities as an enlarged Bowman’s space with a disorganized glomerular architecture was observed. The absence of podocyte-GFP expression in Tmem234 morphants suggested that their pronephric glomerulus and podocytes were affected. Although 48% Znf185 morphants presented slight to moderate pericardial edema, podocin-GFP expression in Znf185 morphants was unaffected. In the pronephric glomerulus, a minimal enlargement of the Bowman’s space was evident but the capillary tuft appeared normal.
Analysis of Tmem234 morphants

As Tmem234 morphant fish exhibited obvious pronephric glomerulus abnormalities, we analyzed the morphants in more detail. We co-injected Tmem234 morpholino with a mouse Tmem234 mRNA. GFP expression was partially rescued with the mouse mRNA as 81% of embryos co-injected with Tmem234 mRNA expressed GFP. This experiment indicated that the loss of GFP was due to the knockdown of Tmem234.

The histological analysis was complemented by counting manually the number of cells in pronephric glomeruli. This showed a significant reduction in the number of pronephric glomerular cells in Tmem234 morphants.

Tmem234 morphants were also evaluated in electron microscopy; foot processes effacement was evident in morphant pronephric glomerulus. The slit diaphragms interconnecting remaining foot processes, the glomerulus basement membrane and endothelial cells appeared unaffected.

To elucidate whether abnormal pronephric glomerulus structure in Tmem234 morphants had functional consequences to the filtration barrier, we injected zebrafish larvae with FITC-labeled 500kDa dextran into the common cardinal vein. In wild type zebrafish, only 10kDa dextran was detected in the tubuli. In contrast to this, FITC-labeled 500 kDa dextran was detected in the tubuli of Tmem234 morphants. Thus, knockdown of Tmem234 in zebrafish compromised the functional integrity of the pronephros filtration barrier.

4.4 MED22 IS NEEDED FOR THE MAINTENANCE OF THE GLOMERULUS FILTRATION BARRIER

Med22 is expressed in the kidney glomerulus and pronephros

The expression of Med22 was analyzed using RT-PCR that showed it to be ubiquitously expressed in all tissues tested in both human and mouse. In the kidney, however, the expression of Med22 was enriched in the human and mouse glomerulus as compared to the kidney fraction devoid of glomeruli. In situ hybridization of zebrafish larvae showed expression in the zebrafish pronephros. Staining of normal human kidney with Med22 showed staining in the cytoplasm of cell nucleus and at the major processes of podocytes. Double staining with the slit diaphragm marker nephrin did show any colocalization.

Knockdown of Med22 in zebrafish produces pronephros abnormalities and proteinuria

We knocked down Med22 in podocin-GFP zebrafish. This resulted in pericardial edema and tubular edema. Interestingly, Med22 morphants did not lose their GFP expression. Instead, these morphants showed defective capillary loop formation. Histologically, we observed that the GFP positive cells, as seen in the gross morphology, concentrated in the midline of the zebrafish larvae. In electron microscopy, a reduced number of podocytes was detected. Remaining podocytes had some remnant foot processes and slit diaphragms. Functionally, Med22 larvae leaks...
large proteins into proximal tubules as detected by the leakage of fluorescently labeled 500kDa dextran molecules.

**Mice lacking Med22 in podocytes develop progressive kidney disease**

The global Med22 KO mice die during embryonic development. Mice where Med22 was specifically inactivated in podocytes (Med22PodKO) developed normally and followed Mendelian distribution in their genotypes. During the first 10 weeks they were comparable with their littermates in size and weight. However, albuminuria was detected in few Med22PodKO animals at 8 weeks of age, and this progressed later to massive albuminuria in all PodKO animals. Hematuria was also detected in most PodKO animals. Blood urea nitrogen levels were normal at 8 weeks of age, but increased progressively from 12 weeks of age and all Med22PodKO mice died by 18 weeks of age due to renal failure.

**Histological analysis of Med22PodKO animals shows formation of vesicles in podocytes**

Kidney histology appeared normal in Med22PodKO mice at 8 weeks of age at light microscopic level. In electron microscopic evaluation, few mice exhibited mild foot process effacement at 8 weeks of age. Occasionally, we found enlarged vesicles in podocytes.

At 12 weeks of age, PodKO animals showed severe morphological abnormalities in glomeruli. Podocytes exhibited large vesicles that were detected in both light and electron microscopy. Often these vesicles appeared to be larger than podocyte nuclei. In light microscopy, these vesicles appeared empty and they were negative in oil-red staining (lipid staining, data not shown). In electron microscopy, these vesicles appeared in most cases empty. Occasionally, we observed an accumulation of electron-dense vesicles in the cytoplasm of podocytes.

Besides podocyte vesicles, we detected focal segmental glomerulosclerosis at 12 weeks of age. This was probably secondary to podocyte loss. In addition, tubuli were often dilated and protein casts were detected as a sign of massive albuminuria. These changes progressed and at 16 weeks of age the global glomerulosclerosis was obvious along with advanced secondary changes in tubuli.

**Immunofluorescence studies in Med22PodKO mice**

Immunofluorescence for podocyte foot process markers nephrin, podocin and synaptopodin gave continuous linear staining pattern along glomerular capillary loops in Med22PodKO and control mice at 8 weeks of age. Wt1, a marker for podocyte nuclei, showed also normal number of podocytes at 8 weeks of age. However, at 12 weeks of age, the staining for all podocyte foot process proteins had nearly completely disappeared. Only few Wt1 positive podocytes were detected in knockout glomeruli indicating a significant loss of podocytes. At 16 weeks of age, no foot process markers were detected and wt1 positive nuclei were only rarely detected.

Next, we analyzed the molecular nature of podocyte vesicles in Med22PodKO mice. Immunofluorescence staining for 12 week old kidneys showed significant upregulation of endosomal marker caveolin while clathrin, marker for clathrin-mediated
endocytosis, was not upregulated. Lysosomal marker LAMP2 was also clearly upregulated in knockout glomeruli. Thus, podocytes vesicles seemed to be originating from caveolin-mediated endocytosis pathway.

**RNAseq confirms downregulation of podocyte markers preceeding proteinuria.**

We performed RNA sequencing on glomeruli isolated from 8-week old Med22PodKO mice. The sequencing revealed 126 differentially expressed genes of which 78 were up- and 48 down regulated. Known genes involved in endosomal and lysosomal trafficking were not differentially expressed. However, we detected the downregulation of podocyte markers podocin and synaptopodin that was in line with our immunofluorescence data.

### 4.5 NEGATIVE RESULTS

In science, negative results often remain unpublished. In my thesis work, I many times ended up with negative results. The reasons for this can be many: redundant or synonymous proteins, compensation mechanisms, lack of tools for detection or optimization, etc.

In the original thesis plan, the purpose was to study both Hip1 and Nfasc KO mouse lines. Hip1 KO mice were published in 2007 (Khatchadourian et al. 2007) and were brought into the lab to analyze their kidneys. Similarly, Nfasc mice were also imported to our lab. Neither of these mouse lines exhibited any obvious kidney phenotype (data not shown).

During my thesis work we identified another new podocyte-enriched molecule, Tmeff2 (data not shown). We imported a mouse line for Tmeff2 to our lab but this turned out to be wrongly targeted. Then, a group published a knockout model for Tmef22 (Chen et al. 2012). Tmeff2-deficient mice were smaller in size and died soon after weaning. We imported also this mouse line to our lab but it never showed an obvious kidney phenotype, even after backcrossing to the 129Sv background, which increased their survival from 4 to 7 weeks, but no obvious anomalies where found.
5 DISCUSSION

5.1 HIP1, NFASC AND OLFML2A IN PODOCYTES

We identified Hip1, Nfasc and Olfml2a as new molecular components of podocyte major processes. We used these new markers to validate previous studies (made mostly in mouse) that show that podocytes contribute to the formation of glomerular crescents. We did not include any functional studies in the published work. However, we followed up this study by importing Hip1 and Nfasc KO animals to our lab. Neither of these mice exhibited any obvious renal phenotype indicating that Hip1 and Nfasc are not essential for the development of normal kidney filtration barrier.

5.2 DENDRIN IN PODOCYTES

We discovered dendrin through a large-scale microarray screen in which over 300 glomerulus-enriched transcripts were identified. (Takemoto et al. 2006). Shortly after, we showed that dendrin had a very restricted expression as we detected it only in the brain and the kidney glomerulus (Patrakka et al. 2007). In the glomerulus, we localized dendrin to the cytoplasmic face of the slit diaphragm. Furthermore, we observed that in patients with minimal change nephrotic syndrome, dendrin was redistributed from the slit diaphragm region to the podocyte cytoplasm (Dunér et al. 2008). These findings motivated us to generate a KO mouse line for dendrin. Our studies in dendrin-deficient mice indicated that dendrin is not essential for the normal development or maintenance of the kidney filtration barrier.

Recently Weins et al. reported that double knockout animals for Cd2ap and dendrin survived longer than Cd2ap KOs. In the study, the absence of dendrin delayed the onset and severity of proteinuria, mesangial expansion and podocyte loss. (Weins et al. 2015). Thus, in this genetic model of podocyte disease, dendrin seemed to have injury promoting effects.

In our study, we also showed that dendrin can interact with Wtip and Gadd45a. Both of these have been shown to mediate gene transcription in the nucleus. These results are well in line with a study reporting that dendrin can relocate to nucleus in response to glomerular injury and promote podocyte apoptosis, thus adding to the injury-promoting role of dendrin. (Campbell et al. 2013)

5.3 ZEBRAFISH SCREEN IDENTIFIES SEVERAL CANDIDATE GENES

Morpholino-based gene silencing is a quick way to knock-down genes in zebrafish and with the recent development of a GFP transgenic for podocyte-specific protein podocin (He et al. 2011), we decided to use the zebrafish model as a screening tool. In the project 3, we observed that silencing of a new podocyte protein Tmem234 in zebrafish resulted in proteinuria and foot processes effacement, as well as loss of podocyte GFP expression. Morpholino experiments in zebrafish have yielded even other candidates that our group is currently working on in mouse models (data not shown). In addition, zebrafish knockdown of Med22 was critical for its identification as a candidate for mouse KO studies. This emphasizes the importance of the zebrafish
model. It seems that many critical genes have a conserved function in zebrafish and therefore this model is useful for studying human biology.

Recently, Kok et al. reported that only less than 20% of the phenotypes found in morpholino knockdowns in zebrafish could be reproduced with mutants. In our hands, so far, the renal phenotypes have correlated with morphants, but we are in the process of developing mutants for several genes that we have generated morpholino knockdowns, among them, Med22 and Tmem234. It is important to elucidate the correlation of morphant to mutant phenotypes in order to make a correct interpretation of these models. I feel that although CRISPR technology facilitates the high throughput generation of mutants, there is still space for the use of morpholinos, at least in the renal field, due to the quickness of morphant models.

5.4 MEDIATOR COMPLEX AND MED22

Lastly, we identified a mediator complex subunit that is imperative for the homeostasis of the kidney filtration barrier. The mediator complex is a huge complex (1.2 MDa for the human mediator complex) composed of about 30 subunits. It is divided into 4 sub-structures, the CDK8 module, the middle module, the head and the tail. Of the 10 subunits knocked out in mouse (www.mousephenotype.org search July 2016), 9 are embryonically lethal. The heterozygous deletion of a subunit can also result in pathology. For instance, haploinsufficiency for Med13/Med13L results in transposition of the great arteries. (Muncke et al. 2003; Reza Asadollahi 2013; Sato et al. 2004)

It is no doubt that the mediator complex is critical for the normal function of cells. However, it is still necessary to elucidate exactly what subunits are expressed in each tissue and under what conditions each subunit functions. Knocking out each subunit does not result in the same phenotype in hypomorphic conditions, so it is probable that there are different interactions with particular subunits in specific cellular processes.

Med22 is part of the highly conserved Surfeit locus complex (Surf1-6)(Garson et al. 1995). It is ubiquitously expressed consistent with being a housekeeping gene. (Angiolillo et al. 2002) Our results confirm this, as Med22 deletion in all cells in mouse results in an embryonic lethality. Although embryology is not the focus of this thesis, it could be that elucidating the cause for embryonic death will give important insights into how Med22 works in cells.

As we detected Med22 to be enriched in podocytes, we generated a podocyte-specific KO mouse. The deletion of Med22 in podocytes does not affect the development of the glomerulus but results in accumulation of cytoplasmic vesicles in adult mice. These vesicles get larger during age and are likely the cause of concomitantly observed podocyte loss. Following loss of podocytes, a process of glomerulosclerosis initiates leading to ESRD and death by 18 weeks of age.

We have seen that vesicles in podocytes are positive for caveolin suggesting that they originate from the endocytic pathway. Normally, endocytic vesicles are either directed to lysosomes and degradation or to be recycled. In the absence of Med22, the vesicular trafficking seems to fail. We do not yet understand the mechanism behind it but with further experiments, both in vivo and in vitro, we hope elucidate this interesting phenomenon.
6 CONCLUSION

I Hip1, Nfasc and Olfm12a are new molecular components of podocyte major processes.

II Dendrin is not imperative for the development or maintenance of the glomerular filtration barrier

III Tmem234 is essential for the zebrafish pronephric filtration barrier function.

IV Med22 is essential for the maintenance of the glomerulus filtration barrier.
7  FUTURE PERSPECTIVES

7.1 NEURAL PROTEINS IN PODOCYTES

Dendrin, Hip1, Olfm12a and Nfasc are neural proteins that we have discovered to be expressed by podocytes. We have analyzed the biological role dendrin, Hip1 and Nfasc in KO animals and results indicate that they are not essential for the development or maintenance of the glomerulus filtration barrier. This was somewhat disappointing as a lot of efforts were made to generate and analyze these lines. Obviously, these studies do not exclude the possibility that these genes would have pathogenic roles in the development of glomerular disease processes. In fact, we have collaborated with Dr Kirk Cambell’s group and they published recently a paper showing that dendrin-deficiency slows the progression of glomerular disease in Cd2ap KO animals. Previously, dendrin has been reported to promote apoptotic signaling in podocytes. It would be interesting to study in more detail the role of dendrin in acquired kidney diseases. Additionally, challenging dendrin KO animals with different disease models, like inducing diabetes and analyzing how the nephropathy progresses in these animals may help elucidate if dendrin plays a pathogenic role in disease models in which podocyte apoptosis occurs. Also, more detailed analyses of expression and subcellular localization in common acquired glomerular diseases are needed. Perhaps dendrin is relocated to nucleus in some diseases and promote there pro-apoptotic signaling? Similarly, analogous studies with Hip1 and Nfasc PodKO animals or double KO/PodKO with other relevant genes might help explain why these proteins are so podocyte enriched

7.2 ZEBRAFISH AND TMEM234

In project 3 we present poorly characterized proteins that are highly enriched in podocytes. We studied further the candidates in zebrafish using morpholinos. However, in this project we did not pursue the proteins that did not have a zebrafish ortholog. It could be interesting to study these candidates (Lrcc49 and Slfn5) in more detail in mammalian models.

As recent study reported a discrepancy between morpholino and mutant phenotypes in zebrafish, we have started a project to knock out in zebrafish several known proteins critical for the filtration barrier, like Nphs1 and Nphs2, but also Tmem234. It will be interesting to see whether for instance Nphs1/Nphs2 mutants exhibit a similar phenotype to corresponding morphants. The use of mutants provides us a more stable model to study in more detail molecular mechanisms in zebrafish.

7.3 THE MEDIATOR COMPLEX AND KIDNEY BIOLOGY

We describe for the first time a role for a component of the mediator complex in the kidney. Although Med22 is clearly essential for the maintenance of the filtration barrier homeostasis, many open questions remain. First, we should elucidate the
specific mechanisms behind vesicle formation in Med22PodKO mice and pinpoint the specific pathway behind it. More importantly, could we figure out a target to reverse or delay this phenotype? Another obvious experiment would be to delete Med22 in another cell type in the kidney, would that give a similar pathology or is the vesicle formation something specific to podocytes, and what can that tell us about Med22’s role in cell survival and homeostasis.
8 ACKNOWLEDGEMENTS

First and foremost I would like to thank my supervisor, Jaakko Patrakka for believing in me at a time when I had lost all faith in science. I am sure we both agree that it was a risk worth taking. I appreciate that you share my curiosity for the unknown so you let me take risks and follow my ideas. THANK YOU!

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Thanks to those at ICMC that have also helped me: Bora and Malin, I couldn’t get most of the data seen on my 4th project, if it weren’t for you. Thanks for your invaluable help. Thanks to Christina and Agata from the Department of Laboratory Medicine, you have barely met me and still helped me, your help is not seen in the 4th manuscript, but it has helped us greatly, it will show. Thanks again!

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My mentor: **Lotte**, who always believed in me, even when I came as a clueless child to her science arms. I would have stayed with you just for the joy that was working with you. As a team we worked beautifully! You are like a second mom: Thanks for all the dinners and lunches, for the science advice, and lately, motherhood. Thanks for helping with the Swedish summary of this thesis.

My dear friends **Sara** and **Alecia** we started working together and the beers have become dinners or tea and always really interesting talks. Thanks for taking me in when I first came to Sweden. A shout out to **Masako**, thanks for all the great dinners and interesting talks, it was nice to have someone to talk about anything or nothing, you are a truly special person.

All those that I shared on my spared time, but now I can’t recall your name, sorry! I have your faces in my heart.

I tried to remember everyone, but if I did not mention you, I am sorry!

Now, a few words to my Spanish-speaking tribe, *because we all need a tribe.*

Muchos están lejos, pero aún así han logrado darme apoyo desde la distancia o me han recibido en sus hogares cuando posiblemente mi cerebro estaba en huelga de hambre con tanto trabajo. Estar con ustedes y poder hablar de lo que siempre hablamos me ayudó a poner mi trabajo en perspectiva y poder continuar en esta lucha, los quiero: **Fernando y Norma.**

A los de Karolinska, **Ilais, Florencia y Germán**. Gracias por todos los cafés y las cervezas, cuando hacían falta los abrazos y los regaños. **Cecilia**, la vida nos va alejando y acercando a su antojo. ¡A por el futuro! **Óscar** gracias por la ayuda con el resumen en español de la tesis. ¡A disfrutar Suecia y la vida en Suecia! **Arancha**: será una aventura ver lo que el futuro tiene para nosotras. Gracias por todo: las cenas, las charlas, los juegos, los viajes. **Spyros** y tu son de mis parejas favoritas. ¿*Me adoptan?*
Obviamente no puedo dejar de agradecer a mi familia. Los quiero a todos, a mis hermanos, Celso, Férnan y Andrés. A mi mamá, Zaida, que me distrae con todo lo que pasa en Puerto Rico. Me gusta saber que todavía soy parte de la familia, que no estoy aquí tan lejos, y de que alguien piense que todo sería mejor si estuviera en Puerto Rico. Pero también a mis tías, Titi Gloria, Titi Geli y Titi Mayi (QEPD), porque con su sabiduría siempre, sin darse cuenta, me ayudan con mis dudas. A mi abuela, Patria, que siempre tiene un buen consejo sobre la vida, y me transporta a esos tiempos ahora solo en libros, y me permite ver que lo que tengo es precioso y que la mujer que soy está en el momento perfecto.

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Finalmente a mi papá, Celso, porque sin él jamás estaría donde estoy. Trabajar contigo me preparó para el mundo exigente de la ciencia. Si no fuera porque me enseñaste a estar en movimiento, nunca podría haber tenido el impetu de seguir empujando, a veces más lento y a veces más rápido, pero nunca detenerme. Gracias a ti soy una mujer cabal, que puede arreglar un carro, que pudo poner bombillas y arreglar equipos electrónicos en el laboratorio, e incluso poder usar una segueta (¡En el laboratorio!) sin problemas. Gracias por dejarme seguir mis sueños, aunque no estabas de acuerdo. Y gracias que cuando dudé me empujaste a seguir adelante, aunque significara unos años más sin mí. Creo que recuerdas cada llamada, desde la primera en CROEM, ¿te acuerdas?

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Y a mi peque, Miri, que no lo sabe pero me ha enseñado que el trabajo no lo es todo, que tengo que ser efectiva porque la vida es corta y ella crecerá muy rápido. Gracias porque siempre quiero volver a casa para estar contigo y papá. Mami te ama.
9 REFERENCES


Available at:


10 RESEARCH ARTICLES
PUTTING THE PIECES TOGETHER: ELUCIDATION OF PODOCYTE BIOLOGY IN THE HOMEOSTASIS OF THE KIDNEY FILTRATION BARRIER

Patricia Mª Rodríguez Rivera

Stockholm, Sweden 2016
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**On the Cover:** *A mouse glomerulus, isolated and in culture for 96hrs. Picture and experiment by Patricia Mª Rodríguez Rivera*

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Putting the pieces together: Elucidation of podocyte biology in the homeostasis of the kidney filtration barrier

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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SUMMARY OF THE THESIS

End-stage renal disease (ESRD) is a devastating condition that can only be treated with chronic dialysis or kidney transplantation. ESRD treatment costs account for up to 10% of healthcare budgets in the Western world. The 5-year survival for patients in dialysis is only about 40%. Glomerular disease processes are the main cause of ESRD. Despite this, our basic knowledge on the biology and disease processes of the glomerulus is poor. As a result, we still lack effective therapy options to stop the progression of glomerular diseases.

In this thesis we have identified a number of candidate genes and proteins that could have an essential role in the glomerular homeostasis. In the first project, we identified a group of neural proteins, Hip1, Nfasc and Olfml2, which are enriched in podocytes. We used these markers to provide further evidence that podocytes are present in glomerular crescent lesions that occur in inflammatory diseases of the glomerulus.

In project 2, we studied the functional role of another neural protein, dendrin, in the kidney by generating and characterizing a knockout (KO) mouse line. Previously, we and others have shown that dendrin is very specific to podocytes and interacts with cd2ap and nephrin, two podocyte proteins imperative for the maintenance of the kidney barrier. The KO mouse model showed that dendrin is not needed for the development or maintenance of the glomerulus filtration barrier. Furthermore, the outcome of glomerular disease in two injury models was unaffected by the absence of dendrin. This suggests that dendrin does not have a role in the development of glomerular damage in these two models.

In project 3, we identified Tmem234, Slfn5, Lrrc49 and Znf185 as highly podocyte-enriched molecules. Morpholino knockdown experiments in zebrafish showed that the silencing of Tmem234 results in podocyte foot effacement and proteinuria in pronephros. Thus, Tmem234 seems to have an important role in the formation of functional filtration barrier in zebrafish pronephros, and therefore it is reasonable to speculate that it can have also an important role in the mammalian kidney.

Lastly, in project 4 we identified a mediator protein subunit, Med22, to be essential for the kidney filtration barrier. In zebrafish pronephros, Med22 morphants exhibit defective capillary loop formation and leak large proteins to tubuli. In mice, full KO mice die during embryonic development. In podocyte-specific KO animals kidney development proceeds normally. However, these mice exhibit proteinuria starting from 8 weeks of age that progresses to ESRD by 16 weeks of age. Histological analysis shows the accumulation of caveolin-positive vesicles in the cytoplasm of podocytes. As these vesicles became larger, we detected the loss of podocytes that leads to glomerulosclerosis and ESRD. Thus, Med22 seems to regulate vesicular trafficking in podocytes and be essential for podocyte survival.

This thesis provides novel insights into podocyte biology and obviously opens up new possibilities to study these candidate genes in glomerular function and pathology.
POPULÄRVETENSKAPLIG SAMMANFATTNING


I den första artikeln identifierade vi proteiner som har beskrivits förut så som specifika för nervsystemet, Hip1, Olmf2 och Nfasc - men som också uttrycks i glomeruli. Vi kunde konstatera att i en sjukdomsprocess i glomerulus, och när andra särskilda podocyt proteiner försvinner, finns det fortfarande podocyter i glomeruli. Vår artikel visar att podocyter i glomeruli är kvar längre än tidigare beräknat.


RESUMEN DE LA TESIS (CIENCIA POPULAR)

La enfermedad renal en estado terminal se describe como el fallo renal sin posibilidad de mejoría. Las principales causas de enfermedad renal son la hipertensión y la diabetes, ambas en alza a nivel mundial. Menos de la mitad de los pacientes en diálisis sobreviven después de 5 años en tratamiento, todos a la espera de un trasplante de riñón. Por tanto, es importante encontrar tratamientos que ayuden a re-establecer la función renal y a protegerla, para poder evitar la enfermedad renal terminal.

El riñón se compone de dos partes, la médula y la corteza. En la corteza se encuentra la unidad de filtración del riñón. La sangre entra por los capilares, pasa por la barrera de filtración (llamada glomérulo) donde se filtran toxinas, sales y otros elementos hacia el espacio urinario para producir la orina primaria. El glomérulo está compuesto por tres capas, un epitelio con poros, una membrana basal y los podocitos. Los podocitos son células especializadas que no se dividen, y en más del 70% de los casos de fallo renal, la pérdida de podocitos es la responsable de la insuficiencia renal.

En esta tesis buscamos entender cómo funcionan los podocitos, qué proteínas expresan y cuáles son importantes para que el podocito, el glomérulo y el riñón funcionen adecuadamente.

En el primer artículo identificamos proteínas que se habían descrito anteriormente como específicas para el sistema nervioso (Hip1, Olmf2 y Nfasc) y que también se expresan en el glomérulo renal. Hemos podido determinar que cuando hay un proceso de enfermedad en el glomérulo, y se pierden proteínas específicas para podocito renal, quedan podocitos en el glomérulo, ya que encontramos Hip1, Olmf2 y Nfasc en glomérulos con daño. Nuestro trabajo demuestra que los podocitos permanecen en el glomérulo más tiempo de lo que antes se pensaba.

En el segundo artículo hicimos un modelo en ratón de una proteína identificada por nuestro grupo anteriormente, Dendrin, que también se consideraba específica del sistema nervioso y que igualmente se expresa en el glomérulo renal. En éste artículo vimos que los ratones sin Dendrin no tienen ningún problema de salud. También vimos que los marcadores específicos de podocitos permanecen intactos y aunque Dendrin interactúa con otras proteínas importantes para el riñón, Dendrin, per se, no es indispensable para el glomérulo renal.


LIST OF PUBLICATIONS


II. Xiao, Z., Rodríguez, PQ., He L., Betsholtz C., Tryggvason T., Patrakka J. Wtip- and Gadd45a-interacting protein dendrin is not crucial for the development or maintenance of the glomerular filtration barrier. PLOS ONE. 2013; 8(12): e83133.


IV. Rodríguez PQ, Jahnukainen T., Guo J., Ebarasi L., Tryggvason K., Patrakka, J. Mediator complex protein 22 regulates vesicular trafficking in podocytes and is essential for the maintenance of the glomerular filtration barrier. Manuscript

Other Publications not included in the thesis


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1 AIMS OF THE THESIS

I. Characterize the expression of neural proteins Nfasc, Hip1 and Olfm2a in the kidney.

II. Develop a KO mouse model for podocyte-enriched protein dendrin and analyze its function in the kidney glomerulus *in vivo*. Challenge KO mice to determine the role of dendrin in disease models.

III. Characterize the expression and function of Tmem234, Znf185, Lrrc49 and Slfn5 in the kidney.

IV. Develop and analyze a KO mouse line for mediator complex protein Med22 in order to determine its role in the glomerular filtration barrier.
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACR</td>
<td>Albumin Creatinine Ratio</td>
</tr>
<tr>
<td>BUN</td>
<td>Blood Urea Nitrogen</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>Cas9</td>
<td>CRISPR Associated protein 9</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic Kidney Disease</td>
</tr>
<tr>
<td>CNF</td>
<td>Congenital Nephrotic Syndrome of the Finnish Type</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered Regularly Interspaced Short Palindromic Repeats</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>dpf</td>
<td>Days Post-Fertilization</td>
</tr>
<tr>
<td>ES</td>
<td>Embryonic Stem Cell</td>
</tr>
<tr>
<td>ESRD</td>
<td>End-Stage Renal Disease</td>
</tr>
<tr>
<td>FSGS</td>
<td>Focal Segmental Glomerulosclerosis</td>
</tr>
<tr>
<td>Gadd45a</td>
<td>Growth Arrest And DNA Damage Inducible Alpha</td>
</tr>
<tr>
<td>GBM</td>
<td>Glomerular Basement Membrane</td>
</tr>
<tr>
<td>GLOM</td>
<td>Glomerulus</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human Embryonic Kidney Cells 293</td>
</tr>
<tr>
<td>hpf</td>
<td>Hours Post-Fertilization</td>
</tr>
<tr>
<td>IP</td>
<td>Immuno Precipitation</td>
</tr>
<tr>
<td>KD</td>
<td>Knockdown</td>
</tr>
<tr>
<td>KO</td>
<td>Knock-out (Gene)</td>
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<tr>
<td>LPS</td>
<td>Lipopolysacharide</td>
</tr>
<tr>
<td>Lrrc49</td>
<td>Leucine Rich Repeat Containing 49</td>
</tr>
<tr>
<td>Med22</td>
<td>Mediator Complex Subunit 22</td>
</tr>
<tr>
<td>NEO</td>
<td>Neomycin</td>
</tr>
<tr>
<td>Nfasc</td>
<td>Neurofascin</td>
</tr>
<tr>
<td>NZW</td>
<td>New Zealand White (Rabbits)</td>
</tr>
<tr>
<td>Olfml2a</td>
<td>Olfactomedin Like 2A</td>
</tr>
<tr>
<td>Pdlim2</td>
<td>PDZ And LIM Domain 2</td>
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<td>PodKO</td>
<td>Podocyte Specific Knock Out</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>ROK</td>
<td>Rest of Kidney: Kidney fraction without glomerulus</td>
</tr>
<tr>
<td>ROF</td>
<td>Rest of Fish without pronephros</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcription Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SD</td>
<td>Slit Diaphragm</td>
</tr>
<tr>
<td>Slfn5</td>
<td>Schlafen Family Member 5</td>
</tr>
<tr>
<td>T2D</td>
<td>Type 2 Diabetes</td>
</tr>
<tr>
<td>Tmem234</td>
<td>Transmembrane protein 234</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>WT-1</td>
<td>Wilmer’s Tumor 1</td>
</tr>
<tr>
<td>Wtip</td>
<td>Wilms Tumor 1 Interacting Protein</td>
</tr>
<tr>
<td>Znf185</td>
<td>Zinc Finger Protein 185</td>
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INTRODUCTION

2.1 THE KIDNEY AND ITS FILTRATION BARRIER

The kidney is our body’s filtration machine. Through the kidney passes approximately 20% of our heart blood volume per minute to be cleaned from toxins and restored with electrolytes. The ultrafiltration occurs through the glomerular capillary wall. This barrier works in a size and charge selective manner. Glomeruli generate about 180 liters of primary urine a day. Most of this filtrate is reabsorbed back into the systemic circulation in the tubular system and we ultimately produce 0.5-2.5L of urine a day.

![Renal glomeruli](image)

Fig. 1. Renal glomeruli are located in the kidney cortex. The three-layered glomerular capillary wall, with endothelial cells, the glomerular basement membrane and podocyte foot processes interconnected by slit diaphragms, forms the ultrafiltration barrier.

The glomerulus filtration barrier is formed of three layers (Fig. 1). The innermost layer is formed of fenestrated glomerular endothelial cells. These cells have fenestrations of 70-100nm in size and their health is dependent on the vascular endothelial growth factor (VEGF). The glomerular endothelium is in charge of synthesizing growth factors for the normal renal function and for this it is in constant interaction with podocytes. The middle layer of the filtration barrier is the glomerular basement membrane (GBM), which is an acellular matrix whose main components are type IV collagens, proteoglycans, laminins and nidogens. (Hudson et al. 1993; Hudson et al. 2003) Mutations in the GBM components are associated with human kidney diseases, such as Alport and Pierson syndrome, highlighting the importance of this structure.

Lastly, the podocyte layer envelops the glomerular capillary loops and forms the final filtration barrier in the glomerulus. Podocytes are specialized epithelial cells that are terminally differentiated. Morphologically, podocytes have prominent cell bodies that lie in the urinary space. From there they send large cytoplasmic extension called
major processes. These extensions further divide to smaller processes, termed foot processes, closer to the capillary wall. Foot processes attach podocytes to the GBM. Interdigitating foot processes are connected by highly specialized cell-cell junctions called slit diaphragms just above the GBM. (Tryggvason & Wartiovaara 2005)

### 2.2 Podocyte Foot Processes and Proteinuria

Podocytes are very dynamic cells with a prominent cytoskeleton that can effectively respond to changes in their environment. The cytoskeleton of foot processes is composed mainly of actin filaments. This actin-based machinery is vital for a sustained kidney filtration, as defects in actin-regulating proteins of podocyte foot processes, such as alpha-actinin-4, result in proteinuria. Importantly, the foot process cytoskeleton is reorganized in almost all proteinuric states, the phenomenon known as foot process effacement. The effacement is believed to be a key event in the pathogenesis of proteinuria.

Basally foot processes are attached to the GBM through a number of adhesion molecules, including integrins. In podocytes, heterodimer α3β1 is the most highly expressed integrin. The proper adhesion of podocytes to the GBM is essential for the kidney filter as mutations in α3β1 integrins result in proteinuria and progressive glomerular disease in both man and mouse.

![Fig. 2 Key molecular components of the podocyte](image)

Fig. 2 Key molecular components of the podocyte: Two interdigitating foot processes are shown emphasizing structural and functional regions: cytoskeletal components, adhesion complexes and the slit diaphragm. Additional gene products include, PGr (prostaglandin receptor) subtypes and podocalyxin, along with its interacting partners NHERF-2 and ezrin. V, vinculin; T, talin; P, paxillin. (Michaud et al. 2007)

### 2.3 The Slit Diaphragm

The slit diaphragm is the highly specialized cell-cell junction of podocytes. It plays a central role in the maintenance of the filtration barrier function. This is highlighted by the fact that mutations in molecular components of the slit diaphragm, such as nephrin and podocin, result in massive proteinuria in both man and mouse. The structure is connected to the actin cytoskeleton of foot processes via a number of
linker molecules, including nck proteins and cd2ap. This link is also essential for the filtration barrier function as KO animals for nck and cd2ap proteins develop massive proteinuria. Dendrin is a cytoplasmic component of the slit diaphragm that interacts directly with nephrin and cd2ap. However, its functional significance in the glomerulus is unknown.

2.4 PODOCYTE MAJOR PROCESSES

Major processes are cytoplasmic extensions between podocyte cell body and foot processes. Bundles of microtubules and intermediate filaments support major processes. However, besides microtubules and the intermediate filament protein vimentin, the components that form these cellular projections are largely unknown.

2.5 MOUSE MODEL FOR KIDNEY DISEASES

Mice are commonly used as an animal model to study kidney biology and diseases. The physiology of the kidney seems to be very similar between man and mouse. Manipulation of genome is well established in mouse and this makes it often the most obvious choice as an animal model. In addition, there are several well-established disease models in mouse that can be used in studies on kidney disease. However, there are also several drawbacks in using mice as a model. The reproductive cycle in mice is rather long which slows the research down. Additionally, the maintenance of mice is reasonably expensive. The relevance of many disease models in mouse to human disease processes can also be questioned. Another problem is that the genetic background of mice affects kidney disease progression and sensitivity; making the interpretation and standardization of experiments difficult. (Gurley et al. 2006) (Wang et al. 2000).

2.6 ZEBRAFISH AS A MODEL ORGANISM

The zebrafish (Danio rerio) is a tropical sweet water fish that is very popular as an aquarium fish but also widely used as a vertebrate model organism in research. There are several reasons why zebrafish has become so useful in scientific research:

1) The zebrafish genome has 71.4% gene orthology with the human genome. The orthologs are even higher if we look at disease-causing genes (82%, Mendelian Disease Database).
2) Zebrafish has the same major organs as humans and physiology of these tissues seems to be very similar to that of humans. This conservation is sometimes even better than in mouse (see Figure 3 - representation of electrocardiogram)
3) Zebrafish eggs are fertilized outside the mother’s body. This facilitates studies in early development.
4) Zebrafish embryos are almost transparent that helps researchers to visualize internal structures.
5) Zebrafish are much cheaper to maintain than mice.
6) Zebrafish produce hundreds of offspring at short intervals providing quickly plenty of material for studies.
7) Zebrafish grow and develop fast, speeding up analyses.
8) A large number of drug metabolism pathways are well conserved making it a suitable model for pharmacological studies.
9) Manipulation of gene expression is relatively easy through use of morpholinos.
10) The zebrafish genome has been fully sequenced. This facilitates the genetic manipulation of zebrafish genome.

Fig. 3 Zebrafish has very similar genome, physiology, drug metabolism and pharmacology compared to humans. (Barros et al. 2008)

2.7 ZEBRAFISH AS A MODEL TO STUDY PODOCYTES

Zebrafish embryos develop quickly with most organs being fully developed at 48 hours post fertilization (hpf). (Drummond et al. 1998) In the case of the kidney, the zebrafish larvae have a pronephros, a single glomerulus formed of two glomeruli that fuse at the midline (Fig. 4).
Fig. 4 Schematic view of the zebrafish pronephric morphology. From each pronephric duct (purple) develops the pronephros (green) that will fuse at the midline of the zebrafish larva connected by the pronephric tubules (blue). Image from: (Drummond et al. 1998)

The pronephros is responsible for the blood filtration and osmoregulation in the zebrafish larvae. It has been estimated that filtration capabilities of pronephros start from about 72 hpf (Drummond 2003). Morphologically, the zebrafish pronephros is very similar to that of mammalian glomerulus. Endothelial cells are fenestrated in the pronephros; podocyte foot processes with slit diaphragms envelope the GBM, identical to that seen in humans. (Fig. 5). Moreover, podocytes in pronephros seem to express the same critical molecules as those in mammalian kidney, including nephrin and podocin. Gene silencing experiments using morpholinos have shown that even their function is conserved between the species as knockdowns of nephrin and podocin in zebrafish larvae results in protein leakage.

Fig. 5 Electron microscopy pictures of human (Hs), mouse (Mm) and zebrafish (Dr) glomerular filtration barrier. The morphology is very similar in all three species. (Modified pictures from following publications: Lefèvre et al. 2010; Fukuyo et al. 2014; Jalanko & Holmberg 2009)

2.8 MANIPULATION OF GENE EXPRESSION IN ZEBRAFISH

The use of morpholinos has been the most popular way to manipulate gene expression in zebrafish. Morpholinos are chemically modified oligos with properties similar to those of genetic material. Morpholinos bind and block translation of mRNA in vitro and in vivo. (Nasevicius & Ekker 2000) When designed properly they are thought to have a limited amount of non-specific effects in silencing other genes. However, the major drawback of them is that one can get only a transient effect on gene expression lasting only up to 2-3 days.

The other popular way to alter gene expression in zebrafish has been the use of genetic mutants. In the past, these mutants were generated for instance by high
throughput N-ethyl-N-nitrosourea (ENU) mutagenesis. More recently, the targeted genetic manipulation has become easier in zebrafish with the introduction of CRISPR-technology (“clustered regularly interspaced short palindromic repeats”). The CRISPR/Cas9 is a type of prokaryotic immune system that confers resistance to foreign genetic material by cutting it. This system is now being exploited by delivering Cas9 nuclease and an appropriate guide RNA into a cell to cut the gene at a desired location. With the methodology one can for instance induce deletions or add genes. The advantage of mutants in comparison to morpholinos is that it is more specific and that one generates a permanent mutant line. This allows more sophisticated mechanistic studies in zebrafish. The major drawback of this methodology is that it is much slower than the use of morpholinos.
3 RESEARCH APPROACH

Following is a summary of the techniques used for the thesis project; techniques used in subsequent papers are not repeated. More details about specific procedures, primers and antibodies are available on the published papers and the manuscripts at the end of this thesis.

3.1 NEURONAL PROTEINS IN PODOCYTE MAJOR PROCESSES AND THEIR EXPRESSION IN GLOMERULAR CRESCENTS

Reverse Transcriptase-PCR
The expression of candidate genes in the mouse kidney was studied by RT-PCR using cDNA isolated from glomerular and the kidney fraction devoid of glomeruli. The glomerular isolation was performed as previously described. (Takemoto et al. 2002)

Antibody Generation
To generate polyclonal antibodies against the human Hip1 and Olfm12a, NZW rabbits were immunized with corresponding purified recombinant proteins.

Western Blotting
Lysates of glomerular tufts and the kidneys fractions lacking glomeruli were obtained from human cadaver kidneys unsuitable for transplantation due to vascular abnormalities (from the fourth Department of Surgery of Helsinki, Finland). Glomeruli were isolated using a standard sieving method.

Immunofluorescence
Normal human kidney samples were collected from human cadaver kidneys (as described above). Human fetal kidney samples were from a 20-week old fetus, non-viable due to neural tube defects and hydrocephalus (University Hospital of Helsinki, Finland). Fresh frozen sections of human kidney were fixated with acetone at -20°C, and blocked with 5% normal goat or donkey serum prior to staining with the corresponding antibodies.

Immunoelectron microscopy
Tissue sample preparations and immunolabeling experiments were carried out as described previously (Wernerson et al. 2003).

Immunoperoxidase staining
Paraffin-embedded renal biopsies were processed using standard procedures.

3.2 DENDRIN IS NOT CRUCIAL FOR THE GLOMERULAR FILTRATION BARRIER
**Generation of dendrin-deficient mouse line**

Dendrin KO mice were generated using Velocigene technology. Correctly targeted ES line was used to generate chimeric male mice that were then bred to C57BL/6 female to generate N1 mice. The mice used in this study were backcrossed over 5 generations onto the C57BL/6 background.

**Immunohistochemistry and histological analysis**

For histological analysis, mouse kidney samples were fixed in 4 % paraformaldehyde followed by dehydration and embedding in paraffin. Samples for immunohistochemistry were collected by snap-frozen kidneys on dry ice. The dendrin antibody and immunohistochemistry procedure has been described previously (Patrakka et al., 2007).

**Urine analysis**

Urine samples were collected from dendrin KO and control mice for up to 1 year of age. The presence of albuminuria was analyzed by running 2 μl of urine on SDS-PAGE gel stained with Coomassie blue or PAGE-Blue stain.

**Mouse disease models**

We induced glomerular injury and proteinuria in dendrin KO mice by LPS-injection and albumin overload as previously described (Xiao et al., 2011). Each mouse was injected with 13-μg/g of body weight of LPS intraperitoneally. For the albumin overload experiment, we injected 200 μg of BSA in 400μl intraperitoneally on four consecutive days.

Adriamycin induced proteinuria model was established in BALB/c mouse that were injected via the tail vein at a dosage of 10.5 mg/kg body weight as previously described (Wang et al. 2000).

**Yeast two-hybrid assay**

The coding sequence of mouse dendrin was cloned to a bait plasmid pGBK7, and used to screen a custom-generated mouse kidney glomerulus cDNA library. The yeast two-hybrid (y2h) screening was performed according to the manufacturer’s instructions. The positive clones were sequenced and analyzed with NCBI’s BLAST database.

**Co-immunoprecipitations**

For coimmunoprecipitations, HEK293 cells were co-transfected with full length myc-tagged Gadd45a and flag-tagged dendrin constructs, or full length myc-tagged Wtip and HA-tagged dendrin constructs, respectively. Experiments were performed according to standard procedures.

**Microarray analysis**

Three dendrin-null and three littermate mice at the age of 13 months were used to profile glomerular transcriptomes. Glomeruli were isolated as described previously.
(Takemoto et al. 2002) and total glomerular RNA was extracted. Microarrays were performed using GeneChip® Mouse Gene 1.0 ST Array.

### 3.3 GENE SCREEN IDENTIFIES TMEM234 AS AN IMPORTANT COMPONENT OF THE FILTRATION BARRIER

**Expression in mouse glomerulus**

qPCR was performed using standard procedures using Fast SYBR® Green (Life Technologies).

**Pronephric glomerulus isolation in zebrafish**

The expression of candidate genes in the zebrafish was studied by RT-PCR using cDNA isolated from pronephric glomerulus and the zebrafish larvae devoid of pronephric glomerulus. Larvae were sacrificed at 96 hpf and the GFP positive glomeruli were microdissected.

**Knockdown of genes in zebrafish using morpholino**

Zebrafish were housed following European and Swedish animal husbandry and ethical guidelines. Morpholino injections were performed using standard procedures on AB wildtype or podocin-GFP transgenic larvae.

**Histological analysis of zebrafish larvae**

The histological analysis of zebrafish pronephric glomerulus was performed on JB-4 embedded larvae using PAS staining. Samples were collected at 72hpf and 96hpf and processed according to standard procedures. Transmission electron microscopy was performed on 96hpf larvae fixed with glutaraldehyde following standard procedures.

**Pronephric glomerulus quantification**

Ten to twelve 96hpf larvae from control and morpholino-injected larvae were histologically processed, stained and imaged. Subsequently the nuclei of each pronephric glomerulus were quantified. Statistical significance was determined using Student’s T-test.

**Proteinuria Assay**

A total of 35 control and 40 morphant 72hpf larvae were successfully injected into the common cardinal vein with 10kDa rhodamine-labeled dextran and 500kDa FITC-labeled dextran in 0.2M KCl as described before. (Drummond et al. 1998)(Ebarasi et al. 2009) Larvae were taken 16hrs post-injection and fixed as described before. (Ebarasi et al. 2009)

### 3.4 MED22 IS NEEDED FOR THE MAINTENANCE OF THE GLOMERULUS FILTRATION BARRIER

Med22 full KO and conditional KO analysis
Med22 mice were acquired from EUCOMM in a C57b/6 129Sv background mix, where they were heterozygous for a Laz/Neo cassette. Heterozygous mice for the cassette were bred with Th-IRES (a global cre deleter as described by Lindeberg et al. 2004) mice to generate the full KO allele. In parallel, mice heterozygous for the cassette were bred with a FLP-deleter line to remove the NEO and LacZ portion of the cassette to generate a floxed mouse line. This line was then crossed with podocin-cre to inactivate the gene exclusively in glomerular podocytes.

**Urine analysis**

Albuminuria was analyzed using Exocell’s Albuwell kit and creatinine using Bioassays kit.

**Blood analysis**

Blood urea and albumin values were determined using Abcams Urea and Albumin ELISA kits.

### 3.5 LIMITATIONS AND ADVANTAGES OF TECHNIQUES USED

**Using Protein Atlas antibodies to screen for proteins: Specificity**

We have had the fortune to collaborate with the Human Protein Atlas project (Uhlén et al. 2005) where they generate antibodies for all human proteins. This has been a great advantage when dealing with orphan proteins, since previously (like in project 2) our own antibodies had to be generated. However, the high throughput generation of antibodies has its limitations, as the confirmation of the specificity of antibodies is perhaps not always optimal. In some cases it may be that the antibody binds unspecifically or the antibody exhibits poor versatility to be confirmed in different techniques. We have tried to avoid these problems by confirming results in both mouse and human tissue whenever possible. Also, we have used both immunofluorescence and Western blottings experiments to detect proteins, as well as validated the results at the mRNA level.

**Using morpholinos to silence genes in zebrafish**

Morpholino technology in zebrafish was at the front of gene expression manipulation for years, making it an easy and affordable way to screen for gene candidates in an appropriate *in vivo* model. Recently, the poor correlation between morphant and mutant phenotypes in zebrafish was reported (Kok et al. 2014), raising concerns about off-target effects of morpholinos. However, this study was only limited to the context of early vascular phenotypes. For renal studies, our basic requirement is intact cardiac and vascular function (so that the pronephros can be formed), which means that we discard larvae with defective circulation from our studies in the first two days post-fertilization. Therefore, it is possible that the co-relation between morphants and mutants could be greater in renal morphants than in vascular morphants.
Morpholino-mediated downregulation of genetic transcripts in zebrafish have been of great use in different fields for many years. However, it is obvious that CRISPR technology (“Clustered regularly interspaced short palindromic repeats”) is now getting more popular also in zebrafish studies. This system is now being exploited by delivering Cas9 nuclease and an appropriate guide RNA into a cell to cut the gene at a desired location, this to induce deletions or add genes. When compared to morpholinos, CRISPR technology allows a permanent and more specific inactivation of genes. This comes with a time-price tag, since the larvae with the desired modification have to be screened to see that the mutation went germ-line. For this, zebrafish have to reach reproductive age, and then their offspring have to be screened. So even though fewer off-targets effects are expected, the speed and convenience of the zebrafish model for analyzing renal mutations could now be comparable to that of mice in a time line. In the scope of this thesis we do not use CRISPR/Cas9 for our studies in zebrafish, although zebrafish mutants for Tmem234 (project 3) and Med22 (project 4) have been generated and in the near future we will start analyzing knockout larvae.
4 RESULTS

Following is a summarized version of the results presented in the projects included in the thesis. For more details, please refer to the respective articles.

4.1 NEURONAL PROTEINS IN PODOCYTE MAJOR PROCESSES AND THEIR EXPRESSION IN GLOMERULAR CRESCENTS

Expression of Hip1, Nfasc and Olfml2a in mouse and human kidney

First, we analyzed the expression of Hip1, Nfasc and Olfml2a in the mouse kidney by comparing the glomerular fraction to the rest of the kidney. The PCR product for Hip1, Nfasc and Olfml2a was only amplified in the mouse glomeruli.

The expression of Hip1, Nfasc and Olfml2a was then studied in the human kidney using Western Blotting. In this approach, we detected the corresponding proteins only in glomerular fractions and no signal was seen in kidney fractions lacking glomeruli.

In immunofluorescence, Hip1, Nfasc and Olfml2a were detected in the glomeruli and located at the side of the urinary space of nephrin. Double labeling with vimentin showed overlapping immunoreactivity in the major processes.

Immunoelectron microscopy was used to confirm the subcellular localization. Gold label for Hip1 and Olfml2a was observed in major processes of podocytes. Taken together, Nfasc, Hip1 and Olfml2a are localized specifically to podocyte major processes.

Expression of Hip1, Nfasc and Olfml2a in human kidney development

The two earliest stages of glomerular development, vesicle and S-shaped stages, showed no Hip1 or Nfasc expression. In the capillary stage glomerulus, a later stage in where the formation of major and foot processes begins, immunoreactivity was observed for Hip1 and Nfasc. At this stage, the staining for these two proteins co-localized with that of vimentin. This subcellular localization suggests an association to developing major processes and supports our findings in the adult kidney.

The expression of Olfml2a during the vesicle stage glomerulus did not show any immunoreactivity. Surprisingly, at the S-shaped stage, Olfml2a presented positive signal in the invading endothelial cells located in the glomerular cleft. In the later stages, more mature endothelial cells did not present any positivity for Olfml2a. Instead, at the capillary stage, a clear signal for Olfml2a was observed in podocytes and this co-localized with that of vimentin. This supports our findings in the adult kidney.

Expression of novel podocyte markers in glomerular crescents

We used our novel podocyte markers to investigate the cellular content of glomerular crescents. Immunoreactivity for Hip1 was observed in 14 of the 26 crescents that were identified in the biopsy materials analyzed. Olfml2a staining was clearly detected in 21 of the 31 glomerular crescents identified. The positive signal for these podocyte markers was detected in less than half of the cells in cellular crescents, and in
these cells, the staining was seen diffusely in the cytoplasm. Anti-Nfasc antibodies did not give any reliable signal.

In order to validate our results, we analyzed the expression of Pdlim2 in glomerular crescents. Pdlim2 is a molecular component of podocyte foot processes, and importantly, Pdlim2 is in the kidney, podocyte-specific (Sistani et al. 2011). We chose Pdlim2 as a marker for podocytes because during podocyte maturation it is expressed earlier than foot process markers nephrin and synaptopodin. In patients with crescentic glomerulonephritis, Pdlim2 presented positive signal in 30 of the 43 glomerular crescents identified. Thus, cells in glomerular crescents showed positivity for three podocyte markers, which strongly supports the idea that podocytes are present in these lesions.

### 4.2 DENDRIN IS NOT CRUCIAL FOR THE GLOMERULAR FILTRATION BARRIER

**Establishment of dendrin-deficient mouse line**

Two coding exons of dendrin and the intervening intron were replaced with an eGFP cassette and the elimination of dendrin in homozygous mice was confirmed by immunohistochemistry. No signal for dendrin was observed in -/- mice whereas littermate control mice showed strong dendrin expression in the glomerulus.

**Lack of dendrin does not affect glomerular development or barrier function**

Previously, others and we have located dendrin specifically to the cytoplasmic side of the podocyte slit diaphragm (Asanuma et al., 2007; Patrakka et al., 2007). Light and electron microscopic examination showed that the glomerulogenesis proceeded normally in dendrin KO mice and mature glomeruli showed normal morphology. Urine analysis of dendrin knockout mice did not show significant albuminuria.

Furthermore, immunostaining patterns for slit diaphragm proteins nephrin and podocin, as well as foot process protein synaptopodin and major process marker vimentin were not altered in dendrin-deficient glomeruli in comparison to controls.

**Expressional profiling of dendrin-deficient glomeruli**

We performed microarray profiling of glomeruli from dendrin KO mice. As expected, the dendrin expression was downregulated in KO glomeruli. Besides the downregulation of dendrin, the expression profiles showed little changes. No significant expressional changes were detected in well-known podocyte genes, including slit diaphragm genes that have been shown to bind to dendrin.

**LPS or BSA-overload induced kidney injury**

To study if dendrin has a role in compensatory mechanisms in the glomerulus, we challenged dendrin KO mice with LPS-injection and BSA overload. Both LPS-injection and BSA overload resulted in significant albuminuria but no difference was observed between the dendrin-deficient and control mice.

**Wtip and Gadd45a bind to dendrin**
To analyze protein-protein interactions of dendrin, we performed a yeast two-hybrid screen. To identify meaningful interactions occurring in the podocyte, we used our own glomerular cDNA library (Sistani et al., 2011) as a prey-library. Two of the identified candidates caught our attention, Wtip and Gadd45a. Wtip has been shown to localize to the slit diaphragm and shuttle to nucleus in injured podocytes, similarly to the previous report for dendrin (Kim et al., 2010). Gadd45a was, on the other hand, significantly upregulated in the dendrin-deficient glomeruli.

The association of dendrin with Gadd45a was confirmed by transfecting HEK293 cells with expression constructs followed by co-immunoprecipitation. Immunoprecipitation of double transfected cells with anti-flag antibody brought down myc-tagged Gadd45a together with flag-tagged dendrin. The other way around, immunoprecipitation with anti-myc antibody co-immunoprecipitated flag-dendrin, whereas the control IP with a non-related expression construct does not bring down flag-dendrin. To get support these results, we analyzed the localization of dendrin and Gadd45a in the glomerulus. In the normal glomerulus, strong immunoreactivity for dendrin was observed as a linear line around capillary loops indicating localization to foot processes. In addition, a weak reactivity in nuclei of podocytes was observed. No dendrin was detected in nuclei of other glomerular cells. Staining for Gadd45a was detected in nuclei of all glomerular cells, including podocytes. Importantly, the staining for dendrin in podocyte nuclei overlapped with that of Gadd45a. In adriamycin-induced nephropathy, immunoreactivity for dendrin in nucleus was increased as 95% (124/133 in 10 glomeruli) of podocyte nuclei showed reactivity for dendrin.

We confirmed the interaction between dendrin and Wtip using co-immunoprecipitation experiments. We transfected HEK 293 cells with HA-tagged dendrin and myc-tagged Wtip. Dendrin co-immunoprecipitated with Wtip in double-transfected cells, whereas Wtip was not detected in the control immunoprecipitation. To get support for these results, we tried to perform immunostaining experiments with various Wtip antibodies. However, in our hands, no reliable signal for Wtip was detected in immunohistochemical experiments and thus no colocalization studies could be made with dendrin.

### 4.3 GENE SCREEN IDENTIFIES TMEM234 AS AN IMPORTANT COMPONENT OF THE FILTRATION BARRIER

RT-PCR and immunofluorescence analysis identifies Tmem234, Znf185, Lrrc49 and Slfn5 as new molecular components of the podocyte

RT-PCR and qPCR experiments showed that all four transcripts were enriched in the glomerulus in comparison to the kidney fraction lacking glomeruli. The amplification of the Tmem234 transcripts gave significant signal also in the kidney fraction lacking glomeruli, whereas other transcripts gave only minimal or no signal in the non-glomerular fraction.

Staining of adult human kidney sections with antibodies directed against Tmem234, Znf185, Lrrc49, and Slfn5 gave strong glomerular immunoreactivity, and only weak signals were detected in rest of the kidney. Double labeling with nephrin, a foot process marker showed overlapping reactivity for Tmem234, Znf185, Lrrc49 and
Slfn5. Staining with major process marker vimentin showed colocalization only with Znf185.

**Expression during glomerular development**

To validate the association of these proteins with podocytes, we analyzed their expression pattern during glomerulogenesis. Staining of human fetal kidney sections gave strong immunoreactivity in developing podocytes. The expression of all four proteins was first detected at the capillary stage glomerulus. At this stage the formation of foot and major processes begins and the earliest expression of podocyte markers, such as synaptopodin and podocin, is observed. (Nagata et al. 1998; Mundel et al. 1997) At the capillary stage, Tmem234, Znf185, Lrrc49 and Slfn5 were colocalizing with nephrin at the basal aspects of pre-podocytes. Znf185, on the other hand, colocalized also with vimentin during podocyte development. To summarize, studies in glomerulogenesis confirmed the association of Tmem234, Lrrc49 and Slfn5 to foot processes, as well as the association of Znf185 to both foot and major processes.

**Zebrafish orthologs and their expression**

Zebrafish was chosen as a model to study the functional role of new proteins in the glomerular filter. For Tmem234 and Znf185 orthologs were identified, whereas no zebrafish orthologs were found for Slfn5 and Lrrc49. Therefore, no further studies were performed with Slfn5 and Lrrc49 in zebrafish.

The pronephric glomerulus expression of Tmem234 and Znf185 was confirmed using RT-PCR and qPCR. Strong expression for both genes was detected in the pronephric glomerulus, whereas weaker or no signal was observed in the zebrafish fraction lacking glomerulus.

**Knockdown of zebrafish orthologues**

Morpholinos were designed to knockdown the expression of Tmem234 and Znf185 orthologs in zebrafish. To assess whether knockdown affected the pronephric glomerulus the presence of GFP expression in transgenic podocin-GFP larvae was analyzed. A decreased GFP expression in a living fish was considered as a sign of podocyte and pronephric glomerulus injury.

The experiments showed successful downregulation of gene expression for Tmem234 and Znf185. Macroscopically, Tmem234 morphants exhibited mild pericardial edema at 72 hpf that progressed slightly by 96 hpf in comparison with control larvae. GFP-positivity in pronephric podocytes was often lost in the morphants. Light microscopy examination of morphants confirmed the pronephric glomerulus abnormalities as an enlarged Bowman’s space with a disorganized glomerular architecture was observed. The absence of podocyte-GFP expression in Tmem234 morphants suggested that their pronephric glomerulus and podocytes were affected. Although 48% Znf185 morphants presented slight to moderate pericardial edema, podocin-GFP expression in Znf185 morphants was unaffected. In the pronephric glomerulus, a minimal enlargement of the Bowman’s space was evident but the capillary tuft appeared normal.
Analysis of Tmem234 morphants

As Tmem234 morphant fish exhibited obvious pronephric glomerulus abnormalities, we analyzed the morphants in more detail. We co-injected Tmem234 morpholino with a mouse Tmem234 mRNA. GFP expression was partially rescued with the mouse mRNA as 81% of embryos co-injected with Tmem234 mRNA expressed GFP. This experiment indicated that the loss of GFP was due to the knockdown of Tmem234.

The histological analysis was complemented by counting manually the number of cells in pronephric glomeruli. This showed a significant reduction in the number of pronephric glomerular cells in Tmem234 morphants.

Tmem234 morphants were also evaluated in electron microscopy; foot processes effacement was evident in morphant pronephric glomerulus. The slit diaphragms interconnecting remaining foot processes, the glomerulus basement membrane and endothelial cells appeared unaffected.

To elucidate whether abnormal pronephric glomerulus structure in Tmem234 morphants had functional consequences to the filtration barrier, we injected zebrafish larvae with FITC-labeled 500kDa dextran into the common cardinal vein. In wild type zebrafish, only 10kDa dextran was detected in the tubuli. In contrast to this, FITC-labeled 500 kDa dextran was detected in the tubuli of Tmem234 morphants. Thus, knockdown of Tmem234 in zebrafish compromised the functional integrity of the pronephros filtration barrier.

4.4 MED22 IS NEEDED FOR THE MAINTENANCE OF THE GLOMERULUS FILTRATION BARRIER

Med22 is expressed in the kidney glomerulus and pronephros

The expression of Med22 was analyzed using RT-PCR that showed it to be ubiquitously expressed in all tissues tested in both human and mouse. In the kidney, however, the expression of Med22 was enriched in the human and mouse glomerulus as compared to the kidney fraction devoid of glomeruli. In situ hybridization of zebrafish larvae showed expression in the zebrafish pronephros. Staining of normal human kidney with Med22 showed staining in the cytoplasm of cell nucleus and at the major processes of podocytes. Double staining with the slit diaphragm marker nephrin did show any colocalization.

Knockdown of Med22 in zebrafish produces pronephros abnormalities and proteinuria

We knocked down Med22 in podocin-GFP zebrafish. This resulted in pericardial edema and tubular edema. Interestingly, Med22 morphants did not lose their GFP expression. Instead, these morphants showed defective capillary loop formation. Histologically, we observed that the GFP positive cells, as seen in the gross morphology, concentrated in the midline of the zebrafish larvae. In electron microscopy, a reduced number of podocytes was detected. Remaining podocytes had some remnant foot processes and slit diaphragms. Functionally, Med22 larvae leaks
large proteins into proximal tubules as detected by the leakage of fluorescently labeled 500kDa dextran molecules.

**Mice lacking Med22 in podocytes develop progressive kidney disease**

The global Med22 KO mice die during embryonic development. Mice where Med22 was specifically inactivated in podocytes (Med22PodKO) developed normally and followed Mendelian distribution in their genotypes. During the first 10 weeks they were comparable with their littermates in size and weight. However, albuminuria was detected in few Med22PodKO animals at 8 weeks of age, and this progressed later to massive albuminuria in all PodKO animals. Hematuria was also detected in most PodKO animals. Blood urea nitrogen levels were normal at 8 weeks of age, but increased progressively from 12 weeks of age and all Med22PodKO mice died by 18 weeks of age due to renal failure.

**Histological analysis of Med22PodKO animals shows formation of vesicles in podocytes**

Kidney histology appeared normal in Med22PodKO mice at 8 weeks of age at light microscopic level. In electron microscopic evaluation, few mice exhibited mild foot process effacement at 8 weeks of age. Occasionally, we found enlarged vesicles in podocytes.

At 12 weeks of age, PodKO animals showed severe morphological abnormalities in glomeruli. Podocytes exhibited large vesicles that were detected in both light and electron microscopy. Often these vesicles appeared to be larger than podocyte nuclei. In light microscopy, these vesicles appeared empty and they were negative in oil-red staining (lipid staining, data not shown). In electron microscopy, these vesicles appeared in most cases empty. Occasionally, we observed an accumulation of electron-dense vesicles in the cytoplasm of podocytes.

Besides podocyte vesicles, we detected focal segmental glomerulosclerosis at 12 weeks of age. This was probably secondary to podocyte loss. In addition, tubuli were often dilated and protein casts were detected as a sign of massive albuminuria. These changes progressed and at 16 weeks of age the global glomerulosclerosis was obvious along with advanced secondary changes in tubuli.

**Immunofluorescence studies in Med22PodKO mice**

Immunofluorescence for podocyte foot process markers nephrin, podocin and synaptopodin gave continuous linear staining pattern along glomerular capillary loops in Med22PodKO and control mice at 8 weeks of age. Wt1, a marker for podocyte nuclei, showed also normal number of podocytes at 8 weeks of age. However, at 12 weeks of age, the staining for all podocyte foot process proteins had nearly completely disappeared. Only few Wt1 positive podocytes were detected in knockout glomeruli indicating a significant loss of podocytes. At 16 weeks of age, no foot process markers were detected and wt1 positive nuclei were only rarely detected.

Next, we analyzed the molecular nature of podocyte vesicles in Med22PodKO mice. Immunofluorescence staining for 12 week old kidneys showed significant upregulation of endosomal marker caveolin while clathrin, marker for clathrin-mediated
endocytosis, was not upregulated. Lysosomal marker LAMP2 was also clearly upregulated in knockout glomeruli. Thus, podocytes vesicles seemed to be originating from caveolin-mediated endocytosis pathway.

**RNAmseq confirms downregulation of podocyte markers preceding proteinuria.**

We performed RNA sequencing on glomeruli isolated from 8-week old Med22PodKO mice. The sequencing revealed 126 differentially expressed genes of which 78 were up- and 48 down regulated. Known genes involved in endosomal and lysosomal trafficking were not differentially expressed. However, we detected the downregulation of podocyte markers podocin and synaptopodin that was in line with our immunofluorescence data.

### 4.5 NEGATIVE RESULTS

In science, negative results often remain unpublished. In my thesis work, I many times ended up with negative results. The reasons for this can be many: redundant or synonymous proteins, compensation mechanisms, lack of tools for detection or optimization, etc.

In the original thesis plan, the purpose was to study both Hip1 and Nfasc KO mouse lines. Hip1 KO mice were published in 2007 (Khatchadourian et al. 2007) and were brought into the lab to analyze their kidneys. Similarly, Nfasc mice were also imported to our lab. Neither of these mouse lines exhibited any obvious kidney phenotype (data not shown).

During my thesis work we identified another new podocyte-enriched molecule, Tmeff2 (data not shown). We imported a mouse line for Tmeff2 to our lab but this turned out to be wrongly targeted. Then, a group published a knockout model for Tmef22 (Chen et al. 2012). Tmeff2-deficient mice were smaller in size and died soon after weaning. We imported also this mouse line to our lab but it never showed an obvious kidney phenotype, even after backcrossing to the 129Sv background, which increased their survival from 4 to 7 weeks, but no obvious anomalies where found.
5 DISCUSSION

5.1 HIP1, NFASC AND OLFML2A IN PODOCYTES

We identified Hip1, Nfasc and Olfml2a as new molecular components of podocyte major processes. We used these new markers to validate previous studies (made mostly in mouse) that show that podocytes contribute to the formation of glomerular crescents. We did not include any functional studies in the published work. However, we followed up this study by importing Hip1 and Nfasc KO animals to our lab. Neither of these mice exhibited any obvious renal phenotype indicating that Hip1 and Nfasc are not essential for the development of normal kidney filtration barrier.

5.2 DENDRIN IN PODOCYTES

We discovered dendrin through a large-scale microarray screen in which over 300 glomerulus-enriched transcripts were identified. (Takemoto et al. 2006). Shortly after, we showed that dendrin had a very restricted expression as we detected it only in the brain and the kidney glomerulus (Patrakka et al. 2007). In the glomerulus, we localized dendrin to the cytoplasmic face of the slit diaphragm. Furthermore, we observed that in patients with minimal change nephrotic syndrome, dendrin was redistributed from the slit diaphragm region to the podocyte cytoplasm (Dunér et al. 2008). These findings motivated us to generate a KO mouse line for dendrin. Our studies in dendrin-deficient mice indicated that dendrin is not essential for the normal development or maintenance of the kidney filtration barrier.

Recently Weins et al. reported that double knockout animals for Cd2ap and dendrin survived longer than Cd2ap KOs. In the study, the absence of dendrin delayed the onset and severity of proteinuria, mesangial expansion and podocyte loss. (Weins et al. 2015). Thus, in this genetic model of podocyte disease, dendrin seemed to have injury promoting effects.

In our study, we also showed that dendrin can interact with Wtip and Gadd45a. Both of these have been shown to mediate gene transcription in the nucleus. These results are well in line with a study reporting that dendrin can relocate to nucleus in response to glomerular injury and promote podocyte apoptosis, thus adding to the injury-promoting role of dendrin. (Campbell et al. 2013)

5.3 ZEBRAFISH SCREEN IDENTIFIES SEVERAL CANDIDATE GENES

Morpholino-based gene silencing is a quick way to knock-down genes in zebrafish and with the recent development of a GFP transgenic for podocyte-specific protein podocin (He et al. 2011), we decided to use the zebrafish model as a screening tool. In the project 3, we observed that silencing of a new podocyte protein Tmem234 in zebrafish resulted in proteinuria and foot processes effacement, as well as loss of podocyte GFP expression. Morpholino experiments in zebrafish have yielded even other candidates that our group is currently working on in mouse models (data not shown). In addition, zebrafish knockdown of Med22 was critical for its identification as a candidate for mouse KO studies. This emphasizes the importance of the zebrafish
model. It seems that many critical genes have a conserved function in zebrafish and therefore this model is useful for studying human biology.

Recently, Kok et al. reported that only less than 20% of the phenotypes found in morpholino knockdowns in zebrafish could be reproduced with mutants. In our hands, so far, the renal phenotypes have correlated with morphants, but we are in the process of developing mutants for several genes that we have generated morpholino knockdowns, among them, Med22 and Tmem234. It is important to elucidate the correlation of morphant to mutant phenotypes in order to make a correct interpretation of these models. I feel that although CRISPR technology facilitates the high throughput generation of mutants, there is still space for the use of morpholinos, at least in the renal field, due to the quickness of morphant models.

5.4 MEDIATOR COMPLEX AND MED22

Lastly, we identified a mediator complex subunit that is imperative for the homeostasis of the kidney filtration barrier. The mediator complex is a huge complex (1.2 MDa for the human mediator complex) composed of about 30 subunits. It is divided into 4 sub-structures, the CDK8 module, the middle module, the head and the tail. Of the 10 subunits knocked out in mouse (www.mousephenotype.org search July 2016), 9 are embryonically lethal. The heterozygous deletion of a subunit can also result in pathology. For instance, haploinsufficiency for Med13/Med13L results in transposition of the great arteries. (Muncke et al. 2003; Reza Asadollahi 2013; Sato et al. 2004)

It is no doubt that the mediator complex is critical for the normal function of cells. However, it is still necessary to elucidate exactly what subunits are expressed in each tissue and under what conditions each subunit functions. Knocking out each subunit does not result in the same phenotype in hypomorphic conditions, so it is probable that there are different interactions with particular subunits in specific cellular processes.

Med22 is part of the highly conserved Surfeit locus complex (Surf1-6)(Garson et al. 1995). It is ubiquitously expressed consistent with being a housekeeping gene. (Angiolillo et al. 2002) Our results confirm this, as Med22 deletion in all cells in mouse results in an embryonic lethality. Although embryology is not the focus of this thesis, it could be that elucidating the cause for embryonic death will give important insights into how Med22 works in cells.

As we detected Med22 to be enriched in podocytes, we generated a podocyte-specific KO mouse. The deletion of Med22 in podocytes does not affect the development of the glomerulus but results in accumulation of cytoplasmic vesicles in adult mice. These vesicles get larger during age and are likely the cause of concomitantly observed podocyte loss. Following loss of podocytes, a process of glomerulosclerosis initiates leading to ESRD and death by 18 weeks of age.

We have seen that vesicles in podocytes are positive for caveolin suggesting that they originate from the endocytic pathway. Normally, endocytic vesicles are either directed to lysosomes and degradation or to be recycled. In the absence of Med22, the vesicular trafficking seems to fail. We do not yet understand the mechanism behind it but with further experiments, both in vivo and in vitro, we hope elucidate this interesting phenomenon.
6 CONCLUSION

I Hip1, Nfasc and Olfml2a are new molecular components of podocyte major processes.

II Dendrin is not imperative for the development or maintenance of the glomerular filtration barrier

III Tmem234 is essential for the zebrafish pronephric filtration barrier function.

IV Med22 is essential for the maintenance of the glomerulus filtration barrier.
7 FUTURE PERSPECTIVES

7.1 NEURAL PROTEINS IN PODOCYTES

Dendrin, Hip1, Olfln12a and Nfasc are neural proteins that we have discovered to be expressed by podocytes. We have analyzed the biological role dendrin, Hip1 and Nfasc in KO animals and results indicate that they are not essential for the development or maintenance of the glomerulus filtration barrier. This was somewhat disappointing as a lot of efforts were made to generate and analyze these lines. Obviously, these studies do not exclude the possibility that these genes would have pathogenic roles in the development of glomerular disease processes. In fact, we have collaborated with dr Kirk Cambell’s group and they published recently a paper showing that dendrin-deficiency slows the progression of glomerular disease in Cd2ap KO animals. Previously, dendrin has been reported to promote apoptotic signaling in podocytes. It would be interesting to study in more detail the role of dendrin in acquired kidney diseases. Additionally, challenging dendrin KO animals with different disease models, like inducing diabetes and analyzing how the nephropathy progresses in these animals may help elucidate if dendrin plays a pathogenic role in disease models in which podocyte apoptosis occurs. Also, more detailed analyses of expression and subcellular localization in common acquired glomerular diseases are needed. Perhaps dendrin is relocated to nucleus in some diseases and promote there pro-apoptotic signaling? Similarly, analogous studies with Hip1 and Nfasc PodKO animals or double KO/PodKO with other relevant genes might help explain why these proteins are so podocyte enriched.

7.2 ZEBRAFISH AND TMEM234

In project 3 we present poorly characterized proteins that are highly enriched in podocytes. We studied further the candidates in zebrafish using morpholinos. However, in this project we did not pursue the proteins that did not have a zebrafish ortholog. It could be interesting to study these candidates (Lrcc49 and Slfn5) in more detail in mammalian models.

As recent study reported a discrepancy between morpholino and mutant phenotypes in zebrafish, we have started a project to knock out in zebrafish several known proteins critical for the filtration barrier, like Nphs1 and Nphs2, but also Tmem234. It will be interesting to see whether for instance Nphs1/Nphs2 mutants exhibit a similar phenotype to corresponding morphants. The use of mutants provides us a more stable model to study in more detail molecular mechanisms in zebrafish.

7.3 THE MEDIATOR COMPLEX AND KIDNEY BIOLOGY

We describe for the first time a role for a component of the mediator complex in the kidney. Although Med22 is clearly essential for the maintenance of the filtration barrier homeostasis, many open questions remain. First, we should elucidate the
specific mechanisms behind vesicle formation in Med22PodKO mice and pinpoint the specific pathway behind it. More importantly, could we figure out a target to reverse or delay this phenotype? Another obvious experiment would be to delete Med22 in another cell type in the kidney, would that give a similar pathology or is the vesicle formation something specific to podocytes, and what can that tell us about Med22’s role in cell survival and homeostasis.
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9 REFERENCES


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