A Podocyte view on RhoGTPases and actin cytoskeleton regulation

Lovisa Bergwall

Department of Physiology
Institute of Neuroscience and Physiology
Sahlgrenska Academy, University of Gothenburg

UNIVERSITY OF GOTHENBURG

Gothenburg 2020

Cover illustration: “Fotfolket” by Lasse Bergwall

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lovisa.bergwall@neuro.gu.se

ISBN 978-91-7833-908-2 (PRINT)
ISBN 978-91-7833-909-9 (PDF)

Printed in Gothenburg, Sweden 2020
Printed by Stema Specialtryck AB

Till Elias

ABSTRACT

Proteinuria is a hallmark symptom of chronic kidney disease, that if left to persist constitutes a risk for progression of disease. Symptomatic treatment aiming at decreasing proteinuria is therefore standard practice. Curative treatments for the underlying cause of disease are however lacking and treatments currently in use to induce disease remission are associated with unfavorable side effects. Dysregulation of the podocyte actin cytoskeleton underlies the pathological process called foot process effacement (FPE), which is one of the leading causes of proteinuria. The studies included in this thesis have focused on podocyte actin cytoskeleton regulation and a group of proteins called RhoGTPases, known to be involved in actin cytoskeleton regulation in podocytes. In the first study, glomerular microarray analysis showed an increase in the expression of the melanocortin 1-receptor (MC1R) in renal diseases focal segmental glomerulosclerosis and membranous nephropathy. Subsequent mass spectrometry analysis in combination with pathway and biochemical analysis revealed the podocyte protective effects of MC1R stimulation *in vitro*. Activation of MC1R proved to be stabilizing the podocyte actin cytoskeleton through inhibition of the epidermal growth factor receptor (EGFR) and maintenance of the actin associated protein synaptopodin. In the second study, the depletion of the prenylation enzyme Geranylgeranyl transferase type I (GGTase-I) in podocytes led to the development of proteinuria and FPE in mice due to an imbalanced RhoGTPase activity and disruption of the actin cytoskeleton. These findings suggest that GGTase-I activity is essential for podocyte function. In the last study, a guanine nucleotide exchange factor (activator of RhoGTPases) named βpix was identified to be modulated in podocytes following treatment with a renal stressor, using mass spectrometry analysis. Gene silencing of βpix protected against actin cytoskeleton remodulation in a model of podocyte injury, demonstrating the importance of βpix for podocyte actin cytoskeleton regulation.

In conclusion, the results in this thesis confirm the importance of actin cytoskeleton regulation for podocyte integrity. Further on, the results provide new information on actin cytoskeleton regulatory pathways involving RhoGTPases in podocytes, which can be of importance for future attempts in finding targeted treatments of proteinuria and chronic kidney disease.

Keywords: Podocyte, RhoGTPases, actin cytoskeleton regulation
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SAMMANFATTNING PÅ SVENSKA

Kroniska njursjukdomar uppkommer till följd av sjukdomar som diabetes och högt blodtryck. Ytterligare en orsak utgörs av sjukdomar som drabbar det kärlnystan i njuren där filtration av blod sker vid produktionen av urin. Detta kärlnystan kallas glomerulus och sjukdomarna kallas följaktligen för glomerulonefriter. Oavsett orsak till den kroniska njursjukdomen, leder den i sig till en förlust av viktiga proteiner ut i urinen hos den drabbade, samt en avtagande njurfunktion. Förlusten av proteiner i urinen leder sekundärt till komplikationer i form av ödem, en ökad risk för blodproppar, åderförkalkning och infektioner.

En av orsakerna till förlusten av proteiner i urinen är en skada på en celltyp som befinner sig i den barriär som blodet filtreras över under produktionen av urin. Denna cell kallas podocyt, och agerar under vanliga omständigheter som en grindvakt som hindrar för stora molekyler, så som proteiner, från att nå ut i urinen. När podocyterna skadas sker en omstrukturering av det cellskelett som håller dom uppe, vilket leder till att de inte kan upprätthålla sin funktion och proteinerna läcker ut i urinen. I denna avhandling har vi studerat processer som reglerar cellskelettet i podocyterna, med fokus på en grupp proteiner som deltar i denna reglering benämnda RhoGTPaser.

I den första studien har en receptor kallad MC1R studerats. Aktivering av denna receptor visade sig kunna skydda mot skador på podocyternas cellskelett. Genom aktivering av MC1R motverkades signalering via en annan receptor, EGFR, som tidigare visat sig kunna skada podocyterna, och cell-skelettet stabiliserades med hjälp av RhoGTPaser.

I den andra studien identifierades ett enzym som reglerar RhoGTPaser och vars aktivitet tycks vara viktig för podocyternas funktion. När uttrycket av detta enzym minskades i podocyter utvecklade möss njursjukdom med förlust av protein ut i urinen. Vävnadsanalys och cellstudier avslöjade att podocyternas form och cellskelett var förändrad, och att aktiviteten hos RhoGTPaserna var påverkad, vilket kunde förklara uppkomsten av njursjukdom hos mössen.

I det tredje och sista projektet, har ett protein kallat βpix identifierats som en regulator av RhoGTPas-aktivitet och visat sig ha en viktig roll i cellskelettets reglering. Minskat uttryck av detta protein skyddade mot omstrukturering av podocyters cellskelett i en modell av njursjukdom.

Sammantaget har dessa studier identifierat nya signaleringsvägar för reglering av podocyternas cellskelett. Denna kunskap kan bidra till förståelsen för hur dessa processer bidrar till njursjukdom och hur man med hjälp av läkemedel kan komma att motverka njursjukdom och förlust av protein i urinen.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Amplification of the Melanocortin-1 Receptor In Nephrotic Syndrome Identifies a Target for Podocyte Cytoskeleton Stabilization

Bergwall L, Wallentin H, Elvin J, Liu P, Boi R, Sihlbom C, Hayes K, Wright D, Haraldsson B, Nyström J and Buvall L.

Scientific Reports (2018) 8 (1), 15731

II. Podocyte Geranylgeranyl transferase type I is essential for maintenance of the glomerular filtration barrier function

Bergwall L, Boi R, Akula M.K, Ebefors K, Bergo O. M, Nyström J, Buvall L.

Manuscript

III. The role of βpix in podocyte Rac1 activation and cytoskeleton rearrangement

Bergwall L, Wallentin H, Boi R, Svensk S, Lövljung V, Sihlbom C, Weins A, Ericsson A, William-Olsson L, Granqvist B. A, Ebefors K, Nyström J, Buvall L.

Manuscript

CONTENT

ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
ADR	Adriamycin
βpix	P-21 activated kinase-interacting exchange factor β
cAMP	Cyclic adenosine monophosphate
Cdc42	Cell division control protein 42 homolog
CKD	Chronic kidney disease
Cre	Cyclization recombinase
DN	Diabetic nephropathy
EC	Endothelial cell
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
ESL	Endothelial surface layer
ESRD	End stage renal disease
FTase	Farnesyl transferase
FP	Foot process
FPE	Foot process effacement
FPP	Farnesyl pyrophosphate
FSGS	Focal segmental glomerulosclerosis
GAP	GTPase activating protein

GBM	Glomerular basement membrane
GEF	Guanine nucleotide exchange factor
GFR	Glomerular filtration rate
GGPP	Geranylgeranyl pyrophosphate
GGTase-I	Geranylgeranyl transferase type I
GGTI	Geranylgeranyl transferase inhibitor
IL-1,2,6,8	Interleukin 1,2,6,8
MCR	Melanocortin receptor
MC1R	Melanocortin 1 receptor
MCD	Minimal change disease
MCP-1	Monocyte chemoattractant protein 1
MN	Membranous nephropathy
PAK	P-21 activated kinases
PAN	Puromycine aminonucleoside
PKA	Protein kinase A
PS	Protamine sulphate
Rac1	Ras-related C3 botulinum toxin substrate 1
RhoA	Ras homolog family member A
RhoGDI	Rho guanine nucleotide dissociation inhibitor
SD	Slit diaphragm
TNF-alfa	Tumor necrosis factor alfa

1 INTRODUCTION

Renal disease can be defined as either acute or chronic, where the onset and duration of symptoms constitutes the divide between them. Diseases that fall under the two categories do however share common traits, as to the influence on renal function and damage to the renal tissue (1).

Chronic kidney disease is defined by a gradual loss of renal function and increasing tissue damage, that with time can develop into end stage renal disease (ESRD). Severity and progression of disease is assessed through the measurement of glomerular filtration rate (GFR) and albuminuria, and stages of chronic kidney disease are classified by these two parameters (2). The main underlying causes of chronic kidney disease are hypertension and diabetes, but about 20% of cases is due to an underlying glomerulonephropathy (1, 3).

Approximately 10% of the world population is believed to suffer from chronic kidney disease (CKD). Chronic kidney disease contributes to an increased morbidity and mortality, both directly and indirectly through the increased risk of cardio-vascular disease. It also entails an economical burden for our societies, due to the often delayed diagnosis of disease and costly treatments for ESRD (4). The lack of specific and curative treatments for the underlying diseases also pose a problem, since it increases the risk of CKD progression (3).

Although the etiology of disease differs in CKD patients, the alterations in renal tissue morphology that occur are similar. A common final pathological observation in CKD patients is the presence of glomerular sclerosis (2). This is in turn brought on by the damage to the glomerular cells that the underlying disease has caused.

The focus of this thesis has been the podocytes, resident cells of the glomeruli, the filtration units of the kidney. Podocyte-specific intracellular processes contribute to the glomerular morphological changes and loss of glomerular function observed in CKD, secondary to both glomerular nephropathies and diabetic and hypertensive disease (2, 5). The aim of the work presented in this thesis was to investigate podocyte intracellular signaling processes, as a means of improving the current knowledge of podocyte biology. Such knowledge could in the larger perspective bring the community one step closer to finding new and viable treatments for patients suffering from CKD.

1.1 The kidney

The kidneys possess various functions, all essential for the maintenance of body homeostasis. Most well-known is the function of filtration of blood and excretion of waste from our bodies. Other functions are those that regulate volume balance and electrolyte equilibrium, acid-base balance, blood pressure, and the production and secretion of different hormones such as erythropoietin, important for erythropoiesis (6).

Located to the upper, dorsal part of the abdomen, the paired kidneys are protected by the ribcage and several layers of adipose and fibrous tissue. It is a well perfused organ, with approximately 20% of the cardiac output being led to the kidneys via the renal arteries. The renal tissue is divided into mainly two anatomical regions: the cortex and the medulla. Within these two regions, the filtration of blood and modulation of urine content is performed in the functional units called nephrons (1, 7).

About 1 million nephrons can be found in each kidney, were filtration of blood occurs in the glomerulus. Subsequent urine modification is achieved through secretion and reabsorption processes in the extensive tubular system of the nephron, which is mainly located within the cortex. Electrolytes, glucose and smaller molecules are freely filtered over the filtration barrier, along with small metabolites such as creatinine and toxic drug waste. In the tubular system, water, most of the electrolytes and glucose are reabsorbed, whilst waste and excess ions can be secreted. Absorbed solutes are brought back to the circulation via peritubular capillaries and the vasa recta. Through these processes, the 180 liters of primary urine filtered each day is reduced to the approximate 1.5 liters that constitute the final urine (6, 7).

1.2 The glomerulus

The glomerulus is a capillary tuft enclosed in a sheet-like tissue called the glomerular capsule or Bowman's capsule (7). As previously mentioned, the glomerulus is the site for filtration of blood, which occurs over the glomerular filtration barrier. This barrier consists of the endothelial cells of the capillaries, the glomerular basement membrane and the glomerular visceral epithelial cells, known as podocytes. Covering the surface of Bowman's capsule are the parietal epithelial cells. The composition of the filtration barrier is constructed to allow for the passage of smaller molecules and water, whilst retaining other components of the blood such as erythrocytes and larger proteins like albumin (8). To achieve this efficient filtration, the barrier is highly selective on the grounds of size and charge of molecules. This selectivity is attained by the

contribution of each of the layers in the filtration barrier, further discussed below.

The endothelial cells in the glomerular capillaries are highly fenestrated, allowing for passage of water, electrolytes, glucose and other small molecules (9). The endothelial cells have a negatively charged and relatively thick endothelial surface layer (ESL) that consists of a glycocalyx and an endothelial cell coat. The ESL is composed of glycoproteins, glycosaminoglycans and proteoglycans, that contributes to the charge and size selectivity of the glomerular filtration barrier (10, 11). The glomerular basement membrane is a specialized and thick basement membrane, arising through the fusion of the basal membranes of the endothelial cells and the podocytes. Components of this layer consist of laminin, collagen IV and nidogen (12). Podocytes cover the outer surface of the glomerular capillaries, and will be discussed in detail in the section below.

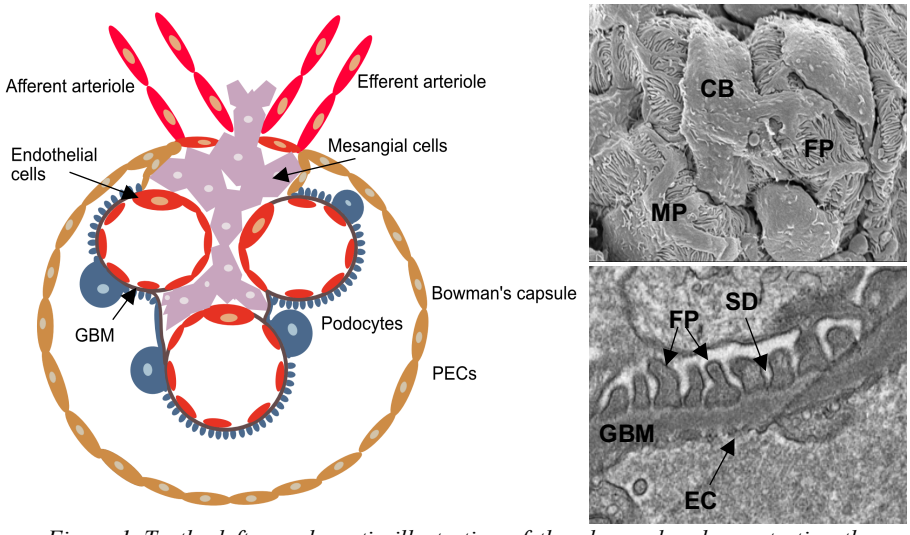

Figure 1. To the left: a schematic illustration of the glomerulus demonstrating the capillaries with endothelial cells, the glomerular basement membrane (GBM) and podocytes covering the outer surface of the capillaries. Depicted is also Bowman's capsule lined with parietal epithelial cells (PECs) and the afferent and efferent arterioles leading the blood to and from the glomerulus. Upper right: SEM micrograph showing the podocytes seen from the urinary space, CB= podocyte cell body, MP = major process, FP= foot processes. Picture courtesy of Dr Kerstin Ebefors. Lower right: TEM micrograph showing the glomerulus and filtration barrier. SD = slit diaphragm, EC = endothelial cells.

A third cell type expressed in the glomerulus is the mesangial cell. The mesangial cells and their matrix provide the glomerular capillaries with structural support. Through their smooth muscle cell like contractile properties, they can regulate the capillary blood flow. The mesangial cells also contribute to the glomerular crosstalk between cells, seemingly necessary for the maintenance of the glomeruli (13, 14).

1.3 The podocytes

The podocyte is a specialized epithelial cell with an intricate morphology. From its cell body, there are major processes expanding that are dividing in an arborizing fashion into the foot processes, the most distal parts of the podocyte relative to the cell body. The foot processes (FP) cover the outer surface of the glomerular capillary in an interdigitating pattern. Specialized protein complexes called slit diaphragms (SD) span the gap between the neighboring foot processes (15). The SD is a specialized intercellular junction reminiscent of an adherens junction (16, 17), consisting of a zipper-like formation of proteins, where nephrin is a core protein (18). Urine filtrate passes over the SD surface, and due to the size of the pores formed by the network of SD-proteins, the selectivity of the filtration barrier is upheld by hindering larger molecules, such as albumin, to enter the urinary space in Bowman's capsule (15).

Major processes contain microtubules and intermediate filament whilst the podocyte foot processes are upheld by a bundled actin cytoskeleton, with contractile properties and a cortical network of actin fibers (19, 20). Podocyte FPs are divided in different domains, the apical, the SD and the basal domain (19). Each of these domains are connected to the actin cytoskeleton of the podocytes, either through focal adhesion proteins in the basal domain or the actin binding SD proteins. A negatively charged glycocalyx, located to the apical domain, causes a repellant anionic charge that provides additional support for the foot process structure (9).

Foot processes, along with their SDs, need to adapt to the changes that occur in the glomerular milieu, in order to maintain an intact filtration barrier. Dynamic regulation of the actin cytoskeleton is therefore important for the maintenance and plasticity of the foot processes. Interacting actin binding proteins, focal adhesion proteins and SD proteins stabilizes the actin cytoskeleton and partake in the signaling cascades that regulate actin cytoskeleton dynamics (21).

Harm to the podocyte or interference with any of the domains of the foot processes, results morphologically in what is known as foot process effacement

(FPE). FPE constitutes a flattening and retraction of podocyte foot processes, during which a dynamic reorganization and disruption of the actin cytoskeleton is observed. In the process, the SD structures are lost as well. As a consequence, the size selectivity of the filtration barrier is lost, and proteinuria arises (22, 23).

1.3.1 Actin cytoskeleton

The podocyte actin cytoskeleton is a dynamic entity, which is continuously regulated in response to cellular or environmental changes. The actin cytoskeleton of podocytes consists of F-actin polymers, together with myosin-II, α-actinin-4 and synaptopodin. F-actin and myosin-II together form units also known as stress fibers. Through the bundling of these fibers by α-actinin-4 and synaptopodin the contractile actin bundles that allow for foot process dynamics are formed (19, 24). Through focal adhesion proteins such as talin, the actin cytoskeleton is connected to the α3β1-integrins, the main mediators of podocyte attachment (25). Furthermore, the actin cytoskeleton is connected to essential SD proteins such as nephrin through several adaptor proteins.

Besides acting as a physical support for the actin cytoskeleton, the SD and focal adhesion (FA) compartments with their proteins constitute two important sites for actin cytoskeleton regulation (26). Slit diaphragm proteins like podocin and nephrin are both known to mediate extra- and intracellular signals that leads to actin cytoskeleton regulation (16). The importance of nephrin for SD integrity was revealed by the finding that a mutated form of the protein causes the nephrotic syndrome of the Finish type, a disease in which no SDs are formed (18, 27). The cytoplasmic tail of nephrin mediates interaction with actin cytoskeleton associated proteins such as CD2AP and Nck, that in turn regulate actin dynamics through interaction with proteins such as cortactin and Arp2/3, that stimulate actin polymerization (19). Through adaptor proteins such as CD2AP and MAGI-1, nephrin is also connected to the actin regulating protein synaptopodin. Besides the actin bundling effect described previously, synaptopodin plays an important role in the regulation of actin cytoskeleton dynamics as well. Loss of synaptopodin renders the podocyte depleted of stress fibers, demonstrating the importance of this protein for actin cytoskeleton formation (24). Synaptopodin has been found to regulate stress fiber formation through the inhibition of proteasomal degradation of Nck and RhoA, a GTPase known to promote stress fibers (28, 29).

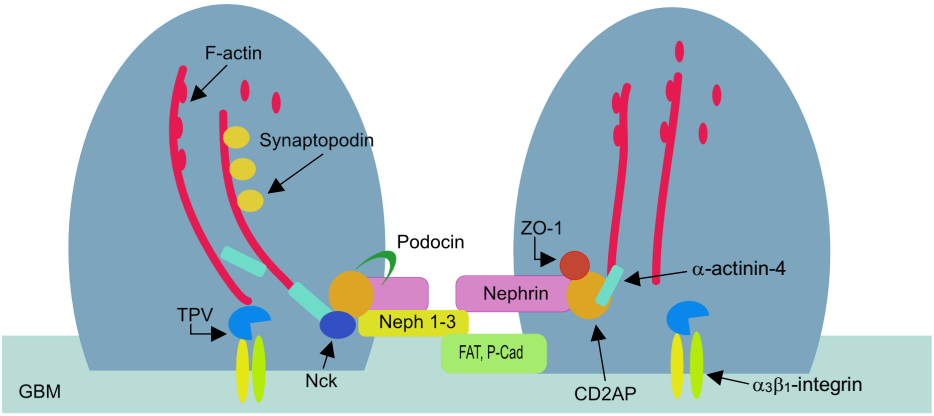

Figure 2. Schematic illustration of podocyte foot processes and the intermediate slit diaphragm. Depicted are the proteins pertaining to the slit diaphragm, as well as adhesion proteins and the actin cytoskeleton with actin binding proteins. These proteins are important for maintenance of podocyte structure and function. (TPV = talin, vinculin, paxillin)

The importance of α3β1-integrin mediated adhesion for foot process integrity is proven by the findings that podocyte-specific depletion of either subunit leads to foot process effacement and proteinuria, with early onset of renal failure in mice (30, 31). α3β1-integrins bind to several adaptor proteins that mediate their interaction with the actin cytoskeleton and proteins regulating actin cytoskeleton dynamics such as cortactin. These adaptor proteins also mediate the interaction with RhoGTPases, a group of well-known actin cytoskeleton regulating proteins (26).

1.3.2 RhoGTPases

The Rho family of small GTPases are a group of proteins pertaining to the larger family of Ras-related small GTPases, known to be involved in the regulation of several intracellular processes. One of their most prominent roles is the role as actin cytoskeleton regulators (32). Out of the 22 proteins that constitutes the family of RhoGTPases, RhoA, Rac1 and Cdc42 are the most well-described regulators of actin cytoskeleton dynamics, and since their discovery in the early 90's these proteins have been the subject of research in many areas of cell biology (33).

Through the study of fibroblasts, it was found that RhoA regulates the formation of stress fibers and focal adhesions (34). Rac1 was identified as a

regulator of lamellipodial formation (35) whilst Cdc42 was shown to induce filopodial protrusions (36).

The RhoGTPases act as molecular switches that cycles between a GDP-bound, inactive state and a GTP-bound, active state. Once in the GTP-bound state, the RhoGTPases signals to downstream effectors for the mediation of intracellular processes, there amongst actin cytoskeleton regulation (33). Regulation of RhoGTPase activity is mediated through three groups of proteins: Guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs) and Guanine nucleotide dissociation inhibitors (Rho-GDIs). GEFs catalyzes the exchange of GDP for GTP for the activation of RhoGTPases. GAPs increase the intrinsic GTPase activity and thereby inactivates the GTPases (37). RhoGDIs sequesters the inactive RhoGTPases, and control their intracellular localization and maintains a readily available source of inactive RhoGTPases in the cytosol (38). Other forms of RhoGTPase activity regulation exists as well, such as prenylation (39), a post translational modification that will be discussed later.

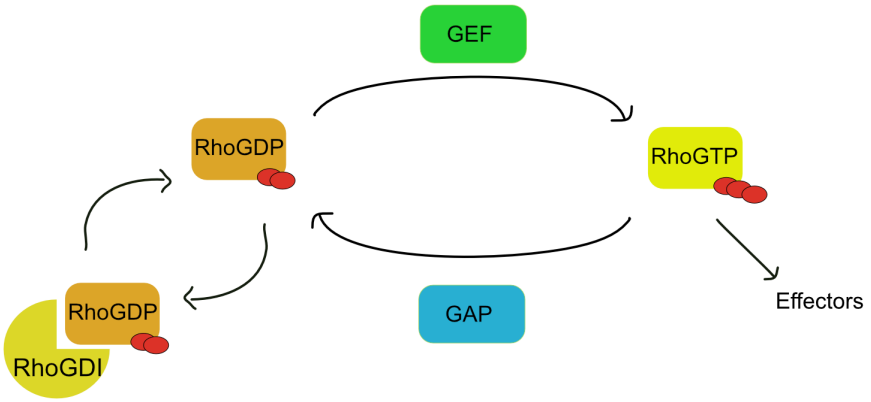

Figure 3. Schematic illustration of regulation of RhoGTPase activity. Inactive Rho-GDP is harbored by Rho guanine nucleotide dissociation inhibitors (RhoGDIs) in the cytosol. Activation is performed by guanine nucleotide exchange factors (GEFs) that catalyzes the exchange of GDP for GTP. GTPase activating proteins (GAPs) inactivate the RhoGTPases by increasing innate GTPase activity.

Considering that a dynamic actin cytoskeleton is a necessity for podocyte health, the role of RhoGTPases as actin cytoskeleton regulators has rendered them the focus of investigation for the past decades. Collectively, this research has shown that a balanced activity between RhoGTPases is necessary for the health of podocytes (40).

Overexpression experiments of Rac1 and RhoA in podocytes have both been shown to cause actin cytoskeleton dysregulation with FPE and proteinuria in mice (41-44). Loss of podocyte Rac1 has not been demonstrated to cause an overt renal phenotype, but has proved to regulate the glomerular response to stressful stimuli in different ways (45). The loss of Cdc42 was however observed to cause both early onset nephrotic syndrome and congenital nephropathy in mice (45, 46). Podocyte specific depletion of RhoA did not cause renal pathology (47), however, the expression of dominant negative RhoA led to the effacement of FPs and proteinuria in mice (44). Collectively these studies show the importance of each individual RhoGTPase for podocyte integrity.

The identification of mutations that lead to an altered RhoGTPase activity in human glomerular disease, further demonstrate the importance of these proteins for podocyte function. Mutations in Arhgap24, a GAP regulating Rac1, was identified in cases of familial FSGS with increased Rac1 activity in podocytes (48). Similarly, a mutation found in ARHGDIA, a RhoGDI, was found to cause childhood onset and congenital nephrotic syndrome and an increase in GTP-loading of Rac1 and Cdc42 (49). Mutations in INF2, commonly known to cause familial forms of FSGS, have further been recognized to cause increased RhoA-mediated signaling in podocytes (50, 51).

RhoGTPase activity in podocytes is stimulated by several pathways, through the activity of membrane receptors and ion channels as well as cytoplasmic proteins (15). A central protein in RhoGTPase signaling in podocytes is synaptopodin. As previously mentioned, synaptopodin increases the activity of RhoA by inhibiting proteasomal degradation of RhoA (29) but it is also known to regulate Cdc42 by suppressing its activity (52). Stable synaptopodin expression is needed for RhoA activity, which is regulated by the binding to 14-3-3 that protects against synaptopodin degradation (53). Degradation of synaptopodin is regulated by the calcineurin mediated dephosphorylation of synaptopodin, which disrupts the binding to 14-3-3 (53). Activity of calcineurin can be induced by TRPC5 (54), a Ca^{2+} ion channel known to induce Rac1 activity and induce proteinuria in mice (55). The binding of calcineurin to synaptopodin was recently shown to be further enhanced by the tyrosine kinase Src, acting downstream of the epidermal growth factor receptor (EGFR), a receptor also known to regulate TRPC5 membrane insertion (54). In this study, it was shown that synaptopodin acts as a regulator of the balance between RhoA and Rac1 activity, by integrating the synaptopodin degradation signaling induced by TRPC5 and EGFR with signaling from synaptopodin stabilizing kinases (54). This pathway of RhoGTPase regulation is just one of

many intricate RhoGTPase signaling pathways that have been identified in podocytes.

1.4 Glomerular disease

Glomerular diseases do often not present themselves with specific symptoms, and are rather more often found in routine medical examinations or during the investigation of general symptoms such as anemia and fatigue (3). In some cases, the presentation is clearer, and patients present with edema and massive urinary protein loss, in what is known as the nephrotic syndrome. The underlying cause of glomerular disease varies, depending in part on geographical and socioeconomic factors. Genetic composition, autoimmunity, malignant disease and infectious diseases are all known causes of glomerular disease (3, 56).

Renal tissue biopsy is key to diagnosis, and is deemed the most important diagnostic tool in glomerular disease with the exception for glomerular disease in children (57). Classification of disease is based on the visual assessment of glomerular morphology, taking into account the involvement of specific cell types, changes in structures in the glomerular barrier, deposition of immunoglobulins, presence of sclerosis and to what extent that the disease affects the glomeruli (58). In some diseases, circulating factors in plasma are connected to disease and can be measured in order to diagnose or follow progression of disease. Proteinuria of varying degree is present in all glomerular diseases, and does in combination with hypertension constitute a risk factor for progression of disease (56).

The sequential findings of mutations in podocyte proteins as leading causes in nephrotic disease identified the podocyte as a major contributor in the development of glomerular disease and proteinuria. One such finding was the identification of mutated nephrin as a cause of nephrotic syndrome of the Finish type (27). Subsequent findings later identified the important role of the podocyte actin cytoskeleton in development of glomerular disease, such as the identification of mutations in α-actinin-4 in familial cases of focal segmental glomerulosclerosis (FSGS) (59). Since then, several mutated proteins important for podocyte function have been identified in cases of FSGS and nephrotic syndrome (21).

1.4.1 Nephrotic syndrome

Urine normally only contain small amounts of protein, in a range between 40-80mg/day. This protein consists mainly of low molecular weight plasma proteins that are filtered over the filtration barrier. Albumin normally constitute around 30% of the urinary protein content. Modification of urinary protein content is achieved through the active reabsorption of both smaller proteins and the filtered albumin by the cells in the tubular system (60, 61).

Disruption of the glomerular filtration barrier leads to what is known as glomerular proteinuria, which entails a loss of proteins with a larger molecule weight, such as albumin. The subsequent increased protein content within the tubular system will induce inflammatory and fibrotic processes that affects renal function and leads to a progression of disease (60).

Nephrotic syndrome is a pathological state caused by glomerular disease, with heavy proteinuria, largely consisting of albumin. The syndrome is defined by a daily urinary loss of proteins over 3.5g, with subsequent development of edema, hypoalbuminemia and hyperlipidemia. The syndrome does also entail an increased risk for thrombotic events and predispose for infections (62). Several of the glomerular diseases discussed below can lead to or present themselves through the nephrotic syndrome (63).

1.4.2 Focal segmental glomerulosclerosis

Focal segmental glomerulosclerosis (FSGS) is a term used to both describe pathologic changes in glomerular morphology as well as the name of a group of glomerular diseases (3). Within the group of FSGS are different subgroups formed based on the pathogenesis of the disease. Primary or idiopathic disease describes those cases where a cause for pathology remains unidentified. The remaining cases are divided in hereditary or genetic FSGS, adaptive FSGS, drug induced FSGS and FSGS secondary to viral infections (64). Patients suffering from primary FSGS tend to present with heavy proteinuria, whilst remaining groups usually show milder degrees of proteinuria. Primary FSGS is believed to be caused by a circulating factor, since observations have been made of recurrent FSGS disease in renal transplants (65).

Central to the development of FSGS is the damage to podocytes and processes of scarring within the glomeruli that leads to formation of sclerotic lesions. These lesions are distributed within and amongst the glomeruli in a focal and segmental pattern, as indicated by the name (66, 67). FSGS mainly affects adults, but the disease shares some pathophysiological traits with minimal

change disease (MCD), the most common glomerular nephropathy observed in children (64).

1.4.3 Membranous nephropathy

Membranous nephropathy (MN) primarily affects adults, and onset of disease is usually associated with heavy proteinuria, often in the form of nephrotic syndrome. MN is characterized by subepithelial immune complex depositions and thickening of the GBM (3). The disease is divided in two groups: a primary, autoimmune form of MN and MN seen in connection to systemic lupus erythematosus (SLE), or induced by infectious or malignant disease. The autoimmune form of MN is characterized by circulating antibodies directed against podocyte antigens, PLA2R and THSD7A (57).

1.4.4 Diabetic nephropathy

Diabetic nephropathy (DN), often referred to as diabetic kidney disease (DKD), develops secondary to diabetes mellitus type 1 and 2 (DM1 and DM2), and constitutes one of the major causes of ESRD worldwide (68). Somewhere between 30-40% of patients suffering from diabetes develop DN, with increasing risk attributable to insufficient glycemic control and hypertension (69). DN affects all compartments of the glomerular filtration barrier, with GBM thickening, mesangial cell expansion, loss of endothelial fenestrations and podocyte FPE. Eventually, glomerular sclerosis and tubulointerstitial fibrosis arises (69).

1.4.5 Treatments in glomerular disease

Central aims in treatment of glomerular disease are the relieving of symptoms and the management of factors that induce a risk for progression of disease. Hypertension and proteinuria are two symptoms as well as risk factors for disease progression that covaries with each other. Blood pressure control is mainly attained through the use of blockers of the renin-angiotensin system (RAS-blockers). As proteinuria to a large extent is alleviated by the reduction in blood pressure, RAS-blockers are used in the treatment of proteinuria as well. Hyperlipidemia is targeted through the treatment with statins, reducing the cardiovascular risk associated with renal disease (3, 57). In nephrotic patients with increased risk for thrombotic complications, the treatment with anticoagulants can be needed.

Immunosuppressants constitute the main agents used to target the underlying glomerular disease. Diseases such as MCD and FSGS are both initially treated with cortisone. Second line treatments in adult FSGS patients non-responsive to steroid treatment is the use of either calcineurin inhibitors or mycophenolate

mofetil (64). In MN, the initiation of immunomodulatory treatment is recommended to await until the point where renal function is starting to decline whilst simultaneously optimizing the treatment of risk factors such as proteinuria and hypertension. Since spontaneous remission can be observed in some cases, it is wise to wait with the secondary treatment, which in MN mainly consists of treatment with alkylating agents such as cyclophosphamide. At times, calcineurin inhibitors are used as well (3, 57).

Rituximab and adrenocorticotropic hormone (ACTH) are two additional treatments that have been trialed in treatment of both MN and FSGS (3). Rituximab has shown promising effects on reduction of proteinuria in patients with MN in a recent randomized controlled trial (70). Used as a treatment of nephrotic disease in the 1950's and 60's, ACTH was discontinued as a therapy when more easily administered cortisone medications became available (71). In 1999 however, it was found that ACTH had positive effects on glomerular function and proteinuria in patients with MN (72). Since then, ACTH has been used in treatment of several glomerular diseases with positive effects on disease remission (73-75), however there is still a need for randomized controlled trials to establish the role of ACTH as treatment in glomerular disease.

The immunosuppressant therapies used in treatment in glomerular disease do however pose risks and complications for patients. Side effects associated to cortisone treatment are well known and include hypertension, glycemic instability and development of diabetes mellitus and osteoporosis, to name a few (76). Alkylating agents such as those used in MN entail an increased risk for malignant transformation and infertility. Further on, they all cause an increased risk for infections (57). Collectively, this prompts the search for new and targeted therapies, with less adverse effects.

1.5 Melanocortin 1 receptor

The melanocortin receptor family (MCR) consist of 5 different G-protein coupled receptors (GPCRs) that show a differential distribution within tissues of the body. The receptors are targeted by the melanocortins, peptide hormones derived from the precursor pro-opiomelanocortin (POMC). Through proteolytic cleavage, POMC gives rise to ACTH, α-MSH, β-MSH and γ-MSH, hormones that bind to the 5 receptors with varying affinity (77).

Named in the order they were identified, melanocortin 1 receptor (MC1R) was first identified to be expressed in melanocytes and melanoma cells, but has further been identified in macrophages and neural tissue. MC2R, which binds

specifically to ACTH, is located to the adrenal glands and stimulation of the receptor induce steroid synthesis. MC3R and MC4R, located in different parts of the nervous system, are both known to affect processes related to energy metabolism and food intake. Mutations in MC4R are a known underlying cause of obesity. MC5R is expressed in most tissues, and stimulate processes in lacrimal and sebaceous glands (78).

MC1R is expressed in melanocytes where it regulates the production of melanin that gives rise to skin pigmentation. Upon stimulation, the MC1R redirects the production of the melanin from the reddish-feomelanin to the brown-black eumelanin (79). Stimulation of MC1R has also been found to mediate protective effects through the reduction of UV-induced oxidative stress and through regulation of DNA-damage repair (80-82).

Activation of MC1R stimulates an increase in cyclic AMP, that further stimulates the activity of protein kinase A (PKA) and downstream transcription factors, mediating the effects described above (79). Secondary signaling pathways downstream of MC1R entail signaling through ERK1/2, whose activity following MC1R activation can be initiated in both cAMP dependent and independent ways, and that has a role in regulation of cell proliferation (83).

Following the findings by Berg et al. regarding beneficial effects of ACTH treatment in MN (72), our research group set out to identify the possible mechanism of action of ACTH in reducing proteinuria and improving glomerular filtration. In our studies, we identified MC1R to be expressed in human podocytes and demonstrated the protective effect of MC1R-specific stimulation in the Passive Heyman nephritis-model of renal disease (84). These findings led to the proposition that MC1R mediated the beneficial effects of ACTH treatment in glomerular disease. Subsequent in vitro studies showed that MC1R specific stimulation in podocytes protected against PAN induced damage through reduction of oxidative stress and increased RhoA activity, leading to a stabilization of stress fibers (85).

1.6 Epidermal growth factor receptor

The epidermal growth factor receptor was one of the first receptor tyrosine kinases (RTKs) to be identified, and is expressed in many different cells throughout the body (86). Activation of the receptor results in autophosphorylation of the receptor and phosphorylation of interacting proteins, that initiates the signaling cascades downstream of the receptor. These signaling cascades include MAPK and PI3K signaling pathways (87). The receptor regulates proliferative processes, differentiation and migration in cells, qualities that has made the receptor the center in cancer research and in treatment of malignant disease (86, 87). Activation of EGFR also mediates the membrane insertion of the Ca^{2+} regulating ion channel TRPC5 (88).

The EGFRs consists of a family of four receptors, ErbB1-4, which can be found differently expressed in cells of the kidney, and that play an important role in renal development. EGFR, also known as ErbB1, is known to be expressed in podocytes (87). Several ligands can stimulate the EGFR, there amongst epidermal growth factor (EGF) and transforming growth factor-α (TGF-α). Interestingly, some of these ligands have been identified in renal disease models (89) and an atypical EGFR activator, the polycation protamin sulphate (PS), is a commonly used agent in renal research (90, 91). The signaling through EGFR has also been implicated in processes that results in renal disease. EGFR signaling in podocytes has been suggested to contribute to the development of rapidly progressive glomerulonephritis (92). Further on, it has been proposed that EGFR signaling plays an important role in the development of DN (87). In support of this hypothesis, it was shown in a study by Chen et al. that depletion of EGFR in podocytes protected against development of DN in the streptozotocin-model of type 1 diabetes (93).

In a recent publication, EGFR activity was further shown to affect podocyte physiology by the disruption of actin cytoskeleton formation. Activation of EGFR potentiated the TRPC5 induced calcineurin mediated synaptopodin degradation. This led to the inhibition of RhoA activity due to destabilization of synaptopodin and loss of stress fibers. EGFR activity also induced Rac1 activity, which further influenced the formation of stress fibers (54). Hence, EGFR signaling in podocytes seem important for actin cytoskeleton regulation and is also a likely contributor to glomerular disease.

1.7 Prenylation

Prenylation, at times referred to as isoprenylation, is a post-translational modification of proteins that occur in all eukaryotic cells (94). It comprises a covalently bound lipid-modification that is important for membrane localization and interaction of proteins, as well as regulation of protein activity (95). Three enzymes are responsible for the prenylation of proteins, farnesyl transferase (FTase), geranylgeranyl transferase type I (GGTase-I) and geranylgeranyl transferase type II (GGTase-II). FTase and GGTase-I are considered to belong to the same subclass of prenyl-transferases since they target proteins that harbor a C-terminal CAAX-motif, whilst GGTase-II specifically targets the Rab family of proteins, containing the CXC or CCXX motif (94). FTase is responsible for the transfer of a 15-carbon farnesyl isoprenoid group to the CAAX-motif of proteins such as RasGTPase proteins and laminin B. GGTase-I regulates the attachment of a 20-carbon geranylgeranyl isoprenoid group to CAAX-proteins such as Rho and RasGTPases. Following the attachment of the prenyl-group, two additional enzymatic steps are performed that enhance the membrane localization of the proteins. First, the -AAX motif is cleaved by the endoprotease Ras-converting enzyme 1 (RCE1), followed by methylation of the prencysteine by isoprenyl-cysteine carboxylmethyl-transferase (Icmt) (96).

The lipid substrates used for prenylation, farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), are products of the mevalonate pathway. The mevalonate pathway produces sterols and isoprenoids, such as FPP and GGPP, that are important for cellular function and survival (97). Mevalonic acid is first formed by the HMG-CoA synthase and HMG-CoA reductase using acetyl-CoA and acetoacteyl-CoA as substrates. Through further processing, mevalonic acid becomes isopentenyl diphosphate (IPP), that is further processed into FPP (98). Through condensation, FPP is transformed into GGPP. FPP and GGPP are thereafter used as substrates in prenylation, but do also constitute substrates in the production of cholesterol and ubiquinone or dolichol, respectively (99).

The FTase and GGTase-I share a common α-subunit but differ in their β-subunit, which provides selectivity for their isoprenoid substrate. Recognition of correct protein substrate for prenylation is partly regulated by the X amino acid in the CAAX-motif, and the enzymes are normally selective in their choice of protein substrate. There is however some cross reactivity regarding protein substrate between the two enzymes (94).

The process of prenylation has become of interest in therapeutical contexts. Direct inhibitors of prenylation, FTase inhibitors (FTIs) and GGTase-I inhibitors (GGTIs), have been developed with the intention of inhibiting Ras and Rho-protein activity in malignant disease (99). GGTIs have further been proposed as therapy for inflammatory and autoimmune diseases (95), such as multiple sclerosis (100), since RhoGTPases are known to regulate inflammatory processes and immune cell functions (101). Indirect interference with prenylation can further be observed secondary to treatment with statins, which inhibit HMG-CoA reductase (102), as well as nitrogen containing bisphosphonates (N-BPs), agents used to treat osteoporosis and myeloma, that target the farnesyl diphosphate synthase and inhibits the formation of FPP (103). The inhibition of prenylation secondary to these treatments are believed to regulate the pleiotropic anti-inflammatory effects of statins (104) and the inhibitory effects of N-BPs on the formation of osteoclasts and bone resorption, respectively (103).

1.8 βPIX

βpix, also known as Cool-1, p85SPR, and Arhgef7, is a guanine nucleotide exchange factor that was first identified in 1997 as a focal adhesion associated protein in epidermoid carcinoma A431 cells (105). In the following years, an intense investigation of βpix was conducted that led to several sequential findings. βpix was found to induce activation of Rac1 and to be binding PAK, an effector protein of Rac1, through a SH3-domain (106). Further studies showed that βpix was interacting with GIT1 in a protein complex, that also included the focal adhesion protein paxillin (107). Thereafter it was described that βpix was bound to and targeted Rac1 to the cell membrane, where it induced Rac1 activity and formation of membrane protrusions (108). Since then, the protein complex consisting of βpix, GIT, PAK and paxillin, has been described to cycle between intracellular compartments (109), where phosphorylation of paxillin homes the complex to focal adhesions (110). GIT1 is a GAP for ADP-ribosylation factor (Arf) small GTP-binding proteins, proteins that are involved in cell adhesion and membrane trafficking. At focal adhesions, GIT acts to decrease Arf-signaling whilst βpix stimulates Rac1/Cdc42 activity. These characteristics allow the protein complex to act as a regulator of cell adhesion and actin cytoskeleton dynamics in processes such as cell migration (111), where the protein complex has been suggested to regulate cell polarity and provide migratory direction (112).

βpix mediated regulation of focal adhesion and actin cytoskeleton dynamics occur in many different cell types, both under normal physiological settings

and during pathological processes. In colorectal adenocarcinoma, βpix is described to drive tumor metastasis through actin cytoskeletal regulated migration of cells (113). Similar descriptions of βpix have been made in lung cancer cell migration, where βpix besides regulating actin dynamics, also regulates focal adhesion formation (114). The role of βpix in regulation of focal adhesion formation has been described in additional studies, where it influenced the motility of keratinocytes and fibroblasts (115, 116).

Besides localizing to focal adhesions for regulation of cell migration, the complex has also been found in intracellular vesicles and neuronal synapses (111). Actin polymerization regulated by βpix has been found important for the localization of synaptic vesicles in rat brain (117). Further on, the activity of βpix and regulation of Rac1 activity has been found important for the spinogenesis in neural cells in the development of synapses (118). In said article, site-specific phosphorylation of βpix promoted affinity for Rac1 and regulation of Rac1 activity during spinogenesis. GEF activity has been found to be regulated by phosphorylation (39) and site-specific phosphorylation of βpix has been described to differently regulate the activity of βpix towards Rac1 and Cdc42 in several studies (119-122). The phosphorylation pattern that determines βpix affinity for downstream RhoGTPases seemingly depend on the stimulating agent and kinases activated downstream of the initiating stimulus.

Expression of βpix in kidney cells has been described in tubular epithelial cells, mesangial cells and podocytes. In collecting duct cells, βpix is described to regulate the activity of sodium channels, ENaCs (123), whilst it regulates endothelin mediated actin cytoskeleton dynamics in glomerular mesangial cells (120, 124). In podocytes, it was recently described that one function of βpix was to maintain filtration barrier integrity through mediating Cdc42 activity and inhibition of podocyte apoptosis (125).

Following the first identification of βpix, a homologous protein called αPix was identified (106). αPix and βpix are encoded by the genes *ARHGEF6* and *ARHGEF7*. The two GEFs are similar in structure and share all domains except for the calponin domain at the N-terminus of αPix. Whilst only one form of αPix has been described (106), several isoforms of βpix have been identified (126, 127). The isoforms are however still not fully characterized and mainly one of these have been investigated in the renal cells described above (128).

2 AIMS

The overall aim of the work performed in this thesis was to investigate actin cytoskeleton regulatory pathways in podocytes, with a focus on regulation of RhoGTPase proteins.

The specific aims of the studies were:

Paper I: To elucidate the mechanisms by which melanocortin receptors mediate renoprotection, by investigating MC1R expression profile in renal biopsies from CKD patients and defining how activation of the MC1R stabilizes podocyte actin cytoskeleton.

Paper II: To investigate the role of prenylation in the regulation of RhoGTPase activity and actin cytoskeleton regulation in podocytes *in vivo* and *in vitro.*

Paper III: To identify guanine nucleotide exchange factors, GEFs, important for regulation of RhoGTPase activity in podocytes, with the special focus on the GEF βpix.

3 METHODOLOGICAL CONSIDERATIONS

This section presents and discuss the selected research methods in this thesis. For precise descriptions of methodological steps and protocols, please refer to the methods section in respective papers.

3.1 Ethics

The experiments conducted with human material (Paper II) were performed in accordance with the declaration of Helsinki. Ethical approval for collecting human biopsies was given by the Gothenburg regional ethical board (#R110-98) and an informed consent was signed by the patient prior to the collection of renal biopsies. For experiments performed on mice, (paper II and III), ethical approval was given by the Regional Laboratory Animal Ethics Committee of Gothenburg (#67-2016 and #109-2012). The experiments performed on rats (Paper I) were approved by the Mallinckrodt Pharmaceuticals Institutional Animal Care and Use Committee (#15-06), and performed according to guidelines by the Institutional Animal Care and Use Committee, IACUC, in the United States.

3.2 Patient material

Renal biopsy material was obtained from tumor associated nephrectomies performed at Sahlgrenska University Hospital. A part of the non-affected cortex of the kidney was collected and thereafter cryo-preserved in optimal cutting temperature compound (OCT). Cryo-sections of renal cortex sections were used in paper II in order to study the glomerular localization of Geranylgeranyl transferase type I, described in more detail in section 3.6.6.

3.3 Animal studies

The general approach in research is to reduce the use of animals in experiments. *In vitro* experiments can provide a vast amount of information on the function of a protein or a gene on an intra- and intercellular level, why these methods should be applied to a large extent. However, information regarding how the gene or protein of interest affects, or is affected, by factors in their i*n vivo* environment, cannot be obtained from experiments *in vitro*. For such information, *in vivo* animal models are still the standard method to use. Through the use of animal models, it is possible to understand the physiological and functional relevance of a gene or protein, as in paper I-III.

Although humans share many physiological and biological properties with animals used in research, it should be noted that differences between species can affect the interpretation and translation of data. Even so, animal models offer a suitable path in the transition from *in vitro* studies to the investigation and implementation of new findings in humans.

3.3.1 Animal models of glomerular disease

There are several animal models available for the study of glomerular disease *in vivo*. In paper I, the Puromycin aminonucleoside (PAN) rat model was used to study the glomerular expression of the Melanocortin 1 receptor in a model of glomerular disease. In paper III, two mouse models of human diabetic nephropathy, the eNOS db/db mouse and the BTBR ob/ob mouse, were used to study the glomerular expression of βpix. Genetically induced models of glomerular disease are also available through the use of the podocin-Cre mice. In paper II we managed to develop a mouse depleted of Geranylgeranyl transferase type I specifically in podocytes, through breeding with the podocin-Cre mouse, resulting in a mouse with glomerular disease.

Puromycin aminonucleoside nephropathy

The PAN rat model was first discovered in the 1950's (129). Since its discovery, the PAN rat has become a frequently used *in vivo* model for MCD and FSGS (91). MCD and FSGS share histological attributes, and are in some ways considered to be a histological continuum of one another.

Sprague Dawley-rats are the prototypical animals used for this model. Administration of puromycin aminonucleoside (PA) is performed through either intravenous, intraperitoneal or subcutaneous injections. PA induced damage is mainly inflicted on the podocytes, that undergo conformational changes due to disturbance of the actin cytoskeleton and slit diaphragm proteins. The rats present with foot process effacement, foot process fusion, proteinuria and eventually podocyte apoptosis and glomerular sclerosis. Depending on the accumulative dose of PAN, the glomerular histology ranges from MCD to FSGS. A single dose injection typically leads to a glomerular histology resembling MCD with proteinuria within 4 days, which then normalizes over time. Repeated injections of PA are necessary to establish a FSGS-like histology (91).

In paper I, the PAN model was established through the administration of sequential intravenous injections of PA, a first injection of 50mg/kg, and then repeated injections of 20mg/kg on day 14, 21 and 28. The rats developed proteinuria on day 7, which peaked at day 28.

eNOS db/db mice

The endothelial nitric oxide synthase deficient mice ($eNOS^{-/-}$) have a reduced vascular eNOS activity and display endothelial dysfunction and hypertension. Through backcrossing of these mice to the db/db mouse, a mouse strain with a genetically inactivated leptin receptor, the eNOS db/db mouse was generated (130). The eNOS db/db mice develop diabetic nephropathy, DN, with early onset albuminuria, decreased GFR, mesangial expansion, thickening of the glomerular basement membrane and arteriolar hyalinosis. This mouse model also demonstrates renal changes associated with advanced DN such as focal segmental and nodular glomerulosclerosis (131). In paper III, the glomerular RNA expression of βpix was analyzed in eNOS db/db and heterozygous eNOS db/+ control mice at 10 and 18 weeks of age.

BTBR ob/ob mice

The BTBR ob/ob mouse is a leptin deficient type II diabetes rodent model with insulin resistance. This mouse model exhibits features of both early and advanced DN with an early onset of progressive proteinuria (132). The mice develop glomerular hypertrophy, focal glomerulosclerosis, mesangial matrix expansion, loss of podocytes and arteriolar hyalinosis. In paper III, the glomerular RNA expression of βpix was analyzed in BTBR ob/ob mice at 8, 14 and 20 weeks of age, on either a regular or a high protein diet, and compared to 20 week old lean control mice.

3.3.2 Cre-lox transgenic mice

The Cre-lox technique allows for targeted modulation of gene expression and is a well-established method of genetic manipulation in mice. The enzyme cyclization recombinase from bacteriophage 1 (Cre) recognizes a 34bp long loxP sequence, which is cut by the enzyme. The gene of interest is flanked by two loxP sequences, at which the gene is referred to as a "floxed" gene. Upon Cre-activity, the gene flanked by loxP is excised, generating a null allele. The system can also generate inversions and translocations of genes and chromosomes. For successful depletion of a gene, either the first exon of the gene or exons encoding functionally important parts of the protein, should be "floxed" (133). Targeted expression of Cre can be accomplished by coupling of the gene to a promotor only active in a specific cell (134, 135). In the transgenic mouse strain for targeted expression of Cre-recombinase to podocytes, the expression is driven by the promotor for the podocyte specific protein podocin, NPHS2 (136). The Cre-lox methodology also allows for temporal control through the use of inducible promotors controlled by agents such as tamoxifen (137).

The spatial and temporal targeting of a specific gene with Cre-lox enables the functional investigation of genes that with other transgenic methods could cause embryonic lethality. It is also a system preferred over other available techniques, such as Flp-FRT, due to a higher efficiency in recombination and site specificity. Although the Cre-lox mouse is favored in genetic studies, it does have its limitations. The expression of Cre can be toxic for cells, as well as act on off-target sequences. Pathology has been described in different organ systems due to expression of Cre alone. In the podocyte specific NPHS2-Cre mouse, the reported adverse effects of Cre-expression are not as severe as reported in other organs, however it can render the mice more susceptible to renal damage (138). Inclusion of mice expressing Cre, and no loxP-transgene, as control in a study is one way of overcoming these adverse effects.

In paper II, the NPHS2-Cre mouse was bred with mice expressing the loxP flanked gene for Geranylgeranyl transferase type I and Farnesyl transferase. In this paper, NPHS2-Cre mice were used as control.

3.3.3 Geranylgeranyl transferase type I depleted mice

The β-subunit of Geranylgeranyl transferase type I dictates substrate specificity of the enzyme and is encoded by the gene Pggt1b, located to chromosome 18 in mice. A conditional loxP-Pggt1b transgenic mouse was generated through the use of a targeting vector inserting loxP sequences in flanking regions of exon 7 of Pggt1b (139). Exon 7 is critical for the enzymatic activity of Geranylgeranyl transferase type I. In paper II, we established a podocyte specific Pggt1b-knockout mouse through the breeding of mixed background Pggt1b ”floxed” mice, B6.CCgTm(Pggt1bfl/fl)295Lbh/J, with mixed background NPHS2-Cre mice, B6.Cg-Tg(NPHS2-cre)295Lbh/J mice. The progenies were born in a Mendelian ratio and were fertile.

3.3.4 Farnesyl transferase depleted mice

The Farnesyl transferase subunit beta is encoded by the gene Fntb, located to chromosome 12 in mice. As for Geranylgeranyl transferase type 1, the β-subunit of Farnesyl transferase dictates the substrate specificity of the enzyme. A conditional Fntb-loxP mouse was established via a targeting vector inserting loxP-sequences adjacent to exon 1 of Fntb (140). Breeding of the Fntb-floxed B6.C-CgTm(Fntb fl/fl)295Lbh/J mice with NPHS-Cre B6.Cg-Tg(NPHS2-cre)295Lbh/J mice, generated a podocyte specific Fntb-knockout mouse. The progeny was fertile and born in a Mendelian ratio. The Fntb-knockout mice were a part of the studies carried out in paper II.

Genotype assessment

To control for the genotype of the mice prior to experiments, genotyping was done. DNA was derived from ear-clippings of mice and analyzed through PCR and subsequent agarose gel analysis of the PCR product. Primers for Podocin-Cre, Pggt1b and Fntb were used in the PCR-reaction and the primer for IL-2 was used as a control. The PCR product of Podocin-Cre is 160bp. The wild type PCR product for Pggt1b is 260bp whilst the floxed gene is 370bp, due to the flanking lox-p sequences. For Fntb, the wild type PCR product is 270bp and for the floxed gene 360bp.

3.3.5 Assessment of renal function

In paper II, we established two strains of podocyte specific knockout mice, the $Pggt1b^{fl/fl}$ and the $Fntb^{fl/fl}$ mice. To evaluate the consequences of these genetic ablations for the integrity of the podocyte as well as the glomerular filtration barrier, urinary loss of albumin and glomerular morphology was analyzed.

Housing of mice

Mice were kept in climatized rooms with 12-hour day-night cycles, and had unlimited access to food and water. For the collection of urine samples from mice, metabolic cages were used. The mice were single-housed in metabolic cages for 24 hours, with unlimited access to water and food. Metabolic cages were kept in climatized units at 21-23°C and 55% humidity. The collection of 24-hour urine is favorable over spot urine collection, since the probability of obtaining a urine sample is higher with this method and a larger volume can be collected for additional analysis.

Measurement of albuminuria

Measurement of urinary albumin-to-creatinine ratio, is an accepted method used for diagnostic and prognostic purposes of renal glomerular disease (57). In paper II, we collected urine from our mice at even intervals, allowing us to assess the phenotype of our genetically altered mice and follow the development of albuminuria over time.

Albumin in the urine from mice was measured using a Mouse Albumin ELISA kit. Creatinine was measured using a Creatinine assay kit with a colorimetric absorbance read out based on enzymatic activity. Concentrations of both albumin and creatinine was measured using a SpectraMax plate reader. An albumin-to-creatinine ratio was calculated based on the concentrations from the readings to obtain a representative value of albumin loss in urine.

The assessment of urinary albumin-to-creatinine ratio allows for a non-invasive analysis of the glomerular function and is a rather uncomplicated

analysis to perform. Due to the ratio against creatinine, the analysis is corrected for urine concentration and constitutes a reliable way of assessing albuminuria.

Gel electrophoresis and staining with Coomassie blue stain is another method for visualization and analysis of urine albumin content. In paper II, prior to the albumin/creatinine assessment, a first analysis of urine samples was done through gel electrophoresis at the time of urine collection, to screen for potential albuminuria. Urine samples were analyzed through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A 7-point albumin standard was run with the samples in the electrophoresis, to create a calibration curve used for the inference of albumin concentration in each urine sample. Obtained urine albumin concentrations were then multiplied with the total urine volume to attain the urinary loss of albumin expressed as μg/24 hours.

Morphological analysis

Glomerular diseases present themselves with similar symptoms, why the assessment of glomerular morphology in renal biopsies is essential for proper diagnosis. Changes in glomerular morphology make the basis for the classification of glomerular diseases, and several of them can be seen in relation to proteinuria (58). To assess the glomerular phenotype and structural changes in our mice in paper II, transmission electron microscopy and light microscopy was performed.

Transmission electron microscopy (TEM) is a reliable method for structural analysis of the glomeruli. It allows for high resolution visualization of the cells and structures pertaining to the filtration barrier, aiding in the assessment of changes in any of these components. Although it is a costly method, both money-wise and time-wise, the information retrieved from the analysis is invaluable.

Light microscopy of histological samples allows for determination of localization of, as well as type of, pathology in glomerular sections. Common histological stains such as hematoxylin-eosin (H&E) reveal information regarding tissue morphology, whilst Periodic acid-Schiff (PAS) staining can provide information on pathological events such as sclerosis of the glomeruli.

At termination of the *in vivo* part of the study (paper II), one kidney per mouse was collected and prepared for TEM. The renal capsule was removed and a longitudinal cut was made through the kidney, before the organ was submerged in Karnovsky fixative. Further preparation of the tissue was done according to standard procedures and are described in detail in paper II. The TalosL120C was used to obtain micrographs of the tissue sections. 3 mice per group were

analyzed and the number of foot processes per μm of glomerular basement membrane was calculated. A minimum of 3800 foot processes per group were counted.

The second kidney was collected and cut at the renal pelvis. One half of the kidney was cryo-preserved. From the renal tissue, 5μm sections were cut and the sections were used for histological analysis and stained with H&E and PAS. Analysis of the tissue sections was performed by a trained renal pathologist. 3 mice per group were analyzed.

3.4 Cell culture

In vitro studies of cells in culture allow for investigation of cellular and intracellular processes, under controlled conditions. The possibility of propagating cells results in a readily available source of material for analysis, in relatively short time, and facilitates reproduction of data. It is a very cost-efficient method in comparison to *in vivo*-studies using animal models, due to the low cost of maintaining cells in culture and their self-propagation.

Cell culture is ideal for mechanistic analysis of protein expression, localization and protein interaction in a specific cell type, where circumstances under which the cells grow can be regulated. The effects of treatments or agonists for receptors can be evaluated and involved intracellular signaling pathways can be identified, as in paper I. The possibility of genetic manipulation, either through over-expression or silencing of a gene, is also easily done in cells in culture and can relay important information on the function of a gene or protein in a cell. An additional advantage of *in vitro* studies is that potential hypotheses can be tested and evaluated before studies are performed on animals or humans.

However, one should keep in mind that cells in culture do not always behave in consistency with their *in vivo* counterparts, which can affect the translation of findings to an *in vivo* setting. Dedifferentiation of cells over time and the loss of protein expression are known side effects of culture of cells. The one-cell type culture of cells that is most common does not reflect the *in vivo* situation, where almost no organ consists of one cell type only. The intercellular communication with other cell types within the organ and in remote organs, is lost. Similarly, the normal physiological stimuli that a cell endures *in vivo*, such as blood flow and pressure changes, are lost *in vitro*. New culture methods such as co-cultures and organoids present possible ways of overcoming these obstacles, at least to some extent. In this thesis, all three

papers include *in vitro* studies where experiments were performed on immortalized murine podocytes in culture.

3.4.1 Immortalized murine podocytes

Podocytes are fully differentiated cells that, with some exceptions, do not normally proliferate well *in vivo*, nor *in vitro*. Cultivation of primary podocytes has been proven hard. Primary podocytes quickly enter into growth arrest, alternatively begin to dedifferentiate, losing the expression of specific proteins and their morphology.

Immortalized murine podocytes were first established in 1997 by Peter Mundel and colleagues and have since become one of the main tools for *in vitro* research on podocytes (141). The immortalized murine podocytes were obtained through glomerular isolation and subsequent cloning of glomerular podocytes from the transgenic H-2K^b-tsA58 mice (142). The H-2K^b-tsA58 mice express a thermo-sensitive form of the SV 40 large tumor T antigen (tsA58) under the control of the H-2K^b promotor. Activity of the H-2K^b promotor can be induced by the use of interferons.

Proliferation of the immortalized murine podocytes is controlled through temperature and the addition of interferon-γ (IFN-γ) to the cell culture media. Under permissive conditions, at a temperature of 33°C and under the influence of IFN-γ, the podocytes proliferate. Differentiation of podocytes is induced through a thermal switch to 37°C and removal of IFN-γ from the cell culture media. This results in an inactivation of the H-2K^b promotor and a temperature regulated degradation of the tsA58 T-antigen. Culturing of cells under these non-permissive conditions results in podocyte growth arrest within approximately one week and differentiation of podocytes. Mature and fully differentiated podocytes are obtained at 14 days post thermal switch.

Although the complex architectural foot processes are not present in cell culture, the differentiated immortalized murine podocytes demonstrate many other characteristics of podocytes *in vivo*. They express podocyte specific marker proteins such as nephrin, synaptopodin, Wilm's tumor 1 and podocin. They also show structural similarities such as modified slit-diaphragms and actin stress fibers (143).

Even though the culture of podocytes has been made easier through the establishment of immortalized murine podocytes, there are still some challenging aspects of the podocyte culture that needs to be considered. The cells are very sensitive to changes in temperature as well as level of confluency,

which can affect cell growth and inhibit differentiation. Proper handling and attentiveness to the cells is therefore of essence.

3.4.2 In vitro models of Glomerular disease

In vitro models of glomerular disease allow for the investigation of the response of a specific cell type to injury. Several nephrotoxic agents are available for use *in vitro*. Commonly used agents used to mimic the injuries endured by podocytes in glomerular disease include puromycin aminonucleoside (PAN), adriamycin (ADR), lipopolysaccharides (LPS) and protamine sulphate (PS).

In glomerular disease, a commonly observed change in podocyte morphology is the effacement and flattening of foot processes, changes that occur secondary to dysregulation of the actin cytoskeleton and interacting proteins of the slit diaphragm. Actin cytoskeletal rearrangement is therefore a commonly used read out method in *in vitro* models of glomerular disease. The abovementioned treatments can induce cytoskeletal rearrangement in podocytes, as well as affect podocyte attachment, cell cycle progression and survival. The different treatments also induce damage to the podocytes in different time spans, allowing for assessment of acute and chronic responses to injury in podocytes (144).

Protamine sulphate

PS is a polycation, that induces morphological alterations in podocytes through the reduction of the podocyte anionic surface coat. In several *in vivo* studies, PS has been shown to cause acute damage to podocytes resembling the damages seen in renal disease and foot process effacement (55, 145, 146). *In vitro,* rearrangement of the actin cytoskeleton and focal adhesions has been observed secondary to PS treatment (144). The effect of PS treatments is immediate and actin cytoskeletal rearrangement can be observed already after 1 hour of treatment (54).

In paper I and III, *in vitro* experiments with PS treatment of podocytes were performed. The cells were treated with 600µg/ml of PS for up to 60 minutes of time.

3.4.3 Melanocortin-1-receptor agonist

In paper I, a melanocortin-1 receptor agonist was used for the activation of the melanocortin-1 receptor (MC1R) in podocytes, in an attempt to identify the intracellular pathways regulated by the receptor. The synthetic agonist MC1R-a, also known as BMS-470539, was used in a previous study where the effect

of the agonist had been evaluated in a PAN-model of glomerular disease in vitro (85). The MC1R-a was chosen since it is a highly selective and stabile agonist of both human and murine MC1R (147, 148).

MC1R-a was dissolved in DMSO, and administered with 0.1% of DMSO during treatment of podocytes *in vitro*. The podocytes were treated with MC1R-a at different time points up to 1 hour, in a concentration of 5.6ng/ml (10nM).

3.4.4 Geranylgeranyl transferase inhibitor

Geranylgeranyl transferase type I (GGTase I) is the enzyme responsible for the prenylation of small GTP-binding proteins, predominantly those belonging to the RhoGTPase subfamily. Naturally occurring inhibitors of GGTase I have been found in fungi and sea living creatures. Synthetic geranylgeranyl transferase type I inhibitors (GGTIs) were developed in attempts of regulating the activity of RasGTPase and RhoGTPase proteins, involved in pathological processes of cancer (149).

In paper II, the inhibitor GGTI-298 was used in *in vitro* experiments in order to study the role of GGTase I in regulating podocyte RhoGTPase activity. GGTI-298 is a CAAX-peptidomimetic synthetic inhibitor with selectivity for GGTase I over other prenylation enzymes such as Farnesyl transferase (FTase) (150). The molecular structure of GGTI-298 renders it lipophilic, making it more cell-permeable than some of the other available GGTIs. Use of GGTI-298 has been reported to be successful in inhibiting prenylation both *in vivo* and *in vitro (151-153).*

GGTI-298 trifluoroacetate was dissolved in DMSO. Further dilution of GGTI-298 in sterile saline was done before administrating the compound to the cells. The podocytes were treated with 10μM GGTI-298 for 5 hours.

3.4.5 Actin cytoskeleton analysis

The actin cytoskeleton maintains cell morphology as well as allows for dynamic cellular processes such as migration, mitosis and phagocytosis. Together with myosin II, and other actin binding proteins, the actin cytoskeleton forms so called stress fibers. To a certain extent, stress fibers are considered to be essential for dynamic processes in cells, where migratory forces are generated through tension of myosin II (32, 154).

Stress fiber quantification

The actin cytoskeleton is important for upholding the podocytes and their foot processes. The consequences of dysregulation or rearrangement of the actin cytoskeleton can be observed as foot process effacement in glomerular disease *in vivo*. *In vitro,* it is possible to study actin cytoskeletal rearrangement through labeling of filamentous actin (F-actin) using fluorescent phalloidin (155).

In vitro, the actin cytoskeleton of murine podocytes normally organizes itself in parallel thin fibers (stress fibers) spanning the width of the cell, with some cortical actin. In disease models, this pattern is disrupted, resulting in either a complete loss of actin, disturbed polarization of stress fibers, increased localization of actin to the membrane or clustering of the fibers.

Quantification of stress fibers can be used as read out method when studying the effects of a treatment on actin cytoskeleton formation. The analysis is based on a visual judgement of the stress fibers of several cells from multiple replicates. There is an element of bias involved in this kind of analysis, due to it being based on a visual assessment made by the researcher. However, by blinding of the experimental replicates and the micrographs obtained, the risk of bias can be diminished. In the assessment, cells with parallel stress fibers are considered as healthy podocytes. Podocytes demonstrating a disrupted actin cytoskeleton, with bent or broken stress fibers, are considered as non-healthy podocytes.

The stress fiber quantification method was used in paper I and III. In Paper I, the actin protective effects of MC1R and involved pathways was studied in cells were PS was used to induce stress fiber rearrangement. In paper III, PS induced stress fiber rearrangement was studied in wild type and genetically modified podocytes to assess the role of βpix in podocyte actin cytoskeleton regulation.

LifeAct

Visualization of the actin cytoskeleton can be done through the use of LifeAct®, a 17 amino acid peptide that binds to F-actin (156). GFP-tagged LifeAct® is introduced into the cells where it binds to the actin cytoskeleton with little or no interference with actin dynamics or actin-interacting proteins. This form of fluorescent labeling of F-actin, enables the real-time analysis of actin cytoskeletal dynamics in live cells and the possibility to assess the effects of actin dynamics in regards to cell size (55). A possible drawback with this method is the risk of inconsistent overexpression of the peptide in the cell population as well as background signal due to binding to globular actin (G-actin) (157).

In paper I, LifeAct®-GFP was overexpressed in podocytes. The cells were also expressing either a control vector, the wild type MC1R or the constitutively active MC1R, E92K. A time lapse series of pictures were obtained to study the rearrangement of actin cytoskeleton in real time, induced by PS treatment, and the actin protective effects of MC1R-activity. Micrographs were obtained on a Zeiss Confocal LSM700 microscope. Using Visiopharm®, the fluorescence of LifeAct®-GFP was quantified and stress fiber dynamics and cell surface area was analyzed.

3.5 Gene expression analysis

Gene expression can be analyzed with a wide range of methods, used in different settings and for different purposes. The available techniques allow for complete analysis of the transcriptome as well as targeted single gene expression analysis. Genes expressed in a variety of concentrations can be quantified and genetic variances and relative expression can be evaluated. In all three papers included in this thesis, gene expression analysis has been performed using one or several of the available methods such as RT-qPCR, ddPCR, RNA-sequencing and microarray analysis.

Most of the methods for analyzing gene expression includes a step of reverse transcription of RNA into complementary DNA (cDNA). RNA is an unstable molecule, easily degraded by RNases, posing potential problems for a reliable quantification of the gene expression. cDNA is a more stable molecule and less disposed to undergo mutation in comparison to the RNA molecule, making it a more suitable template for reactions through which gene expression is assessed.

3.5.1 Microarray

Microarray analysis enables largescale analysis of mRNA expression with parallel analysis of several genes at once. The method is based on the hybridization of complementary cDNA strands, located to a base/array. Synthesized DNA oligomers of either a whole transcriptome or a few selected genes are located to this array. The purified RNA from the samples is reverse transcribed into cDNA and labeled with fluorescent tags. Upon hybridization between the labeled cDNA and the DNA oligomers on the array, a fluorescent signal is released. The fluorescence intensity is proportional to the amount of cDNA in the sample and the relative expression of a gene can be determined through measurement of this intensity (158, 159).

Through microarray analysis, a large amount of data can be obtained, which can be used for different purposes. Many data sets from large scale genomic microarray analyses have been made publicly available due to the amount of data and the various possible applications of it. However, the vast amount of data obtained needs to be further processed, normalized and validated before analysis and comparisons can be done properly. The information received is also limited due to the design of the DNA oligomers in the array, no unknown genes can be quantified.

In paper I, public microarray data from renal biopsies from FSGS and MN patients was analyzed for the glomerular expression of melanocortin receptors (accession number GSE47183, GSE32591 and GSE37460). The data was obtained through the use of the Affymetrix Human Genome U133A and Affymetrix Human Genome plus 2.0 platforms, with microarray analysis performed at five different occasions. Bioinformatic processing was done as described in section 3.5.3. A total of 26 controls, 26 FSGS patients and 21 MN patients were analyzed.

3.5.2 RNA sequencing

RNA sequencing, referred to as RNA-seq, is a method that allows for analysis of the complete transcriptome, including small RNAs other than the traditional protein-coding mRNA. It is a high throughput analysis, where both sequencing and quantification of genes is performed. Smaller RNA-molecules and fragmented RNA molecules are converted into cDNA, tagged with an adaptor, and thereafter sequenced. The method does not require a reference genome unlike the microarray analysis, although a reference can be used for mapping of the sequenced transcripts. New splice variants and isoforms can be identified through RNA-seq and the high resolution of the method permit the identification of sequence variants such as single nucleotide polymorphisms (160, 161).

The RNA-seq is however a sensitive method in regards to the handling of samples and the high risk of RNA-degradation, affecting the reliability of the sequencing. The analysis also results in large data sets, that require both a large digital storage capacity as well as the bioinformatic knowledge in how to handle the data.

In paper III, glomerular RNA-seq data from two diabetic mouse models was obtained from Astra Zeneca. The glomerular gene expression of βpix was analyzed in eNOS db/db and heterozygous control mice at 10 and 18 weeks of

age. In the BTBR ob/ob mice, glomerular expression of βpix was analyzed at 8,14 and 20 weeks of age and compared to lean control mice.

3.5.3 Bioinformatic analysis of Gene expression

The microarray data used in paper I for the analysis of glomerular expression of melanocortin receptors, was processed using bioinformatic and statistical analysis in order to obtain data regarding the differential expression of the receptors. The data processing, performed by a trained bioinformatician, is described in detail in the method section of paper I. Since the data was obtained at five different occasions and using two different platforms, normalization within batches was first performed. Following sample quality check and exclusion of outliers, the data obtained from the two platforms were normalized and merged. Clustering of data from the two patient groups and the control group was done to assess differences between groups and thereafter analyzed using the Significant Analysis of Microarray (SAM). A SAM q-value below 0.05 was set as the cut off for significantly regulated genes.

Bioinformatic analysis of data obtained from RNAseq was done at Astra Zeneca and performed using the Blue Collar Bioinformatics (bcbio-nextgen) pipeline.

3.5.4 Taqman™ Real time qPCR

Taqman™ PCR is a fluorescence based real time quantitative PCR method that allows for relative quantification of gene expression. The assay consists of a forward and reverse primer and a sequence specific Taqman™ probe harboring a fluorescent reporter and a quencher. Suppression of the fluorescent signal is achieved through fluorescent-resonance energy transfer (FRET) between the reporter and the quencher. During gene amplification, the Taq DNA polymerase cleaves the probe, releasing the fluorescent reporter from the quencher, and fluorescence is subsequently detectable. The number of amplification cycles needed to reach a preset fluorescence threshold is called the CT-value, which is inversely related to the amount of cDNA in the sample. The CT-value can then be used to calculate the relative expression of a gene, most commonly through the $2^{-\Delta\Delta CT}$ method (162).

Taqman™ PCR allows for a quick, sensitive and low-cost analysis of gene expression with high accuracy. However, the number of genes analyzed in one session is limited, due to the need of gene specific probes, also limiting the possibilities of detecting splice variants of a gene.

Taqman™ analysis was performed in all three papers included in this thesis. In all three papers, it was used to verify the gene silencing and overexpression in cells following lentiviral transduction. In paper II, the knockout of Pggt1b in glomeruli from genetically modified mice and their controls was verified through Taqman™ analysis.

3.5.5 ddPCR

Digital PCR is a method used for absolute quantification of gene expression based on end-point PCR reactions, where the cDNA sample is diluted or divided into several reactions. The dilution of the samples results in a distribution of the cDNA template leaving some reactions blank of a template. Following the PCR amplification, each reaction is then classified as a positive or a negative based on the presence of a cDNA template. Thereafter, using Poisson statistics, the absolute quantity of cDNA in the input sample can be estimated based on the fraction of positive reactions.

The droplet digital PCR (ddPCR) is a rapid analysis that allows for a precise and sensitive quantification of gene expression. In ddPCR, the cDNA templates are divided into small droplets, also containing ddPCR mix and a PCR assay, in our case the Taqman™ assay. Following end-point PCR, the fluorescence generated by the Taqman™ -probe is read using a droplet flow cytometer, and the droplets classified as positive or negative based on the level of fluorescence (163, 164). In paper I, ddPCR was used to analyze the expression of melanocortin receptors in cultured immortalized murine podocytes treated with PS.

3.5.6 RNAscope™

RNAscope™ is a form of RNA in situ hybridization that allows for the visualization and analysis of RNA expression in intact tissues and individual cells. The assay is based on the use of a pair of Z-shaped RNA recognizing probes, that will hybridize to the region of interest. When the two probes hybridize to the target sequence, they will generate a hybridization sequence recognized by the pre-amplifier probe, which in turn is recognized by the amplifying probe. The label probe will then hybridize to the amplifier-probe. The label probe can either be a fluorescent probe or labeled with horse radish peroxidase (HRP) or alkaline phosphatase, allowing for visualization through either fluorescent or bright field microscopy. Semiquantitative and quantitative analysis of the signal from labeled RNA-molecules can thereafter be done (165).

Although the design of the probe system makes it a sensitive and specific method to use, one should be aware of the challenges it can entail when it comes to preservation of the RNA in the tissues during fixation and sample preparation. In paper I, the glomerular expression of MC1R in paraffin embedded renal tissue sections from PAN-rats was analyzed using RNAscope™.

3.6 Protein expression analysis

Numerous methods for analysis of protein expression are available today, enabling researchers to take on different approaches in their investigation. Methods, such as mass spectrometry, can be applied for a "top-down" approach, starting out with a general analysis and identification of proteins and peptides in a sample. The identified proteins can thereafter constitute the platform for further and more specific investigations of proteins with methods such as western blot. Alternatively, a "bottom up" approach can be used where a specific protein is the starting point for investigation. Through the investigation of interacting proteins and pathways involving the protein of interest, the function of a protein in a general setting can be determined. The available methods are also possible to use for quantitative and semiquantitative analysis of protein expression, which can be relevant for the comparison between samples.

An important aspect of the available methods, is that no single analysis can offer a complete understanding of a protein. Most of the analyses are used in combination to fully understand the function, localization and abundance of a protein. In all three papers included in the thesis, protein expression analyses have been used.

3.6.1 Mass spectrometry

Mass spectrometry analysis with a phospho-proteomic approach was used in paper I and III in order to study effects of MC1R stimulation in podocytes and to identify regulated GEFs in podocytes following treatment with PS. The analysis was performed on an Orbitrap Fusion Tribrid mass spectrometer with an Easy-nLC-1000 liquid chromatograph. The samples were labeled using tandem mass tag-technology, TMT, enabling the relative quantification of proteins in several samples at the same time.

Mass spectrometry allows for identification and quantification of proteins in a sample with high specificity and sensitivity. The sample specific TMT-labeling reduces the risk of variations in between analyses, since several

samples can be analyzed together, making the comparison more robust. Mass spectrometry is a high through-put analysis which provides large amounts of data, that through proper handling can be of great value. It does however require extensive knowledge both in the actual sample preparation and analysis, but also in the post-experimental interpretation of data.

In paper I and III, podocytes in culture were treated with either MC1R-a for 0-60 minutes or PS for 0-60 minutes. Harvest of podocytes was done and protein concentrations were measured using a BCA-assay. 500-1000µg of protein was used for the phospho-peptide analysis and 30µg for the total protein analysis. Samples were prepared according to previously described methods (166). Phospho-peptide enrichment was done using a TiO_2 Phospho-peptide Enrichment and Clean-up kit.

Total protein peptides and phospho-peptides thereafter underwent labeling with tandem mass tags and the LC MS/MS analysis was performed. The data for each TMT-set was then identified through the use of Proteome Discoverer v 1.2 or 1.4, respectively, and database searches done in the Mascot search engine set for *Mus musculus* Swissprot Database. Quantification of peptides was normalized to the protein median and phospho-peptides were evaluated based on individual sequences. Pathway analysis was thereafter performed on the data either using Ingenuity Pathway analysis (IPA) or Database for Annotation, Visualization and Integrated Discovery (DAVID).

Sample preparations and analysis was performed at the Proteomics core facility and Bioinformatics core facility at Gothenburg University and a more detailed description of the analyses can be found in respective paper.

3.6.2 Bioinformatic analysis of Protein expression

Ingenuity Pathway analysis

The Ingenuity Pathway Analysis (IPA) is a software provided by Qiagen used as a tool for bioinformatic analysis of gene and protein expression. Through the use of IPA, proteomics data can be interpreted and visualized in a contextual way. It can provide information on the potential signaling pathways affected in a sample and perform network analysis revealing affected proteins in said pathways. Post-translational modifications, such as phosphorylation, can be analyzed and correlated to the most probable regulated pathways and networks downstream of the modification. Following IPA, the identified pathways and regulated proteins can be explored and confirmed through additional experiments.

In paper I, IPA was used to identify the most probable regulated pathways in podocytes following MC1R stimulation. Network analysis of signaling pathways was also performed, identifying probable proteins involved in the signaling cascades. Changes in the phosphorylation status of peptides in the mass spectrometry data were considered significant if the fold change was 15% or more, based on the inherent error margin of MS analysis. The phospho-proteomic data was thereafter analyzed through the IPA software and significantly affected pathways, as well as networks for each time point, 0-60 minutes, were identified.

Database for Annotation, Visualization and Integrated Discovery

Database for Annotation, Visualization and Integrated Discovery (DAVID) is a web-based bioinformatics program used for the analysis and visualization of gene and protein expression. In paper III, the total protein data from the proteomic mass spectrometry analysis was analyzed using DAVID, to find the most probable regulated pathway in podocytes following treatment with PS.

3.6.3 SDS-page and Western blot

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blot, is a tool used for the identification of the proteins expressed in a sample, their modifications and their relative expression. It is a semiquantitative method for the analysis of protein expression that is widely used in biochemical and medical laboratories, and allows for analysis of almost any kind of biological sample.

The analysis is done in four steps of gel separation of proteins, blotting, probing with antibodies and acquisition and analysis of luminescent signal. As a first step, a bicinchoninic acid assay (BCA) was used to assess the protein concentrations in the samples, to assure equal loading onto the gel. Samples were then mixed with SDS-buffer and reducing agent (dithiothreitol) for denaturation of the proteins and to give the proteins a negative charge. For further denaturation, the samples were boiled at 95°C for 5 minutes. SDS-PAGE was thereafter performed and blotting of the proteins was done onto a polyvinylidene fluoride membrane (PVDF-membrane). A primary antibody targeting the protein of interest was thereafter used to probe the membrane, followed by probing with a HRP-conjugated secondary antibody. Luminescence was measured following the addition of a chemiluminescent reagent to the membrane.

The analysis involves many different steps from sample preparation to the final analysis, which leaves room for unintended errors by the researcher, possibly affecting the result. Awareness of these risks is therefore important for a successful analysis. One of the most important considerations in western blot analysis, is the step of normalization of protein expression to a loading control, to assure that subsequent comparisons between samples are correct. Traditionally this has been done using housekeeping genes such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH), expected to keep a stable expression and therefore being a reliable reference. There is however the possibility that expression of such proteins does change as a result of experimental conditions. An alternative method is the normalization against the total protein load in each lane, which aids in overcoming the problems with housekeeping genes, and has therefore been chosen as the preferred method in this thesis.

In all papers included in the thesis, western blot analysis has been performed to analyze the presence of, and changes in expression or modification of, proteins. In paper I, western blot analysis was used to confirm the pathways and involved proteins following MC1R stimulation in podocytes that were identified through mass spectrometry analysis. In paper II, the method was used to analyze the eluates from activity pull down assays of RhoGTPases following reduction of GGTase-I activity. Lastly, in paper III, western blot was used to identify the expression of βpix in different tissue samples as well as to analyze the samples obtained following immunoprecipitation of βpix and cell fractionation. In paper II and III, western blot has also been used to verify and quantify the degree of knockdown of proteins following lentiviral transduction.

3.6.4 Immunoprecipitation

Immunoprecipitation is a method applied to enrich, purify and characterize a specific protein. In paper III, an immunoprecipitation was performed in order to enrich for the protein βpix and its possible isoforms expressed in podocytes. The method is based on the binding of an antigen to an antibody. The antibody can either be coupled to a bead directly, or be precipitated onto a bead following the binding of the antigen. The antigen and antibody are bound to the beads, and unbound proteins are removed through washing. Following elution of the bound antigen, the sample can be analyzed through the use of SDS-PAGE and western blot to evaluate the expression of the protein(s).

3.6.5 Cell fractionation

Cell fractionation is a method used to subdivide the different compartments of a cell; plasma membrane, cytoplasm and organelles. Through repeated steps of centrifugation with sequentially increasing force and gradient sedimentation, the separation of cellular components is achieved and can be isolated for further analysis. Depending on the level of separation of cellular components that is sought after, the steps of centrifugation and the gradient for sedimentation can be modified, allowing for small molecules such as RNAs to be isolated.

In paper III, cell fractionation was performed in order to investigate the subcellular localization of βpix, more specifically, the localization of isoforms of the protein. Cell fractionation was performed as described in paper III and the fractions were analyzed through SDS-PAGE.

3.6.6 Immunofluorescence

Immunofluorescence (IF) is a widely used and important biochemical method for the detection and localization of proteins in cells and tissues. It can be used to assess the level of protein expression and to visualize the dynamics of proteins under different experimental conditions. Through the use of a primary antibody targeting the protein of interest and a secondary antibody conjugated to a fluorophore that recognizes the primary antibody, detection and visualization of proteins can be done. By using different fluorophore-conjugated secondary antibodies, several proteins can be visualized simultaneously. This can be done in order to investigate the co-localization of different proteins, as done in paper III, or the cell specific expression of a protein, as was done in paper II.

Beside the spatial information of protein localization, it is also possible to quantitatively analyze the expression of proteins through immunofluorescent staining. Fluorescent intensity can be measured, but does require a standardized approach in the acquisition and processing of images. Uneven fluorescence between samples due to unequal distribution of primary antibody during the sample preparation and background fluorescence due to unspecific binding of the secondary antibody are just two things that can affect the fluorescence intensity. Experience in such analysis is therefore of great importance for reliable analyses. Manual quantifications are also possible, and are preferably done in a blinded fashion, minimalizing the risk of a biased assessment.

Immunofluorescent staining was used in paper II and III. In paper III, immunofluorescent staining was used to identify the cellular localization and function of βpix in immortalized murine podocytes. In paper II, the glomerular expression of GGTase-I in mice and humans, was assessed through immunofluorescent staining. It was also used to assess integrin localization following depletion of GGTase-I in immortalized murine podocytes.

3.6.7 Bio-plex Immunoassay

The Bio-plex Pro assay is a bead-based form of immunoassay that allows for identification and quantification of several proteins or biomarkers at once. Antibodies capturing the antigen or protein of interest are conjugated to beads carrying a fluorescent dye. For each protein, the fluorescent color of the bead is different, making it possible to differentiate the proteins in a sample from each other. A biotinylated detection antibody is thereafter bound to the bead-complex, followed by the binding of a streptavidin-phycoerythrin conjugate to the detection antibody. The phycoerythrin acts as a fluorescent reporter.

Through flow cytometry-based analysis on a Bio-Plex 200 system, the beads are subjected to excitation by two lasers. A red laser, excite the bead fluorophores, whilst a green laser excites the fluorescent reporter. This permits for the simultaneous discrimination of the protein through the spectral emission from the beads and the measurement of concentration through the signal obtained from the fluorescent reporter.

The Bio-Plex assay offers a great possibility to quantitatively measure several proteins simultaneously. In some ways, this makes it a better method for protein analysis in comparison to both ELISA and western blot, which both are limited in the number of proteins that can be analyzed at once. However, the possibilities are restricted since the number of available beads are limited in number and at the moment only available for certain proteins such as growth factors and cytokines.

In paper II, the Bio-Plex assay was used to analyze cytokines released from podocytes *in vitro* as a result of lentiviral knockdown of GGTase-I, in order to assess the degree of cell injury that the inhibition of prenylation might have caused the cells.

3.6.8 RhoGTPase Activity assay

RhoGTPases cycle between a GDP-bound, inactive state and a GTP-bound, active state. Due to the many processes regulated by the RhoGTPases, assays have been developed to assess their activity through the analysis of their GTP-loading. The affinity based pull down method used in paper II is a commonly used method for assessing RhoGTPase activity in cells.

The method is a Glutathione S-transferase (GST) - glutathione based pulldown method that utilizes the selective binding between active RhoGTPases and downstream effectors as a means of isolating the GTPases from a lysate. The GTPase binding domains from downstream effectors are GST labeled. For RhoA it is the Rho-binding domain of Rhotekin, for Rac1 and Cdc42 it is the p21-binding domain of p21 activated kinase (PAK1), and for Rap1 it is the Rap1 binding domain of RalGDS. Cell lysates are allowed to incubate with the GST-fusion proteins in the presence of a glutathione agarose resin. Active GTPases will bind to their respective effectors, and the GST will immobilize the bound proteins to the glutathione resin. Through elution of the proteins from the resin into a buffer containing SDS and reducing agent at high temperature, samples containing the active form of the RhoGTPases are obtained. These are then analyzed through SDS-PAGE and western blot. Quantification of active RhoGTPase and total RhoGTPase levels is done based on western blot results and the amount of active RhoGTPase is normalized against the total amount of the RhoGTPase in the cell lysate.

Other assays are available for the analysis of RhoGTPase activity, for example fluorescent methods that can visualize the localization and timely regulation of active RhoGTPases in cells. However, these methods do not provide the possibility of analyzing the RhoGTPase activity in a cell population as a whole, as is possible with the affinity-based method.

In paper II, the activity of RhoGTPases RhoA, Rac1 and Cdc42 as well as the RasGTPase Rap1, was assessed using the activity pull down assay on lysates from cultured immortalized murine podocytes with reduced GGTase-I activity. This was done to assess the effects of prenylation on RhoGTPase activity in podocytes.

3.7 Gene silencing and over-expression

Genetic manipulation through the silencing or over-expression of genes offers a way of studying the function and relevance of a gene and its respective protein. By altering the expression of a gene, it is possible to identify intracellular pathways and processes regulated by a specific gene. Through mutagenesis and over-expression of a mutated gene, regulatory sites in a protein can be studied and functionally evaluated. Interactions between proteins can be the investigated and domains needed for protein function can be identified.

Gene overexpression is accomplished through the introduction of the specific gene into a host cell. Silencing of a gene can be done either through a complete knockout of the gene or a knockdown, depending on the chosen method. In the papers included in this thesis, gene silencing through shRNA-mediated gene knockdown has been performed. With this method, the shRNA targets the specific mRNA for degradation, and is thereby decreasing the expression of the gene.

Different systems can be used for introducing the genetic material needed for gene manipulation into a host cell. The expression of the introduced gene can also be controlled through the chosen method to either be transiently or stably expressed. In the papers included in this thesis, gene overexpression and gene silencing has been done using lentivirus mediated transduction into the host cell, generating a stable expression of the introduced genes.

3.7.1 Lentivirus

The lentiviral gene delivery system based on the HIV-1 virus, is a commonly used technique for the regulation of gene expression. The system is favored for its capability of introducing stable gene expression in both dividing and non-dividing cells, and differentiated cells such as the podocytes. Lentiviruses used for the delivery of transgenes are made safer than their related HIV-1 viruses through the removal of genes needed for viral replication from the transfection plasmids and the division of elements important for virus assembly on several plasmids. This generates viruses capable of a single infection with integration of the transgene into the host genome without further virus replication.

A plasmid containing the transgene, either a gene for increased expression of a protein or a shRNA for the silencing of a gene, was used for production of lentivirus. Assembly of lentiviral particles was done through transfection of HEK293T cells with the transgene plasmid and two different plasmids for the

viral envelope and capsid. The assembled virus was released into the cell culture media which was collected and used for transduction of podocytes.

Lentiviruses were used in all three papers included in this thesis to either over-express or silence genes in immortalized murine podocytes. To confirm the expression of the genes following transduction, Taqman™ PCR, western blot and fluorescence microscopy was performed. For each new batch of virus made, virus titration was done in order to find the optimal virus concentration for experiments.

3.7.2 Gene silencing with shRNA

Silencing of gene expression using lentiviral transduction of shRNA was done in paper II and III to knockdown the Pggt1b and βpix genes in cultured podocytes. The shRNAs were delivered from Sigma Aldrich in pLKO.1 vectors, ready for use. Assembly of lentivirus particles was done as described previously. The knockdown efficiency of several shRNAs for each gene was evaluated through Taqman™ PCR and western blot analysis to establish the most efficient shRNA, used in subsequent experiments.

Transduction of podocytes was done on day 7 post thermal switch by addition of virus-containing media to the cells. To enhance the transduction, hexadimethrine bromide was added to the cells along with the virus. The cells were left to incubate with the virus for 16 hours, and were thereafter kept in normal growth media. On day 7 post transduction, the cells were subjected to experimental treatments and/or harvested for analysis.

3.7.3 Gene over-expression

Over-expression of genes was done in paper I and paper III. In paper I, over-expression of the gene for wild type MC1R and the constitutively active mutant E92K was done in cultured podocytes. Both genes were cloned out of the pcDNA 3.1+ vectors kindly provided by Professor Rosenkilde at University of Copenhagen, into the over-expression vector VVPW-EGFP, kindly provided by Dr Anna Greka at Brigham and Women's Hospital and Harvard medical School. For the Cherry-expressing MC1R vectors used in paper I, the EGFP-sequence in the VVPW-construct was replaced with the gene for mCherry. A VVPW-LifeAct-GFP vector was also used in paper I.

The genes for wild type EGFR and the mutated EGFR T669A were cloned out of the pHEX-vector kindly provided by Professor Roger Davis at Massachusetts Medical School, and inserted into the VVPW-EGFP vector replacing the sequence for EGFP. This replacement was done since a vector

expressing both EGFP and EGFR would become too large for transduction in later experiments. To be able to assure the expression of EGFR in the cells, the cDNA for puromycin resistance and the hPGK-promotor were cloned out of the pLKO.1 TRC vector and inserted in the VVPW vector following the insertion of the genes for EGFR, creating puromycin resistant vectors expressing EGFR and EGFR T669A. Both vectors were used in paper I.

In paper II, the cDNA for the two isoforms of βpix, isoform H and C, were cloned out of murine podocyte cDNA and inserted into the overexpression vector VVPW-EGFP. Sanger sequencing was done on all vectors following cloning, in order to confirm the correct gene expression in the newly assembled vectors.

Lentiviral particles with respective over-expression vector were assembled as previously described. Transduction of cultured immortalized murine podocytes was done on day 7 following thermal switch by the addition of hexadimethrine bromide and virus-media to the cells as described previously. For experiments in paper I, where cells were expressing both EGFR and MC1R, the first transduction with the EGFR-vectors was done on undifferentiated podocytes with subsequent puromycin selection. Since podocytes do not proliferate following differentiation, the puromycin selection had to be done on podocytes in the undifferentiated state, to obtain a large enough cell population with stable expression of the respective EGFR-genes. At 90% confluency following puromycin selection, the cells were subjected to thermal switch, and at day 7 the cells were transduced with the MC1R-vectors. On day 14, cells were used for further experiments.

Cyclic adenosine monophosphate assay

Upon activation of G-protein coupled receptors (GPCRs), like MC1R, an intracellular increase in cAMP can be observed, which stimulates activity in downstream signaling pathways. In order to verify that the two overexpressed receptors, wild type MC1R and constitutively active E92K, were functional, cAMP levels following stimulation with MC1R-a was assessed using the cAMP-Screen® immunoassay system. In paper I, cultured murine podocytes were transduced with lentivirus for the expression of either of the two receptors. Thereafter the cells were treated with different concentrations of MC1R-a for 30 minutes in starvation medium with 0.1% FBS. 3-isobutyl-1-methylxanthine (IBMX) was also supplemented to the medium during the treatment. Cells were harvested and thereafter analyzed using the immunoassay. As a control, forskolin was used. The analysis of cAMP is a good way of verifying the activity and function of an ectopically expressed

receptor, keeping in mind that cAMP levels can be increased through other signaling pathways as well.

3.8 Statistical analysis

The statistical analyses in the papers included in this thesis were performed in Graphpad prism version 6-8. A test for normal distribution was performed where applicable. For multiple comparisons of data following the normal distribution, One-way ANOVA was performed with either Sidak's or Tukey's test for multiple comparisons. For comparisons between two groups, Students t-test or Mann-Whitney U-test was performed. Fishers exact test was done for the statistical analysis of data in the IPA. Microarray data was analyzed using Significant Analysis of Microarray (SAM). All values are presented as mean ± SEM unless stated otherwise. P-values of 0.05 or below were considered significant and annotated as follows; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

4 RESULTS AND DISCUSSION

In the following sections, the results from the three papers included in this thesis will be presented and discussed. Conclusions drawn based on these results will further be presented in the following chapter.

The work in this thesis has focused on the activity of RhoGTPases and actin cytoskeleton regulation in podocytes. In paper I, the renoprotective effects or MCRs was investigated, with a focus on the podocyte actin cytoskeleton stabilizing effect following MC1R activation. In paper II, the prenylation of RhoGTPases in podocytes was investigated in an effort to determine the consequences it has for podocyte function and the integrity of the glomerular filtration barrier. In paper III, we set out to identify guanine nucleotide exchange factors important for RhoGTPase activity in podocytes.

4.1 Paper I: Amplification of the Melanocortin-1 Receptor in Nephrotic Syndrome Identifies a Target for Podocyte Cytoskeleton Stabilization

In 1999, a coincidental discovery was made by Berg et al. when they observed beneficial renal effects of ACTH treatment in patients suffering from membranous nephropathy (72). The treatment, which was meant to primarily affect the hematological lipid status, also proved to increase GFR and reduce proteinuria in the patients. Several clinical studies have since shown beneficial effects of ACTH treatment in patients suffering from glomerular renal disease of different types (73-75). In an attempt to better understand the underlying mechanisms of these renal effects, our laboratory group started to investigate the target receptors for ACTH, the melanocortin receptors (MCRs), and their expression in the kidney. The investigation resulted in the discovery that the main MCR in the kidney was the MC1R, with localization of the receptor to the podocytes. Further work within the group showed that the specific targeting of MC1R reduced proteinuria and improved GFR and glomerular morphology in the Passive Heyman Nephitis (PHN) rat model of membranous nephropathy (167). MC1R activation also reduced oxidative stress in the PHN-model, which later proved to occur in podocytes *in vitro* as well (85). Through MC1R mediated increase in catalase activity, ROS-levels in cultured podocytes decreased causing a dephosphorylation of p190RhoGAP, allowing for the activation of RhoA and promoting stress fiber formation in podocytes. Through the identified pathway, stress fiber integrity was upheld in spite of

PAN-induced actin cytoskeleton rearrangement. In addition, cell viability was improved by MC1R activation in PAN-treated podocytes (85).

In paper I, our aim was to further understand the role of melanocortin receptors in glomerular disease and how they are involved in the restoration of the glomerular filtration barrier and its function. To do so, we first set out to study the glomerular expression of MCRs in glomerular diseases with nephrotic symptomatology. Through analysis of glomerular microarray data from patients with MN and FSGS we found that the only MCR significantly upregulated in nephrotic disease was MC1R, when compared to healthy controls. Expression of the other MCRs, MCR 2-5, was significantly decreased in both patient cohorts, when compared to healthy controls.

In a second step, we then investigated the expression of MC1R in the PAN-rat model of glomerular disease. 7 days following PAN-injections, rats developed proteinuria. Glomerular sections from the rats were analyzed using RNA-in situ hybridization technology (RNAscope™). In the analysis, a significantly increased glomerular expression of MC1R in PAN treated rats was observed in comparison to control rats. Collectively, these findings showed that there was an increase in glomerular MC1R-expression as a consequence of nephrotic disease.

As previously mentioned, our studies have revealed that the glomerular expression of MC1R is mainly located to podocytes (167). The microarray data and the RNA-ish data both showed that glomerular injury led to an increased expression of MC1R. To assess the regulation of MC1R following podocyte injury, podocytes in culture were subjected to treatment with the proteinuric agent PS. PS is, as previously mentioned, known to cause acute damage to podocytes both *in vitro* and *in vivo,* causing rearrangement of the actin cytoskeleton and foot process effacement (55, 145, 146). After 30 and 60 minutes of PS-treatment, gene expression of the five melanocortin receptors was analyzed using ddPCR, and MC1R was the only significantly upregulated receptor following PS treatment. Protein expression analysis using western blot confirmed the increase of MC1R. The same effect was observed in our previous study, were PAN-treatment *in vitro* caused an increase in mRNA and protein expression of MC1R (85).

The observation that MC1R is the most prominently expressed MCR in the glomeruli in addition to our observations of increased MC1R expression in response to glomerular and podocyte injury, strongly suggests that MC1R is the receptor mediating the effects of ACTH-treatment in glomerular disease.

Our next attempt was therefore to assess the podocyte intracellular pathways regulated by MC1R activation.

As mentioned earlier, our previous study identified the actin cytoskeleton regulating pathways downstream of MC1R activation in podocytes treated with PAN, an agent known to cause damage to podocytes through slow processes that extend over several days (85). In order to assess if MC1R activation would protect podocytes in a similar way in the more acute form of podocyte injury induced by PS, LifeAct® analysis was performed. Following overexpression of either the wild type MC1R, or a constitutively active MC1R (E92K), cells were treated with PS. The expression of LifeAct-GFP allowed for the real-time analysis of actin cytoskeleton rearrangement following treatment with PS. Expression of the wild type MC1R was in itself not protective against cytoskeletal rearrangement induced by PS, whilst cells expressing the constitutively active MC1R were partially protected. Pre-treatment of MC1R expressing cells with a specific MC1R agonist, MC1R-a, resulted in protection against actin cytoskeletal rearrangement. Based on calculations of the podocyte cell surface area, it was also found that MC1R overexpressing cells treated with MC1R-a better maintained their cell shape in comparison to the cells overexpressing E92K and the control cells. The results suggest that MC1R activation relays an actin cytoskeleton protective effect also in an acute model of podocyte injury. Due to the probable disparities in injury mechanism, as judged by the time-to-effect difference between PS and PAN, we hypothesized that the protective effect of MC1R-activation probably was mediated through different pathways in the two models of podocyte injury.

In an effort to identify the pathways downstream of MC1R involved in actin signaling, we applied a phospho-proteomic approach. Podocytes over-expressing MC1R were treated with the agonist MC1R-a for 0-60 minutes and thereafter analyzed through LC-MS/MS, allowing for the identification of regulated phosphoproteins. Ingenuity Pathway Analysis of identified phosphoproteins revealed the 10 most significantly regulated pathways following MC1R activation at each of the time points of treatment. At 5 minutes of agonist treatment the top ranked regulated pathway was the actin cytoskeleton signaling pathway. Amongst the identified pathways at this time point was also other signaling pathways closely connected to actin cytoskeleton regulation, such as the integrin signaling pathway, ILK-signaling and PAK-signaling pathways. At 10 minutes of treatment, the top ranked regulated pathways were pathways related to regulation of actin cytoskeleton, such as the PAK-signaling and axonal guidance pathways. After 30 minutes of treatment, the majority of the identified pathways were pathways related to RhoGTPase signaling and actin cytoskeleton regulation. Actin cytoskeleton,

RhoGTPase family of proteins, PAK, RhoGDI, axonal guidance, ILK, Integrin and Paxillin signaling pathways constituted 8 of the 10 identified pathways at this time point. At the same time, activity in the ERK/MAPK-signaling pathway was found among the most regulated pathways as well. At 60 minutes of treatment, ILK-signaling and actin cytoskeleton signaling were in the top of the regulated pathways. Activation of MC1R leads to the increased synthesis of cAMP, which in turn stimulates protein kinase A (PKA) activity (79). At all time points of treatment, the PKA-signaling pathway was amongst the top regulated signaling pathways, confirming the activation of MC1R in our podocytes. Taken together, the phospho-proteomic data supported the idea of MC1R having a role in actin cytoskeleton signaling and indicated it to be involved in several different pathways connected to actin cytoskeleton regulation.

To further elucidate the pathways resulting in actin cytoskeleton regulation downstream of MC1R activation, the identified signaling pathways were analyzed through network analysis. In the analysis, proteins involved in the early time point signaling events following 5 minutes of agonist treatment were identified. Among the identified proteins were synaptopodin, a known actin cytoskeleton stabilizing protein in podocytes, that also regulates the activity of RhoA. Degradation of synaptopodin results in destabilization and loss of stress fibers in podocytes (19). EGFR was a second protein identified in the network analysis. Activation of EGFR has been shown to cause a degradation of synaptopodin in podocytes, resulting in a disruption of stress fibers due to increased Rac1 activity and simultaneous decrease in RhoA activity (54). A third protein identified in the network analysis was MAPK/ERK1/2. ERK1/2 is a kinase known to be activated downstream of MC1R in melanocytes and also known to regulate EGFR activity (83, 168).

The identification of the abovementioned proteins and the knowledge regarding their respective functions, generated the hypothesis that MC1R regulated actin cytoskeleton signaling could be attained through the modulation of EGFR activity. In the phospho-proteomic data, EGFR had proven to be phosphorylated at the inhibitory site T669, a site previously described to be phosphorylated by ERK1/2 (168). Therefore, we set out to first investigate if ERK1/2 mediated phosphorylation could be involved in the stress fiber protective effects of MC1R activity.

First, to assess if MC1R activity resulted in ERK1/2 activity in podocytes as well, treatment of MC1R overexpressing podocytes with the agonist MC1R-a was performed. Western blot analysis on cell lysates revealed an increased phosphorylation of ERK1/2 at 5 minutes of treatment, which gradually

decreased over time. No changes were observed in the total level of ERK1/2. MC1R activation thereby seemed to result in ERK1/2 activity in podocytes as well.

Thereafter, the effects of ERK1/2 signaling on actin cytoskeleton rearrangement was assessed. MC1R overexpressing podocytes were treated with MC1R-a for one hour, followed by the treatment with PS to induce actin cytoskeletal rearrangement. As previously described, MC1R-a treatment protected against the PS induced cytoskeletal disruption. However, in the cells treated with the ERK1/2 inhibitor PD98059, the protective effects of MC1R-a were abolished. Through stress fiber quantification, it was shown that MC1R-a treatment significantly protected against stress fiber rearrangement, whilst the inhibition of ERK1/2 significantly blocked the protective effects of MC1R-a treatment. ERK1/2 activity thereby seemed important for the actin cytoskeleton protective effects of MC1R.

In order to confirm the phospho-proteomic data regarding phosphorylation of the EGFR-inhibitory site T669, western blot analysis was performed on MC1R-a treated MC1R overexpressing cells. The analysis showed an increased phosphorylation of EGFR-T669 as a consequence of MC1R activation at 5-10 minutes of treatment. No changes in phosphorylation of the activating site Y1068 was observed, nor any changes in the total levels of EGFR. MC1R activation thereby seemed to mediate inhibition of EGFR through ERK1/2 phosphorylation of T669.

Activation of EGFR by PS was previously shown to induce Src activity and subsequent synaptopodin degradation, leading to an increased Rac1 activity and loss of stress fibers (54). Due to the role of EGFR in disruption of stress fibers in podocytes the next step was to assess whether MC1R mediated EGFR-inactivation would affect Src activity and synaptopodin levels in accordance with the described pathway. Western blot analysis of MC1R overexpressing cells treated with MC1R-a and PS, showed that activation of MC1R decreased the PS induced activation of Src. Synaptopodin levels were stabilized in the MC1R-a treated cells and phosphorylation of the EGFR-activation site Y1068 diminished following MC1R-a treatment. These results suggested that MC1R activity stabilizes the actin cytoskeleton through the inhibition of EGFR-activity, decreased Src activity and maintained synaptopodin expression, allowing for stress fiber formation.

In the last experiment, the significance of MC1R induced phosphorylation of EGFR T669 was finally evaluated. Podocytes were either overexpressing the wild type EGFR or a phosphorylation resistant EGFR T669A. The cells were

treated with MC1R-a and PS. As previously observed, the treatment with MC1R-a protected the cells against the PS induced stress fiber rearrangement in the cells expressing the wild type MC1R. In the cells overexpressing the phosphor-resistant EGFR T669A however, the protective effect of MC1R-a treatment was abolished. Stress fiber quantification showed a significant reduction in the number of cells with stress fibers in PS treated cells, which was significantly reversed in MC1R-a treated cells expressing wild type EGFR.

General discussion

In previous studies performed by the group, the innate expression of MC1R in wild type murine podocytes posed a problem for the analysis of MC1R signaling. In comparison to the expression in human glomeruli, the expression in murine podocytes was lower. MC1R regulated effects could be observed in wild type podocytes, but the signal was faint. Due to the observed differences, and the finding that PAN-induced podocyte injury did increase expression of MC1R in wild type murine podocytes, the decision was made to conduct experiments on MC1R overexpressing cells to facilitate the study of downstream pathways. Thereby, the use of MC1R overexpressing cells was continued in our experiments. The observed increase in MC1R expression due to glomerular and podocyte injury reported here, further support this decision. This observed increase could hypothetically be a form of protective response from the podocytes, through which they try to maintain their actin cytoskeleton and attachment when stressed.

The overexpressed constructs of MC1R, wild type MC1R and constitutively active E92K, both proved to cause cAMP production, as presented in the supplemental material of paper I. This proves the ectopically expressed receptors as functional, since MC1R signaling in vivo is attained through cAMP. However, the constitutively active E92K, has been reported not to stimulate other signaling pathways regulated by the wild type receptor, such as the signaling through ERK1/2. The partial rescue effect against PS induced actin cytoskeleton rearrangement observed in E92K overexpressing cells could therefore be attributed to the inability of the receptor in generating ERK1/2 activity.

In our study, we chose to investigate the signaling pathways and involved proteins at the earlier time points of MC1R stimulation, revealing our proposed signaling pathway of actin cytoskeleton stabilization. Further analysis of signaling pathways at the later time points might provide us with additional information on the MC1R regulated actin cytoskeleton pathways.

4.2 Paper II: Podocyte Geranylgeranyl transferase type I is essential for maintenance of the glomerular filtration barrier function

The RhoGTPase family of proteins have in the latest decades attracted attention in biochemical research due to their many functions. They have been the interest of cancer research, research on the immune system and in the research of glomerular disease. One of their important functions is the regulation of actin cytoskeleton dynamics, in which three prominent members of the RhoGTPase family have been identified; RhoA, Rac1 and Cdc42 (32). In the renal research field, the interest for the RhoGTPases in their role as actin cytoskeleton regulators is motivated by the importance of the actin cytoskeleton for the structure and function of the podocytes in the glomerular filtration barrier (40). RhoGTPase activity has been shown important for the integrity of the podocytes by the findings that a dysregulated activity of RhoGTPases results in foot process effacement, glomerular disease and nephrotic syndrome in both humans and experimental mice models(41, 43, 46, 48, 49).

RhoGTPase activity is regulated by several upstream proteins and modifications. Guanine nucleotide exchange factors activate the RhoGTPases, GTPase activating proteins inactive them, and RhoGDIs sequesters the GDP-bound, inactive RhoGTPases in the cytoplasm (39). A post-translational lipid modification of RhoGTPases is performed by the Geranylgeranyl transferase type I (GGTase-I) through the attachment of a 20-carbon geranylgeranyl lipid to the CAAX-motif in the C-terminal end of the proteins (94). It has been described to localize the RhoGTPases to the cell membrane as well as regulate their activity (169). Prevention of RhoGTPase geranylgeranylation has been believed to cause a decrease in their activity. However, this has been proven to be complex and studies have found an increase in activity of RhoGTPases following geranylgeranyl transferase type I inhibition (153, 170, 171). The consequences of this modification have not been investigated in the context of glomerular disease and RhoGTPase function in podocytes.

In paper II we therefore set out in investigating the role of geranylgeranylation of RhoGTPases in podocytes and the consequential effects on RhoGTPase activity, foot process and actin cytoskeleton integrity and glomerular filtration barrier function.

First, we wanted to assess the expression of the enzyme GGTase-I in the podocytes and other glomerular cells. No previous description of expression

of the enzyme in podocytes has been made to our knowledge, and only mesangial cells have been described to express the enzyme (172). Human and murine glomerular tissue sections underwent immunofluorescent labelling for geranylgeranyl transferase type I and cellular markers for podocytes and endothelial cells. In the murine sections, a clear co-localization could be observed between the GGTase-I and synaptopodin, a marker for podocyte foot processes. No co-localization was seen with CD31, the marker for endothelial cells. In human sections, the clearest overlap was observed with WT-1, the marker for podocyte cell bodies, though some co-localization could also be seen with synaptopodin. The marker for endothelial cells, Ulex, showed no co-localization with GGTase-I. This led us to conclude that podocytes express GGTase-I and constitute the main cells expressing the enzyme in the filtration barrier.

As a measure to evaluate the importance of geranylgeranylation in podocytes, we created mice with specific depletion of GGTase-I in podocytes. Mice expressing the Cre-recombinase gene driven by the podocyte specific promotor for human podocin were bred with mice expressing lox-p sequences flanking exon 7 of Pggt1b, the gene for the β-subunit of GGTase-I. The breeding gave rise to mice with a podocyte specific knockout of the Pggt1b. The progeny was born in a mendelian ratio with mice that were homozygous for the expression of Cre only, referred to as $Pggt1b^{+/+}$, heterozygous mice expressing Cre and one floxed allele of Pggt1b referred to as $Pggt1b^{fl/+}$, and mice homozygous for both Cre and two floxed alleles of Pggt1b, referred to as $Pggt1b^{fl/fl}$.

In order to assess the integrity of the filtration barrier following depletion of GGTase-I, urine samples were collected from the mice by 24-hour housing in metabolic cages. The mice were kept in metabolic cages at even intervals over a 7 months period of time, allowing for the continuous assessment of glomerular function in the mice. At the point of urine collection, the urines were assessed for albumin content through gel electrophoresis analysis. $Pggt1b^{fl/fl}$ mice developed albuminuria at 1 month of age in comparison to littermate control mice. Over the course of the study, the albuminuria progressed and reached significantly higher levels in the $Pggt1b^{fl/fl}$ mice ($p<0.001$), in comparison to $Pggt1b^{+/+}$ and $Pggt1b^{fl/+}$ mice.

Since Cre-expression alone has proven to cause morphological changes to the glomeruli of mice as well as susceptibility to renal damage, the $Pggt1b^{+/+}$ mice were kept as control in the study, instead of wild type mice. Following the first assessments of proteinuria through gel electrophoresis of urine aliquots, it became clear that the heterozygous mice, $Pggt1b^{fl/+}$ did not develop a significant proteinuria in comparison to the $Pggt1b^{+/+}$ mice. As we resonated,

this was probably due to the remaining allele allowing for the expression of GGTase-I and thereby a residual capacity of modifying the RhoGTPases. In the following analyses, these mice were therefore excluded.

The normalization of albumin concentration in urine to the concentration of creatinine makes for a reliable assessment of albuminuria correcting for the concentration of urine. To assess the albuminuria in our mice with precision we therefore performed albumin and creatinine analysis on the collected urines, focusing on the $Pggt1b^{fl/fl}$ and the $Pggt1b^{+/+}$ mice at the first and the last time point of urine collection. From the measured concentrations, an albumin/creatinine ratio was calculated. The analysis showed an increase in albuminuria in the $Pggt1b^{fl/fl}$ mice at 1 month, that raised to significant levels at 7 months ($p<0.001$), in comparison to the $Pggt1b^{+/+}$ mice. These results suggested that geranylgeranylation is of importance for podocyte function, and thereby for the integrity of the glomerular filtration barrier.

Some proteins in the RhoGTPase family can be post-translationally modified by another form of prenylation, called farnesylation, which is performed by the enzyme Farnesyl transferase (94). The consequences of farnesylation are believed to be the same as for geranylgeranylation, meaning regulation of localization and activity of RhoGTPases. To assess whether farnesylation has the same influence on podocyte and filtration barrier integrity, we established a mouse with podocyte specific depletion of farnesyltransferase. This was done by breeding of Podocin-Cre expressing mice with a mouse expressing the lox-p sequence flanking exon 1 of Fntb, the gene for farnesyltransferase.

Assessment of proteinuria in 4-month-old $Fntb^{fl/fl}$ mice showed no increase in proteinuria in comparison to the $Pggt1b^{+/+}$ control mice. However, a significant increase in proteinuria was found in the $Pggt1b^{fl/fl}$ at the age of 4 months ($p<0.01$). The depletion of farnesyl transferase did not show any consequences for the function of the podocytes or the filtration barrier at 4 months of age, whilst removal of GGTase- clearly did. We thereby speculated that FTase was not as important for podocyte function as GGTase-I and decided to terminate the studies on FTase depleted mice. The results suggested to us that specifically GGTase-I was important for podocytes. Further on, since RhoA, Rac1 and Cdc42 all have been described to be specifically modified by GGTase-I (96), and not to be prenylated by FTase, our results suggest that the phenotype we observed in our mice specifically involved the three mentioned RhoGTPases.

After establishing the presence of albuminuria in the GGTase-I depleted mice, we sought to investigate the morphological changes in the glomerulus and filtration barrier that could explain the albuminuria. Renal cortex tissue was

obtained from mice and prepared for analysis through transmission electron microscopy. In the micrographs of glomeruli from mice at 1 month of age we could observe a tendency of podocyte foot process effacement in the $Pggt1b^{fl/fl}$ mice, which was not present in the control $Pggt1b^{+/+}$ mice. At 7 months of age, the foot process effacement in the $Pggt1b^{fl/fl}$ mice had progressed and presented itself in a segmental pattern. No such development was observed in the control mice. Quantification of foot processes per μm of glomerular basement membrane showed a significant reduction in the number of foot processes per length of basement membrane in $Pggt1b^{fl/fl}$ mice at 7 months, compared to control ($p<0.001$). GGTase-I thereby seem to be of importance for maintaining foot process structure, and a depletion of the enzyme cause a foot process effacement that explain the albuminuria.

In a second step to assess glomerular pathology in the mice, histological analysis was performed on glomerular tissue sections. Tissue sections were stained with periodic acid-Schiff for the evaluation of extracellular tissue deposition such as sclerosis, and with hematoxylin/eosin to assess the tissue and cell morphology. Due to the changes inflicted by cryo-preservation of tissues, the glomerular morphology was difficult to assess on a microscopical level. On a macroscopical level however, the presence of glomerular or tubular sclerosis was excluded. Inflammatory processes in mentioned compartments was also excluded. The results suggest that pathological processes commonly seen in relation to albuminuria were not present in glomeruli of the mice. A closer analysis of morphology in better preserved tissues might however provide additional information regarding glomerular and tubular morphology.

Foot process effacement can be seen as a result of a disrupted actin cytoskeleton, in turn caused by dysregulated activity of RhoGTPases (22). We therefore wanted to assess the activity of RhoA, Rac1 and Cdc42 following depletion of GGTase-I in podocytes. Unfortunately, the isolation of primary podocytes from the genetically modified mice proved to be difficult and we were not successful in getting podocytes to proliferate in culture. Therefore, we resorted to conditionally immortalized murine podocytes in culture. GGTase-I activity was reduced in the murine podocytes through lentiviral shRNA mediated knockdown of the enzyme and through the treatment with a specific inhibitor of GGTase-I, GGTI-298. Reduced activity of GGTase-I was assessed in both experimental modalities through western blot analysis and probing for non-prenylated Rap1. Thereafter followed analysis of RhoGTPase activity using affinity-based activity pulldown and detection kits and western blot analysis. Both the knockdown of GGTase-I and treatment with the inhibitor proved to significantly increase the activity of the three RhoGTPases ($p<0.05 – 0.001$).

Rap1 is a protein belonging to the RasGTPase family. It has been described to be specifically prenylated by GGTase-I, and also to be important in the regulation of β_1-integrin activity in podocyte focal adhesions (173, 174). We therefore wanted to assess the activity of Rap1 following reduced GGTase-I activity. As for the other GTPases, Rap1 activity was found to increase following knockdown of GGTase-I, almost reaching significant levels ($p = 0.0506$). Treatment with the inhibitor resulted in a significant increase in Rap1 activity ($p<0.01$).

Collectively, these results showed that inhibition of geranylgeranylation in podocytes led to an increased activity in RhoGTPases important for actin cytoskeleton regulation and in a RasGTPase important for regulation of β_1-integrin. These *in vitro* results are probably translatable to the *in vivo* situation of GGTase-I depletion in podocytes, and could explain the observed foot process effacement and albuminuria observed in the transgenic mice. Increased activity of both RhoA and Rac1 have been shown to cause foot process effacement and proteinuria in transgenically modified mice overexpressing either RhoGTPase (41-44). Besides regulating β_1-integrin activity and clustering, Rap1 is also known to act as a scaffolding protein for GEFs regulating Rac1 and Cdc42 activity (175). An increase in active Rap1 could thereby further stimulate increased Rac1 and Cdc42 activity in the podocytes. The balanced activity between RhoGTPases would hence be disturbed and the podocyte left in a stressful state with both migratory and stationary forces affecting it. The podocytes normal response to stress is the effacement of foot processes, as we observed in our mice. The influence of GGTase-I on RhoGTPases and Rap1 thereby seem a likely explanation to the pathological processes in the $Pggt1b^{fl/fl}$ mice.

To assess the consequences of increased activity of the abovementioned RhoGTPases and Rap1 for podocyte actin cytoskeleton and β_1-integrin formation, immunofluorescent microscopy was performed. Fluorescent phalloidin labeling of stress fibers was done in Pggt1b knockdown cells. In the GGTase-I knockdown cells, the distribution of the actin cytoskeleton was changed in comparison to control cells. Stress fibers otherwise spanning the width of the cell in parallel, fine fibers was disorganized and polarization of actin fibers was disturbed. The thickness of the stress fibers also seemed increased, and clustering of stress fibers was observed in the GGTase-I knockdown cells. Immunolabeling of active and total β_1-integrin revealed an altered, disorganized distribution of the focal adhesions, distributed in a pattern corresponding to the stress fiber organization. The clusters of active and total β_1-integrin were larger and thicker than observed in the control cells.

The observed changes in stress fiber and β_1-integrin formation resonates with the changes in RhoGTPase and Rap1 activity. The disturbed balance in RhoGTPase activity would explain the inability of the podocytes to form normal actin fibers, as observed in the GGTase-I knockdown cells. A disrupted actin cytoskeleton could also explain the foot process effacement observed in the GGTase-I depleted mice. The results thereby further support the idea that disturbed RhoGTPase activity as a consequence of GGTase-I depletion is the leading cause of foot process effacement and albuminuria in the $Pggt1b^{fl/fl}$ mice. The increased size of β_1-integrin clusters could be explained by the increase in Rap1 activity, which is known to increase the clustering of β_1-integrins (176). The functional consequences this change would entail for podocytes does however need to be further assessed.

Podocytes are known to release cytokines in response to stressful stimuli. In an attempt to assess the podocyte response to the stress inflicted on them by depletion of GGTase-I, a Bio-Plex analysis of cytokines was performed on cell culture media. The cytokines analyzed in the Bio-Plex were cytokines known to be released in connection to inflammatory processes and as a response to toxic metabolites present in glomerular disease (177-180). In the analysis, the release of monocyte chemoattractant protein-1 (MCP-1) and IL-6 were both significantly decreased in GGTase-I knockdown cells in comparison to control ($p<0.05$). Excretion of KC, the mouse equivalent to IL-8, was unchanged, whilst levels of IL-1 and TNF-alfa were below the limit of detection in the analysis.

MCP-1 is a chemoattractant for macrophages. In the glomeruli, several cells excrete the chemoattractant, guiding macrophages to the site (181). Macrophages are in turn known to contribute to inflammatory processes and sclerotic transformation in the glomeruli and tubulointerstitial tissue in glomerular disease (182). The treatment with statins has proven to reduce the renal infiltration of macrophages in the PAN-rat model of renal disease (183). One of the anti-inflammatory effects of statins is the inhibition of MCP-1 expression through the inhibition of geranylgeranylation of proteins (184). It is therefore likely that the reduction in MCP-1 excretion in our podocytes is due to the inhibition of geranylgeranylation through depletion of GGTase-I. Further on, it is possible that the absence of inflammation or sclerosis in the histological analysis of renal tissue from the mice, could in part be ascribed to the decreased expression of podocyte MCP-1 and thereby less infiltration of macrophages in glomerular tissue.

General discussion:

In this study, our focus was on the consequences of GGTase-I inhibition for RhoGTPase activity in relation to the actin cytoskeleton dynamics. However, our results also suggested that other processes besides actin cytoskeleton dynamics were affected by the loss of geranylgeranylation of RhoGTPases. Release of cytokines from podocytes was altered, showing that GGTase-I has an immunomodulatory effect in podocytes as well. Since RhoGTPases affect many signaling pathways, further investigation of processes in the podocytes affected by GGTase-I deficiency should be made to complete the picture. The mechanism behind the increased GTP-loading of RhoGTPases was not addressed in this study, but will also be necessary to investigate.

As mentioned above, statins can affect the geranylgeranylation of proteins. Through blocking of the mevalonate pathway, statins reduce the levels of the substrate for GGTase-I (102). Statins have been implicated in modulation of proteinuria, described to both reduce and increase it (185, 186). Increased proteinuria secondary to treatment with certain statins, was suggested to be due to impaired tubular reabsorption of albumin as a consequence of an inability to prenylate GTPases in tubular cells (187). Our findings suggest that there are other possible reasons for proteinuria related to statin treatment, since geranylgeranylation inhibition in podocytes causes foot process effacement and albuminuria. Reported beneficial effects could in such circumstances be due to immunomodulatory properties of statins (188). A subclass effect between different statins could explain the inconsistent outcomes of statin treatment.

Nitrogen containing bisphosphonates, N-BPs, also affects the mevalonate pathway, and consequently the supply of geranylgeranyl pyrophosphate that is available for GGTase-I (103). Treatment with N-BPs has been found to cause acute kidney injury, nephrotic syndrome and FSGS-like disease in patients (189-192). The underlying cause has not been identified. However, it has been found that N-BPs cause a decrease in geranylgeranylation of RhoGTPases in macrophages and osteoclasts with subsequent increase in their activity (193, 194). Suggestions that similar effects in renal cells would cause the N-BP induced renal disease have been made. Our findings of an increased activity of RhoGTPases secondary to inhibition of geranylation in podocytes support such a hypothesis and offers a partial explanation for the pathological processes in N-BP related renal disease.

4.3 Paper III: The role of βpix in podocyte Rac1 activation and cytoskeleton rearrangement

The focus of paper III was, in similarity with paper II, to investigate regulators of RhoGTPases in podocytes. As previously mentioned, beside the prenylation modification explored in paper II, the RhoGTPases are regulated by three groups of proteins, the GEFs, GAPs and RhoGDIs.

Research has identified both GAPs and RhoGDIs to be involved in glomerular disease in humans. Mutations in the gene for Arhgap24 was identified to cause an inability to inactivate Rac1, leading to a familial form of focal segmental glomerulosclerosis (48). A mutated form of a RhoGDI, ARHGDIA, was also found to cause glomerular disease with congenital and childhood onset nephrotic syndrome (49). Hence, the upstream regulators of RhoGTPase activity are seemingly important in the development of glomerular disease.

Guanine nucleotide exchange factors, GEFs, are proteins responsible for the activation of RhoGTPases through the exchange of bound GDP to GTP. GEFs constitute a group of over 60 proteins in humans (195), most of which are still not identified or investigated in podocytes. However, the importance of balanced RhoGTPase activity for the integrity of podocytes prompts the exploration of this group of proteins.
In paper III we set out to identify guanine nucleotide exchange factors important for the regulation of RhoGTPase activity in podocytes, with a possible role in glomerular disease.

GEF activity has been proposed to be regulated through phosphorylation (39). In our first attempt at identifying GEFs important for podocyte actin cytoskeleton regulation, we applied a phospho-proteomic approach. Immortalized murine podocytes in culture were treated with protamine sulphate (PS), as previously described known to cause podocyte damage and induction of actin cytoskeleton rearrangement. Following phospho-enrichment processing of protein lysates and TMT-labeling of samples, analysis was done by LC-MS/MS. In the analysis, several GEFs with altered total protein level or phosphorylation status were identified. A phosphorylation-ratio above 1.2 or below 0.8 in the comparison of phosphorylation between PS treated samples and control was considered significant.

One GEF that showed an altered phosphorylation status in response to PS was βpix. Three phospho-sites in βpix were identified to be affected by the treatment with PS: Ser340, Ser516 and Thr526. Phosphorylation of Ser340 was already increased at 15 minutes of treatment, but increased over time, reaching

a 2-fold increase at 60 minutes of treatment. In Ser516, the phosphorylation also increased over time, but was not as evident as phosphorylation of Ser 340. In contrast, phosphorylation of Thr526 decreased with time, reaching the lowest phosphorylation level at 60 minutes.

Phosphorylation of βpix Ser340 downstream of EGFR has been shown to increase βpix affinity for, and the activation of, Rac1 (119). Both phosphorylation of the site and increased βpix activity has been observed as a consequence of EGFR activation in several studies (196, 197). Since PS can induce activation of EGFR (90), we speculated that the increase in phosphorylation of βpix Ser340 was due to EGFR-activity in response to PS. Since the site also is known to initiate Rac1 regulation, we hypothesized the PS induced activation of EGFR would lead to the βpix mediated increase of Rac1 activity in the podocytes.

The Ser516 site is also known to regulate βpix affinity for Rac1 (118), however, the phosphorylation ratio at this site did just reach a significant level. This suggested to us that the PS treatment was not affecting the phosphorylation status of this site to the same extent as it did affect Ser340. Thr526 is known to regulate both Cdc42 and Rac1 in collaboration with sites Ser516 and Ser 525, respectively (120-122). The significantly decreased phosphorylation of Thr526 suggested that βpix affinity for, and regulation of, Cdc42 was not regulated by PS stimulation.

Interestingly, a recent study by Matsuda et al. (125), showed that βpix mainly regulated Cdc42 activity in podocytes. The discrepancies between our two studies could be ascribed to the dual capacity of βpix in regulating both RhoGTPases and the respective experimental circumstances. Phosphorylation of the different sites in βpix and downstream events seems to be regulated differently depending on the treatments. Our experimental model using PS stimulation increased the affinity of βpix for Rac1, whilst seemingly reducing the affinity for Cdc42. It is thereby likely that other experimental conditions guide βpix towards Cdc42.

In order to further analyze the mass spectrometry data and map out pathways regulated upon PS treatment, analysis using Database for Annotation, Visualization, and Integrated Discovery was done. In the analysis, the most probable regulated pathway following treatment with PS was the actin cytoskeleton pathway. In the pathway, βpix was found to be a central signaling protein. These findings indicate that βpix is involved in PS induced actin cytoskeleton regulation, which judging by the phosphorylation data probably is done through the regulation of Rac1. Rac1 is since before known to cause

actin cytoskeleton rearrangement downstream of PS (54, 55), further supporting our hypothesis.

Our next step was thereafter to localize the expression of βpix in podocytes. Earlier reports on βpix localized the protein to focal adhesions where it overlapped with the traditional focal adhesion protein paxillin (105). Co-immunolabeling of βpix and classic focal adhesion proteins paxillin and vinculin showed that βpix was located to the focal adhesions in podocytes as well. Some expression of βpix dispersed in the cytosol was also observed. Focal adhesions constitute a central node for cellular attachment and actin fiber formation (26), why localization of βpix to focal adhesions could allow for the regulation of both actin cytoskeleton and focal adhesion dynamics. The importance for βpix in regulation of the actin cytoskeleton had already been suggested to us in the previously described experiments. After having established the localization of βpix to FAs, we therefore wanted to investigate the possible role for βpix in focal adhesion dynamics as well.

Through lentiviral shRNA transduction, βpix was knocked down in immortalized murine podocytes. β_1-integrin is an essential component of the focal adhesions of podocytes, initiating the assembly of these important structures (25). To study the effect of βpix depletion on focal adhesion assembly, immunolabeling of active and total β_1-integrin was done. Depletion of βpix led to a disruption of the otherwise organized β_1-integrin pattern observed in control cells. In the knockdown cells, β_1-integrin clusters were dispersed over the cell surface, seemingly smaller in size than their counterpart in control cells. The number of focal adhesions also seemed to be increased. To properly assess the changes on β_1-integrin conformation and localization, further analysis is needed. However, the current data suggest a role for βpix in regulation of focal adhesion dynamics in podocytes. Similar to our results, reports have been made on changes in FA size, localization and assembly due to decreased βpix activity, where βpix has been suggested to negatively regulate FA maturation (114-116). Whether this is the role for βpix in FA regulation in podocytes and the practical consequences need to be investigated further.

To assess the effects of βpix depletion on actin cytoskeleton formation, labeling with fluorescent phalloidin was done as well. Stress fibers in βpix knockdown cells were thicker with wider ends in comparison to the thin stress fibers in control cells. These findings further support the role of βpix in actin cytoskeleton regulation and led us to the next step in our investigation of the GEF.

As mentioned, PS induce actin cytoskeleton rearrangement, which has been described to occur through increased Rac1 activity. Our phospho-proteomic data suggested that PS treatment increased the activity of βpix and stimulated regulation of Rac1 activity. This led us to hypothesize that PS induced loss of actin cytoskeleton could be due to increased βpix activity and downstream activation of Rac1. In order to test this hypothesis, βpix knockdown cells and control cells were treated with PS for 60 minutes and thereafter labeled with fluorescent phalloidin. In our analysis we found that depletion of βpix protected against PS induced loss of stress fibers observed in the control cells. Stress fiber quantification allowed for the proper assessment of this protective effect which proved that βpix depletion did significantly protect against the insult of PS treatment ($p<0.001$). A similar protection against PS induced damage has previously been described in podocyte Rac1-deficient mice (45). The results confirm the role of βpix in the regulation of actin cytoskeleton dynamics in podocytes, and that PS mediates one way of activating the GEF. Further on, our data also suggest that βpix-controlled actin cytoskeleton regulation is attained through regulation of Rac1. Further experiments are however needed to confirm this hypothesis.

In the article by Matsuda et al. (125) depletion of βpix was found to cause detachment of podocytes further augmented by treatment with Adriamycin. Depletion of βpix did thereby not seem to protect against the damage induced by Adriamycin, as it does against treatment with PS. Adriamycin is known to cause podocyte cell death secondary to DNA-damage (91), whilst PS inflicts damage to the podocytes through disruption of the actin cytoskeleton and dysregulation of RhoGTPases (54, 55). These differences suggest that depletion of βpix is protective in a model of altered RhoGTPase activity, further supporting the hypothesis that βpix is important for actin cytoskeleton regulation in podocytes.

Thus far, the experiments performed *in vitro* suggested a functional role for βpix in actin cytoskeleton regulation in podocytes. As a first step to assess the role of βpix *in vivo*, analysis of glomerular RNA-sequencing data from two murine models of diabetic nephropathy was done.

In the analysis, glomerular βpix expression data from eNOS db/db mice and BTBR ob/ob mice was evaluated. In the eNOS db/db mice, expression of βpix at 10 weeks of age was unchanged in comparison to control mice. However, at 18 weeks of age, there was a tendency of increased glomerular βpix expression in eNOS db/db mice. In the BTBR ob/ob mice, an increase in glomerular expression of βpix was observed at 8 weeks of age, further increasing at 14 and 20 weeks of age when comparing to control lean mice. A high protein diet

further increased the glomerular expression of βpix in the BTBR ob/ob mice. No changes in cortical expression of βpix could be observed in the two models of glomerular disease. Although no significance could be found in the increased expression of βpix in neither of the animal models, the tendency of increased expression over time and in relation to disease progression suggest a role of βpix in diabetic glomerular disease. Whether this is as a cause of disease, or as a protective response cannot be said, and further analysis is needed. However, βpix activity seems to be important for glomerular function, since the analysis showed no changes in the expression of βpix in tubulointerstitial tissue in relation to disease. The investigation of βpix activity in podocytes thereby seem appropriate.

Several isoforms of βpix exist and have been described to be differently expressed in cells of different origin (198). In an effort to further understand the role and expression of βpix in vivo, western blot analysis on murine tissue lysates was done. In the analysis, mainly two isoforms of βpix were identified in whole kidney, glomeruli and podocytes of murine origin. One was the ubiquitously expressed β1pix or C-isoform, previously described to be expressed by podocytes (128). The second, not previously described in either renal tissue or podocytes, was the β1pix-b or H-isoform. The H-isoform appeared to be the most abundant βpix-isoform in podocytes. In other tissues, expression of βpix was found to agree with previously described tissue expression, like C-isoform expression in neural tissue (127). Through immunoprecipitation we further confirmed the expression of these isoforms. Our investigation hereby revealed the expression of a previously unknown βpix isoform in podocytes and confirmed the expression of a previously known isoform.

The two isoforms differ in the C-terminus of the protein, by an insertion of 59 amino acids. The insertion is located between the GBD domain, mediating binding to GIT1, and the coiled coil domain, CC, that enables dimerization of the protein (127, 198). In an effort to investigate the two isoforms further, we over-expressed them tagged with EGFP in murine podocytes. Cloning of either isoform from podocyte cDNA was done and then ligated into an EGFP-tagged overexpression vector. Through immunolabeling of paxillin and fluorescent labeling of the actin cytoskeleton the spatial localization of the two isoforms was assessed. The two isoforms proved to be differently located in the podocytes, the H-isoform was located to the ends of stress fibers, suggesting a focal adhesion localization, whilst the C-isoform seemed to be located along the stress fibers. Interestingly, the localization of paxillin seemed to differ depending on the expressed isoform, co-localizing with the isoforms in their respective localizations. To further investigate the localization of the two

isoforms, cell fractionation of wild type podocytes and subsequent western blot analysis of the subcellular fractions was done. In the analysis, the H-isoform was expressed in whole cell lysate and in the cytosol, organelle and plasma membrane fractions. The C-isoform was however expressed in all compartments but the plasma membrane. Focal adhesions are structures located close to the plasma membrane, why the finding that the H-isoform is found in this fraction is reasonable. Since the C-isoform mainly showed localization to the cytosol and cytoskeleton in the overexpression experiment, the absence of the C-isoform in the plasma membrane fraction is to expect. The differences in localization and influence on paxillin localization suggests that the isoforms serve different functions in the cell and allow for the speculation on the possible implications of these two isoforms of βpix in podocytes. One possibility is pure redundancy between isoforms, where loss in expression or function of one isoform is saved by another. However, it could also be that the two isoforms possess different qualities that renders them important in different situations. The insertion of 59 amino acids could entail a relief of a steric hindrance in the protein, altering the interaction with other proteins or allowing for the di- and oligomerization described as essential for generating GEF-activity (126). These are just speculations on our behalf which needs to be confirmed through further experiments.

General discussion

Some of the results presented in this project are of a preliminary nature and need to be confirmed through addition of experiments. Mass spectrometry analysis was only performed once, and should be repeated in order to obtain additional information that mass spectrometry analysis could yield. However, the analysis that was performed allowed us to find a new probable protein candidate whose activity was assessed through complementary experiments. The experiments performed following identification of βpix did confirm that it was important for actin cytoskeleton regulation as indicated by the pathway analysis. The experiments also confirmed that PS does seem to regulate the activity of βpix, however, further experiments assessing the activity of Rac1 downstream of βpix in this experimental setup are needed. Since activation of βpix could be due to PS induced activation of EGFR it should be investigated whether activation of EGFR with receptor specific ligands yield βpix activation. Further experiments are needed in order to fully understand the role of βpix in focal adhesion assembly and the implications in podocytes. Further on, a closer investigation of βpix *in vivo* is needed to determine the role of the protein in podocytes in both healthy and diseased glomeruli. The expression of βpix in human podocytes also needs to be evaluated, in order to address the possibilities of translating our findings to a human setting, a process that can prove to be hard.

Final discussion

The role of RhoGTPase signaling for actin cytoskeleton regulation in podocytes has been stressed in several summarizing articles during the last decades, where RhoGTPase regulatory proteins and pathways have been reviewed (26, 40, 199). A consensus has formed regarding the need for a controlled and balanced activity of these proteins for appropriate podocyte function, and many have suggested that regulation of the RhoGTPases could constitute the target for future therapy. However, as recently described, the regulation of RhoGTPases in podocytes involves an intricate network, where several pathways intersect at the level of the RhoGTPases (40). Further studies will be needed in order to unravel the components of this complex network.

All three papers included in this thesis underline the importance of a balanced regulation of actin cytoskeleton for podocyte structure and function. Three distinct ways of regulating the activity of RhoGTPases and actin reorganization in podocytes have been delineated in our studies, demonstrating both the sensitivity of this system for alterations in components affecting RhoGTPases and the range of the possible ways of regulating their activity. Directly targeting the RhoGTPases would be difficult and probably lead to adverse effects, why upstream modulators should be of interest in development of future therapies. Some of these regulators have been identified here and will complement the current understanding of the actin cytoskeleton and RhoGTPase regulating pathways in podocytes.

5 CONCLUDING REMARKS

The three papers in this thesis aimed to improve the current understanding of RhoGTPase activity and actin cytoskeleton dynamics in podocytes. In an effort to do so, three different paths to regulation of RhoGTPases were discovered, that in their individual ways provide new information to the research field.

5.1 Paper I

In paper I, the aim was to further elucidate the mechanisms by which melanocortin receptors mediate renoprotection, through investigation of the glomerular expression of melanocortin receptors and the identification of actin stabilizing pathways downstream of MC1R.

The study revealed increased glomerular expression of MC1R in the human nephrotic diseases MN and FSGS, as well as in the PAN-rat disease model of nephrotic syndrome. Increased podocyte expression of MC1R was also observed in the PS model of acute podocyte injury. PS induced actin cytoskeleton rearrangement proved to be reduced in podocytes treated with a MC1R specific agonist, MC1R-a. Further on, the involvement of MC1R in regulation of podocyte actin cytoskeleton dynamics was confirmed through mass spectrometry and IPA analysis. Biochemical analysis thereafter identified the actin cytoskeleton stabilization pathway downstream of MC1R to involve the ERK1/2 mediated phosphorylation and inactivation of EGFR, leading to a decreased Src activity, stabilized synaptopodin levels and promotion of stress fiber formation through RhoA.

Taken together, these results suggest MC1R to be the receptor responsible for mediating the effects of ACTH in treatment of glomerular disease. We thereby propose MC1R as a receptor mediating podocyte protection which is achieved through stabilization of the podocyte actin cytoskeleton.

5.2 Paper II

In paper II, the aim was to investigate the consequences of prenylation of RhoGTPases in podocytes.

The aim to assess the role of geranylgeranylation of RhoGTPases in podocytes was attained through studies performed *in vivo* and *in vitro.* First, we found that podocytes express the enzyme Geranylgeranyl transferase type I, both in humans and in mice. Second, we found that depletion of GGTase-I in

podocytes *in vivo* led to the development of albuminuria in mice, with concurrent foot process effacement – both of which progressed over time. Podocyte depletion of the prenylation enzyme farnesyl transferase in mice did not affect the glomerular integrity, and confirmed the development of albuminuria to be specific for the depletion of GGTase-I. *In vitro* studies performed on immortalized murine podocytes showed that both knockdown and inhibition of GGTase-I led to an increased activity in actin cytoskeleton regulating proteins RhoA, Rac1 and Cdc42, as well as in the β_1-integrin regulating protein Rap1. The increased activity of the RhoGTPases and the RasGTPase provides a possible explanation to the foot process effacement and albuminuria observed in the GGTase-I depleted mice. Stress fiber and β_1-integrin morphology was altered in GGTase-I knockdown cells, as a probable result of imbalanced activity of the RhoGTPases and Rap1, and further explaining the observed foot process effacement in the mice. Finally, it was found that podocytes depleted of GGTase-I excreted significantly lower levels of MCP-1 and IL-6, possibly affecting the development of pathology in renal tissue.

In conclusion, these results provide further evidence that geranylgeranylation is not needed for the activation of RhoGTPases. The results also show that inhibition of geranylgeranylation in podocytes results in an imbalanced activity of RhoGTPases leading to a disruption of the actin cytoskeleton, foot process effacement and proteinuria in mice. We thereby conclude that geranylgeranylation of RhoGTPases is important for podocyte and glomerular filtration barrier integrity and function.

5.3 Paper III

In paper III, the aim was to identify guanine nucleotide exchange factors important for the regulation of RhoGTPase activity and actin cytoskeleton dynamics in podocytes.

Through mass spectrometry analysis of the podocyte phospho-proteome following PS treatment, we identified the GEF βpix. The site-specific increase in phosphorylation at Ser340 suggested βpix to regulate Rac1 activity following PS stimulation. Pathway analysis proposed βpix to be involved in actin cytoskeleton regulation, which was confirmed by experiments where knockdown of βpix altered stress fiber and focal adhesion morphology in podocytes. The actin cytoskeleton regulatory role of βpix was further confirmed through the observation that knockdown of βpix significantly protected against PS induced actin cytoskeleton dysregulation. A tendency of

increased expression of glomerular βpix was observed in animal models of diabetic nephropathy, that was augmented with disease progression.

In conclusion, these results reveal the role of βpix as a regulator of Rac1 activity and actin cytoskeleton dynamics in podocytes, with potential importance in glomerular disease. The findings presented here motivate further investigations of the actions of βpix in podocytes.

6 FUTURE PERSPECTIVES

Glomerular diseases are still somewhat of an enigma for the community of renal researchers and nephrologists. Although intense research has revealed some of the secrets behind glomerular disease, there is still a lot of progress to be made. One area of improvement is the current treatment strategies in glomerular disease. No specific treatments are available, and most treatments entail immunomodulatory agents such as corticosteroids, commonly connected to unfavorable adverse effects. In order to refine the treatment for patients suffering from glomerular disease, the intracellular processes underlying pathology needs to be defined and managed.

The work conducted in this thesis has aimed at addressing this problem and tried to improve the understanding of intracellular processes important for podocyte function. The specific proteins and modifications identified in these studies might not all serve the purpose of acting as drug targets, but offers additional parts in the puzzle in order to fully understand the RhoGTPase regulation and actin cytoskeleton dynamics in podocytes.

In paper I, we continued the investigations to better understand the renoprotective effects of ACTH. As previously mentioned, ACTH has proven beneficial in clinical studies in the treatment of MN, MCD, and some cases of FSGS. ACTH is however associated with adverse effects resembling those seen secondary to cortisone treatment, since ACTH stimulates the release of cortisol from the adrenal cortex. The findings that ACTH mediates renoprotection also in steroid resistant nephropathies suggest that the main effect of ACTH is not the increased release of cortisol, but rather a direct effect on one of the MCRs (73-75). Based on the findings from paper I and the previous work within the group, it is likely that the MC1R is the main target for ACTH in the glomeruli.

The glomerular and podocyte protective effects of MC1R specific stimulation observed in paper I and in previous studies, suggests that specific targeting of MC1R could be a potential treatment strategy in treatment of glomerular disease. By using a specific agonist of MC1R, as the one in paper I, the adverse effects seen with ACTH treatment could be avoided. Therefore, it would be of interest to further study the effects of MC1R specific stimulation in experimental glomerular diseases *in vivo*, as a step on the way to clinical trials of a MC1R-agonist in treatment of human glomerular disease.

Prenylation of RhoGTPases has been viewed as a necessary modification for their activity, why inhibitors of prenylation were developed for the inhibition of RhoGTPase activity in tumor cells (99). Research during the last two decades has however proved that inhibition of geranylgeranylation not necessarily causes an inhibition of RhoGTPase activity (153, 171). The findings in paper II add to these observations and demonstrates not only that inhibition of geranylgeranylation causes increased RhoGTPase activity in podocytes, but that it also impairs the glomerular filtration barrier. How the increased GTP-loading of RhoGTPases is induced is not known and could be of interest in future work. Seeing that the two regularly used medications statins and bisphosphonates both are known to regulate pathways providing the substrate for geranylgeranylation, it would also be interesting to investigate if they produce similar effects in podocytes as the removal of GGTase-I. Through such investigations, the adverse effects of nitrogen-containing bisphosphonates could be explained and changes in treatment strategies could be made to avoid them. Further on, it could explain the variable effect between statins on proteinuria, allowing for a refinement in statin treatment in glomerular disease.

The investigation of βpix in paper III is still at an early stage where further experiments are needed to fully understand the functional role of the protein. The ability of βpix to regulate both Rac1 and Cdc42 needs to be further addressed in future experiments, since it seems as though circumstances dictate which of the two RhoGTPases are targeted by βpix at a given moment and what the consequences are that follows. The findings of a trend in increased glomerular expression of βpix in diabetic nephropathy suggests that there is a role for βpix in the development of glomerular disease. This needs to be investigated further through the additional analyses of βpix in experimental animal models of glomerular disease.

The renal research field is expanding and new findings are being made that bring us forward. The combination of preclinical, biochemical studies with clinical trials will further improve our knowledge and allow us to offer a good care for patients suffering from glomerular disease. The findings described in this thesis will hopefully contribute to the advancement in the field by addition of information that offers insight into three distinct ways of RhoGTPase regulation, complementing the intricate network of RhoGTPase and actin cytoskeleton regulatory pathways that have been identified in podocytes.

ACKNOWLEDGEMENT

Finally, I would like to thank all the people who have been involved in the work included in this thesis, my friends and my family:

My main supervisor, **Lisa Buvall**, for giving me the opportunity to pursue a career in research and obtain a PhD. Thank you for your support and for introducing me to all the things that the scientific world entails, for challenging and motivating me in my work, and allowing me to grow as a researcher.

My co-supervisor, **Jenny Nyström,** for all the inspiration, support and guidance during this time. Thank you for your belief in me as a researcher and the confidence in me to allow me to continue my research career under your supervision.

My second co-supervisor **Kerstin Ebefors**, for always being there to help me and answer all my questions, big or small. For helping out in every situation, also those non-work-related ones. Also, thank you for all the coffee breaks and chit-chats in your office, for being to one to count on for a laugh, and for becoming my friend.

My third co-supervisor, **Börje Haraldsson**, for your inspiring lectures that opened my eyes for the kidney and for first allowing me into the group. Thank you for all those interesting conversations on the occasions we have met at ASN.

I would further like to thank all my current colleagues. **Roberto Boi**, for always being there for me, always helping out without hesitation, for all the discussions about science and music and most of all for becoming a true friend. **Alina Khramova**, my comrade and twin, thank you for all the laughter and the hugs, for teaching me Russian card games and for being the loveliest of roommates (even though you wander at night), **Dalinda Sánchez-Vidaña** for helping me with my Spanish and for sharing your philosophy about life, **Ida Carlsson**, for being a happy and colorful person with such passion for science.

Emelie Lassén, thank you for these years together as colleagues, neighbors and close friends. Your friendship has made every day easier and work has not really been work with you around. Thank you for all our stimulating, serious conversations but also all those silly, crazy moments. And thank you for being the constant optimist countering my cynicism. I am really grateful for you becoming such a close friend.

My former colleagues, **Peidi Liu**, for teaching me things in the lab, **Johannes Elvin**, for the collaboration on projects and **Deman Najar, Aditi Chaudhari,** and **Sabrina Müller-Lühlhoff,** for your support.

Thank you to all the students for helping out with things in the lab and in my projects, and for allowing me to practice my teaching skills on them; **Alva Johansson, Sarah Anjum, Sofie Melin, Paula Hansson, Timothy Johansson.** Also, a warm thank you to **Ella Warnberg** for all the nice conversations about everything and for organizing us all.

Hanna Wallentin, for being the one who first taught me how to do research hands on, for making my first summer in the lab such a fun summer and for always being such a cheerful and happy person with crazy ideas and funny stories. Your guidance and collaboration have been essential in this thesis.

Maria Johansson for all the guidance within the basic medicine program and inspiration as a researcher.

Holger Nilsson, thank you for your guidance in the jungle of physiology and teaching, for sharing your vast knowledge of things and for always having the time for an interesting conversation. **Veronika Golubinskaya**, for your support, kind words, warmth and for always encouraging me as a scientist and a medical doctor.

The rest of the gang at the Department of Physiology, for making the lunchbreaks very enjoyable (and sometimes far to loooong), with funny conversations; **Maria Hammarlund, Filip Mjörnstedt, Saray Gutierrez Sabroso, Marcus Ulleryd, Aura Zelco, Axel Andersson, Giacomo Gravina, Eridan Rocha-Ferreira, Syam Nair, Joakim Ek, Cristiano Santos, Marie Le May, Fiona Peris Sampedro, Iris Stoltenborg.**

Further on, I would like to thank my friends **Lisa Groening** and **Maja Johansson**, for always being there to offer true friendship and support, during all the years we have known each other. For giving me those refreshing breaks and funny moments when we get together. **Mikael Sandstedt** and **Caroline Josefsson**, thank you for all our dinners and conversations, for being true friends and a source of inspiration. Thank you for your kindness and positivity.

I would also like to thank my family: my mom, **Ewa Bergwall Axelsson**, for your unconditional love, your constant belief in me and for always being there, my dad, **Lasse Bergwall**, for always supporting me in my endeavors, for your calmness and belief that things will work out, and for your love.

Mårten Axelsson, for your support and for helping me with everything, for making sure I know how to multiply and for always rooting for me. **Elisabeth Olofsson**, for your guidance and advise on all matters in life. My uncle **Per Andersson**, for being a source of inspiration, for all the "bad" jokes and for always giving so generously. My baby brothers, the famous twins, **Erik** and **Gustaf Bergwall,** thank you for your patience with your big sis', for your belief in me, for all the funny moments and stories and for your warmth and kindness. **Kerstin Ärlemalm**, **Abdel-Krim Chemlal** and **Simon Ärlemalm** for opening up their home and family to me and for always showing an interest in what I am doing.

My grandmother **Lisbeth Bergwall**, and those who are no longer with us; **Sten Bergwall, Ulla Andersson and Arnold Andersson**. Thank you all for pursuing you own paths in life and for building a solid foundation for us to grow from.

And finally, **Elias Ärlemalm**, the person without whom nothing of this would be possible. Thank you for your love and your strong belief in me. For inspiring and challenging me intellectually and for supporting me emotionally, with your warmth and empathy. Thank you for everything we have built together and for being the one I always can count on. I love you ♥.

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