Nephrin Mediated Signaling in Actin Organization of Podocyte

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Abstract

Podocytes are highly differentiated epithelial cells that contribute to the selective filtration barrier of the renal glomerulus. The actin-based podocyte foot processes possess a subplasmalemmal actin network and a central core of actin filament bundles. The dynamic regulation of the podocyte actin cytoskeleton is vital to kidney function. Nephrin, identified as a slit diaphragm protein, connects foot processes from adjacent podocytes, which is critical for the structural and functional integrity of podocytes. It has been reported that tyrosine phosphorylation of nephrin was responsible for its interaction with PI3-kinase and Nck. PI3-kinase and Nck-Pak signalings are well known regulators of actin dynamics. Therefore, we studied the role of PI3-kinase and Nck-Pak signalings activated via the tyrosine phosphorylation of nephrin, in actin cytoskeleton organization of cultured podocytes. We identified that Y1152 of rat nephrin was responsible for nephrin –PI3K interaction. Stable transfection of rat nephrin in the podocytes with podocin led to nephrin tyrosine phosphorylation, PI3K-dependent phosphorylation of Akt, increased Rac1 and Pak activity, and actin cytoskeleton reorganization with decreased stress fiber and increased lamellipodia. These actin cytoskeleton changes were reversed with an inhibitor of PI3K and not seen when the nephrin mutant Y1152F replaced wild type nephrin. Pak was coimmunoprecipitated with nephrin and Nck in vitro and in vivo. The nephrin increased Pak phosphorylation was completely abolished by a nephrin mutantY1208/1224F (fail to bind Nck), but not affected by the Rac1 inhibitor. Dominant negative Nck constructs blocked Pak1 phosphorylation induced by antibody-mediated cross-linking of nephrin. Transient transfection of constitutively kinase active Pak1 into differentiated mouse podocytes decreased stress fibers and increased cortical F-actin, while kinase-dead mutant, kinase inhibitory construct, and Pak2 knockdown by shRNA had the opposite impacts. Finally, in the rat model of puromycin aminonucleoside nephrosis, nephrin tyrosine phosphorylation, nephrin-PI3K association, glomerular Akt and Pak phosphorylation were all decreased. These results suggest that PI3K and Nck-Pak signaling is involved in nephrin-mediated actin reorganization in podocytes.

Disturbed nephrin-PI3K and Nephrin-Nck-Pak interaction might contribute to abnormal podocyte morphology and proteinuria.

Résumé

Les podocytes sont des cellules épithéliales fortement différenciées qui contribuent à la barrière de filtration sélective du glomérule rénal. Les processus de pied de podocyte à base d'actine possèdent un réseau d'actine sous-plasmalemme et un noyau central de groupes de filaments d'actine. La dynamique de la régulation du cytosquelette d'actine podocyte est vitale pour la fonction rénale. La néphrine, identifiée comme une protéine d'une micro-membrane, relie les processus de pied des podocytes adjacents, ce qui est essentiel pour l'intégrité structurelle et fonctionnelle des podocytes. Il a été rapporté que la phosphorylation de la tyrosine de la néphrine est responsable de son interaction avec la kinase-PI3 et le NCK. La kinase-PI3 et la signalisation de NCK-Pak sont des régulateurs bien connus des dynamiques de l'actine. Par conséquent, nous avons étudié le rôle de la kinase-PI3 et la signalisation de NCK-Pak activée par l'intermédiaire de la phosphorylation de la tyrosine de la néphrine, dans l'organisation de l'actine du cytosquelette des podocytes de culture. Nous avons identifié que le Y1152 de la néphrine du rat était responsable de l'interaction PI3K-néphrine. La transfection stable de la néphrine du rat dans les podocytes avec podocine conduit à la phosphorylation de la tyrosine de la néphrine, la phosphorylation de l'Akt dépendant du PI3K, accroît l'activité du Rac1 et du Pak, et la réorganisation de l'actine du cytosquelette avec une diminution du fibres de tensions et l'augmentation de la lamellipode. Ces changements de l'actine du cytosquelette ont été renversés avec un inhibiteur de la PI3K et n'ont pas été observés lorsque le mutant Y1152F a remplacé la néphrine de type sauvage. Le Pak a été coimmunoprécipité avec la néphrine et le NCK in vitro et in vivo. L'activité de la néphrine activée par le Pak a été totalement abolie par une néphrine mutante Y1208/1224F (échec à lier NCK), mais n'a pas été affectée par l'inhibiteur Rac1. La construction dominant négatif de NCK a bloqué l'activation de Pak1 induite par les anticorps servant d'anti-intermédiaires par réticulation de la néphrine. La transfection transitoire de la kinase Pak1 constitutivement active dans les podocytes de souris différenciées a diminué le les fibres de tensions et l'augmentation d'actine F corticale, alors que le mutant mort, la construction

inhibitrice de la kinase, et la diminution du Pak2 par le shRNA a eu l'impact contraire. Enfin, dans le modèle de rat de la néphrose puromycine, de la phosphorylation de la tyrosine de la néphrine, de l'association de la néphrine PI3K, de la phosphorylation glomérulaire de l'Akt et du Pak ont tous subi une diminution. Ces résultats suggèrent que la PI3K et la signalisation du NCK-Pak sont impliqués dans la réorganisation de l'actine dont la néphrine sert d'intermédiare en podocytes. La néphrine PI3K ayant un désordre et l'interaction de la néphrine NCK-Pak pourrait contribuer à une morphologie anormale du podocyte et à une protéinurie.

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Contributions of Authors

Chapter 2. Text and figures in this chapter are produced from "Zhu J., Sun N., Aoudjit L., Li H., Kawachi H., Lemay S., Takano T. Nephrin mediates actin reorganization via phosphoinositide 3-kinase in podocytes. (2008) Kidney Int. 75, 556-66."

I contributed most of this paper. Ningzhi Sun and Hongping Li contributed to the identification of Y1152 of rat nephrin responsible for nephrin-p85 interaction. Lamine Aoudjit contributed to studies of puromycin aminonucleoside nephrosis and the RhoA pull-down assay.

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Publications

1. Li H., **Zhu J**., Aoudjit L., Latreille M., Kawachi H., Larose L., Takano T. Rat nephrin modulates cell morphology via the adaptor protein Nck. Biochem Biophys Res Commun. 2006;349(1):310-6.
2. Zhang H., Cybulsky AV., Aoudjit L., **Zhu J.**, Li H., Lamarche-Vane N., Takano T. Role of Rho-GTPases in complement-mediated glomerular epithelial cell injury. Am J Physiol Renal Physiol. 2007; 293(1):F148-56.
3. **Zhu J**., Sun N., Aoudjit L., Li H., Kawachi H., Lemay S., Takano T. Nephrin mediates actin reorganization via phosphoinositide 3-kinase in podocytes. Kidney Int. 2008;73(5):556-66.
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Abbreviations

ADF, actin depolymerizing factor

AID, autoinhibitory domain

BAD, Bcl-2/Bcl-X_L-antagonist, causing cell death

CD2AP, CD2-associated protein

CIN, chronophin

CNS, central nervous system

DH-PH, Dbl homology-pleckstrin homology

DHR-2, Dock homology region 2

GAPs, GTPase-activating proteins

GBM, glomerular basement membrane

GDIs, guanine nucleotide-dissociation inhibitors

GEC, glomerular epithelial cells

GEFs, guanine nucleotide exchange factors

Grb2, growth-factor-receptor bound protein 2

IQGAP1, IQ motif-containing GTPase-activating protein 1

LIMK, LIM domain kinase

LMW-PTP, low molecular weight protein tyrosine phosphatase

MAGI2, membrane-associated guanylate cyclase inverted 2

MEF, mouse embryonic fibroblast

MLC, myosin light chain

MLCK, myosin light chain kinase

PAKs, p21-activated kinases

PAN, puromycin aminonucleoside nephrosis

PBD, p21-binding domain

PDK1; phosphoinositide-dependent kinase 1

PH-domain; pleckstrin homology domain

PI3-kinase; phosphatidylinositol 3-kinase

PI, phosphatidylinositol

PI(4), phosphatidylinositol 4-phosphate

PI(4,5)P2, phosphatidylinositol 4,5-biphosphate

PI(3)P, phosphatidylinositol 3-monophosphate

PI(3,4)P2, phosphatidylinositol 3,4- biphosphate

PI(3,4,5)P3, phosphatidylinositol-3,4,5-trisphosphate

PIX, Pak-interacting exchange factor

PKB, protein kinase B

PP2A, protein-serine/threonine phosphatase 2A

POPX1 and POPX2, partner of Pix-1 and Pix-2

ROCK, Rho-associated kinase

SH2, Src-homology domain 2

SH3, Src-homology domain 3

SSH, slingshot

PCR, polymerase chain reaction

RNA, ribosome nucleotide acid

TESK, testicular kinases

TPPP, tubulin polymerization promoting protein

WASP, Wiskott-Aldrich syndrome protein

ZO-1, Zonula occludens 1

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Chapter 1. General Introduction
(Literature review)

1.1 **Glomerular filtration barrier**

The main function of the kidneys is ultrafiltration of plasma in renal glomeruli during the formation of the primary urine [1]. In an adult human, the glomeruli produce around 180 liters of primary urine per day [2]. The glomerular filtration barrier that includes the fenestrated capillary endothelial cells, glomerular basement membrane (GBM) and the visceral epithelial cells (podocytes) is responsible for the size and charge selective filtration (Figure 1) [2].

The possible roles of glomerular endothelial cells and GBM in filtration remain poorly understood. The fenestrated endothelium without a diaphragm could act as a barrier for plasma macromolecules. The surfaces of glomerular endothelial cells are negatively charged due to the coating of glycocalyx, which could contribute to the charge-selectivity of the filtration barrier. The GBM is composed of a type IV collagen meshwork linked by laminins, proteoglycans and other matrix components. Laminin is crucial for the filter function because defects in the laminin β2 chain result in massive proteinuria in mice and humans [3].

The podocytes that cover the outer layer of the GBM are highly specialized differentiated epithelial cells, which consist of the cell body and long projections extending to the primary, secondary and tertiary foot processes [3]. Podocytes are polarized, which present with the luminal (apical) and basolateral membrane domains separated by the intercellular junctions of the foot process [3]. The architecture of foot processes is maintained by a complex arrangement of actin filaments, consisting of a subcortical actin network and a central core of actin filament bundles [4]. Neighboring foot processes are laterally interconnected with a highly specialized cell-cell junction, the slit diaphragm. The fact that defects in podocyte actin-associated proteins result in proteinuria indicates that the integrity of the actin cytoskeleton in the foot processes is essential for the glomerular filtration barrier [5].

1.2. Slit diaphragm and nephrin

The slit diaphragm has been described as a zipper-like structure with pores corresponding to the size of albumin molecules [6]. The width of the slit diaphragm is about 40nm [3]. The molecular nature of the slit diaphragm

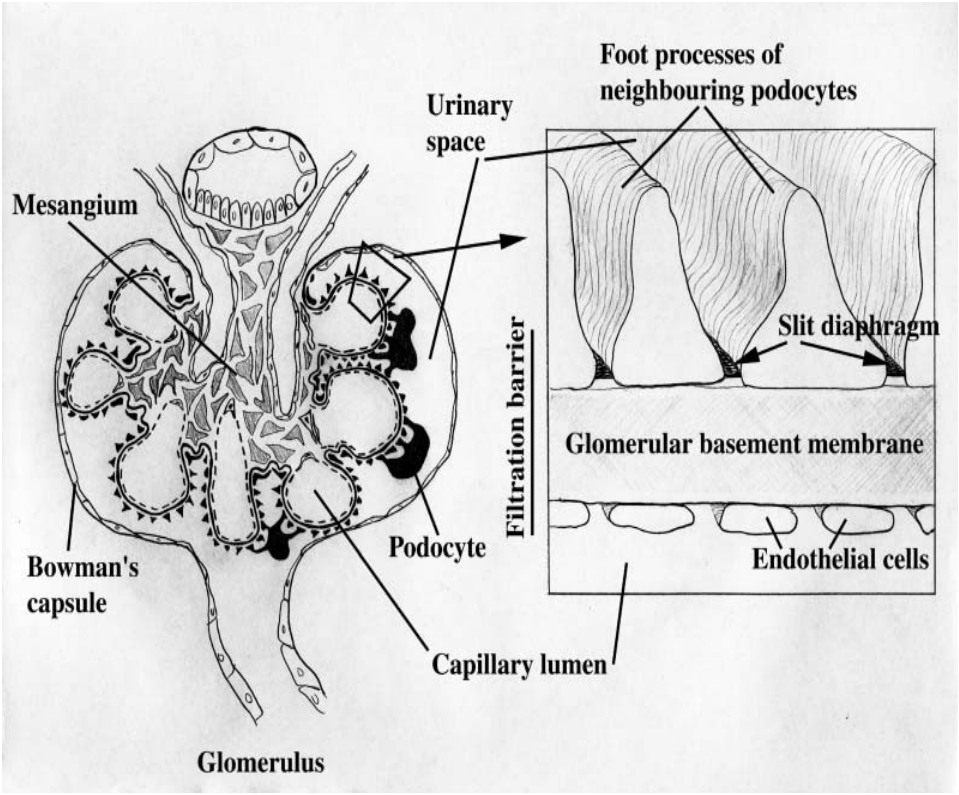

Figure 1. A schematic diagram of glomerulus (left) and the glomerular filtration barrier (right) (Patari-Sampo A. et al. Annals of Medicine 2006;38:483-492)

remained unknown until nephrin was identified. Nephrin was discovered through positional cloning of the gene mutated in patients with congenital nephrotic syndrome of the Finnish type (CNF), a rare autosomal recessive developmental disorder characterized by massive proteinuria and diffuse podocyte foot process effacement [7]. Deletion of nephrin by gene targeting in mice results in a similar phenotype [8]. In a kidney, nephrin is expressed in podocytes. In mature podocytes, nephrin is localized at foot process intercellular junction, where it has been suggested that it contributes to the mechanical filter presumed to function at the slit diaphragm [9] .

In rodents, nephrin promoter activity has been detected in extra-renal tissues, such as the central nervous system (CNS), and β-cells of the pancreas [8, 10]. In addition, nephrin expression has been indicated in mouse testis, spleen, and thymus [11]. In adult human tissues, nephrin has been detected in pancreatic and lymphoid tissue, but not in the CNS [12, 13]. However, the extra-renal function of nephrin has not been clarified.

1.3. Other slit diaphragm proteins

In addition to nephrin, there are other proteins that are localized at the slit diaphragm. The Neph family of transmembrane proteins (Neph1, Neph2 and Neph3), which is structurally related to nephrin, has been suggested to interact with the extracellular domain of nephrin [14]. Neph proteins all have a shorter extracellular domain with five extracellular IgG-like motifs. Nephs are found in many tissues, but in the glomerulus, both NEPH1 and 2 were found to form homodimers and interact with the extracellular domain of nephrin in the slit diaphragm [14-16]. The glycosylation of both NEPH and nephrin is required for the Neph-Nephrin heterodimerization [14]. The fact that injection of a combination of subnephritogenic doses of anti-nephrin and anti-Neph1 antibodies led to proteinuria in vivo suggested the importance of the nephrin-Neph1 interaction for slit diaphragm function [16]. The kidney phenotype of Neph1-deficient mice, which showed proteinuria associated with podocyte foot process effacement [17], is milder than that of nephrin knockout mice, which suggests that nephrin is the principal molecule in the slit diaphragm.

FAT1 and FAT2 are two large cadherins, which co-localize with nephrin in the slit diaphragm [18]. It has been reported that the long extracellular domain of FAT is important for cell-cell adhesion [19]. The cytoplasmic domain of FAT1 has been shown to interacts with Ena/VASP proteins, which regulate the actin polymerization complex [20]. FAT1 knockout mice suffered from proteinuria and perinatal lethality with the loss of slit diaphragm and foot process effacement [21].

Podocin belongs to the stomatin protein family, and associates in the plasma membrane with specialized region (lipid rafts). Podocin is hairpin-shaped integral membrane protein with both ends directed into the intracellular space, serving as a dock for transmembrane signaling complexes. Podocin interacts with nephrin *via* its C-terminus (amino acids 125 to 385) [22]. Nephrin-podocin interaction augments the activation of the AP-1 promoter by nephrin [22]. Mutations of podocin cause autosomal recessive steroid-resistant nephrotic syndrome in childhood [23]. Podocin knockout mice suffer from foot process effacement, absence of slit diaphragms, proteinuria, and early lethality [24].

CD2-associated protein (CD2AP) that was initially found in T-lymphocytes has been reported as a nephrin-associated protein in the podocytes. It can interact with nephrin via its C-terminal domain [25]. CD2AP has been shown to connect cell-membrane receptors and actin-modifying proteins [26]. In addition, the N-terminal domain of CD2AP can bind to p85 and facilitate the nephrin-induced AKT signaling, which protects podocytes from apoptosis [27]. CD2AP knockout mice show defects of the podocyte foot processes and mesangial cell hyperplasia with extracellular matrix depositions, and develop the nephrotic syndrome at the age of 3-4 weeks, which suggests that the function of CD2AP may be compensated for by other proteins [28].

Zonula occludens 1 (ZO-1), a component of the tight junction, is expressed in podocytes and localizes to the cytoplasmic base of foot processes near the slit diaphragm [29]. ZO-1 is a membrane associated multidomain protein containing three PDZ domains that interact with short peptide sequences at the carboxy terminal of the transmembrane proteins [3]. It has been showed that ZO-1 is not directly attached to nephrin [30]. However, ZO-1 was shown to interact with

Neph1[31], by which ZO-1 couples the nephrin–Neph1 complex to the actin cytoskeleton.

1.4. Structure of Nephrin

Nephrin is a transmembrane protein of the immunoglobin superfamily [7]. Nephrin molecule consists of a short intercellular domain, and an extracellular domain with eight distal IgG-like motifs and one proximal fibronectin type III-like motif (Figure 2) [32]. The length of the entire extracellular domain of nephrin is ~35nm. Each IgG-like motif contains two cysteine residues forming a disulfide bridge within the repeat structure [32]. In addition to the two cysteine residues in each IgG-like motif, nephrin has three 'free' cysteines in the extracellular domain: one in IgG motif 1, one in the spacer region between IgG motifs 6 and 7, and one in the fibronectin domain [33]. The three free cysteines are likely to play a role in forming intermolecular disulfide bridges that provide strength to the slit diaphragm. These cysteines are important because their absence results in proteinuria and congenital nephrotic syndrome [34].

Based on the structure and localization to the slit diaphragm, nephrin molecules were proposed to interact in the slit through homophilic interactions forming a porous substructure in the slit diaphragm [9, 32] (Figure 3). The model was supported by the data from immunoelectron microscopy and electron tomography, the distal IgG1 and IgG2 motifs of nephrin were localized to the central region of the slit, and nephrin molecules in the solution were shown to resemble the strands observed in the native slit diaphragm [33].

The extracellular domain of human nephrin has ten potential N-glycosylation sites [33]. It has been shown that nine of them are indeed modified by N-linked glycosylation [35], which might be important to proper folding and localization of nephrin in the plasma membrane [36].

The intracellular domain of nephrin has no significant homology with other proteins. The intracellular domain of human nephrin consists of 154 amino acids, and contains six tyrosine residues conserved in human, mouse, and rat sequences [37](Figure 4). Some of them might become phosphorylated during ligand binding of nephrin [38].

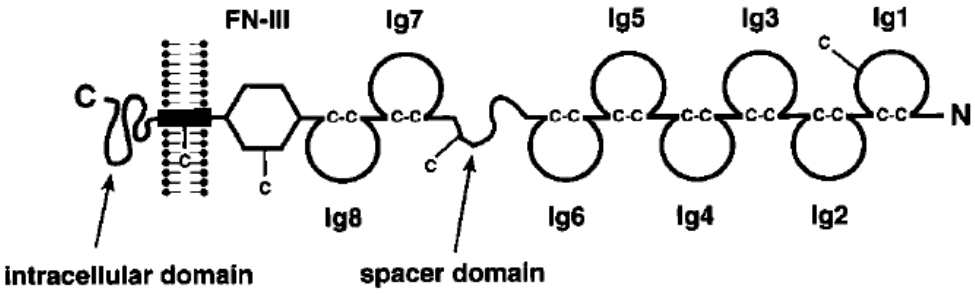

Figure 2. Schematic domain structure of human nephrin. The amino terminus (N) is located in the extracellular space, and the carboxy terminus (C) is intracellular. The Ig repeats are depicted as incomplete circles connected by disulfide bridges (C-C), and numbered from the N terminus. The locations of free cysteine residues are indicated by a -C. (Tryggvason K. J. Am. Soc. Nephrol.1999;10:2440-2445)

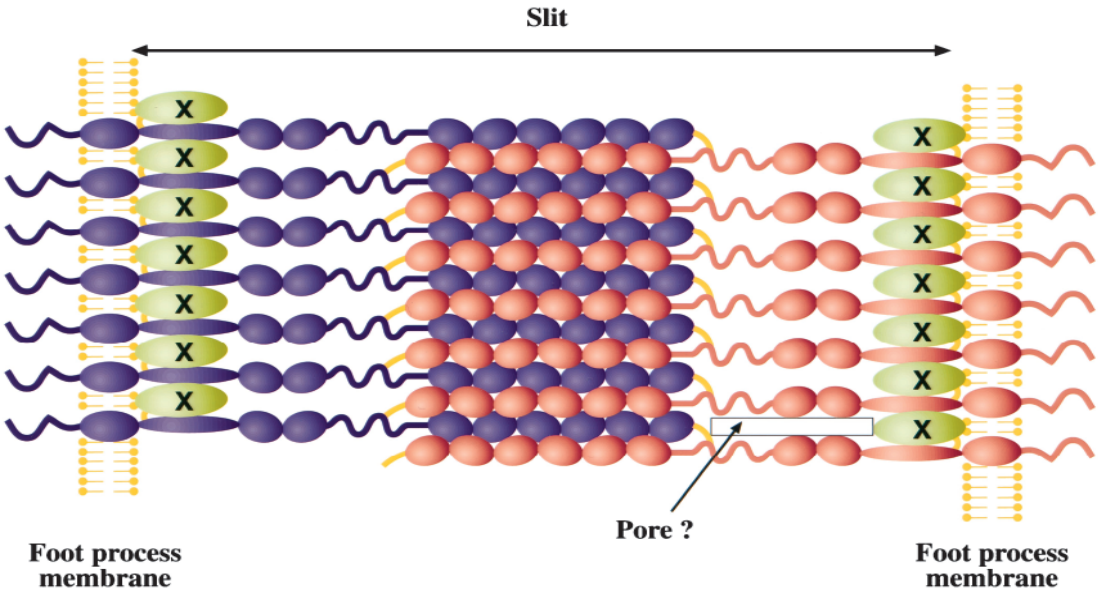

Figure 3. Hypothetical assembly of nephrin assembly into an isoporous filter of the podocyte slit diaphragm where molecules from opposite foot processes are predicted to interact with each other in the center of the slit. For clarity, nephrin molecules from opposite foot processes are shown in different colors. In this model, it is assumed that Ig repeats 1 through 6 of a nephrin molecule from one foot process associate in an interdigitating manner with Ig repeats 1 through 6 in neighboring molecules reaching out from the opposite foot process. Disulfide bonds predicted to be formed between cysteine residues in Ig repeat 1 and in the region between Ig repeats 6 and 7 are depicted by yellow lines. The free cysteine present in the fibronectin domain may interact with a neighboring nephrin molecule, or as depicted with another, as yet unknown protein (X), that may connect with the plasma membrane or cytoskeleton. (Tryggvason K. J. Am. Soc. Nephrol.1999;10:2440-2445)

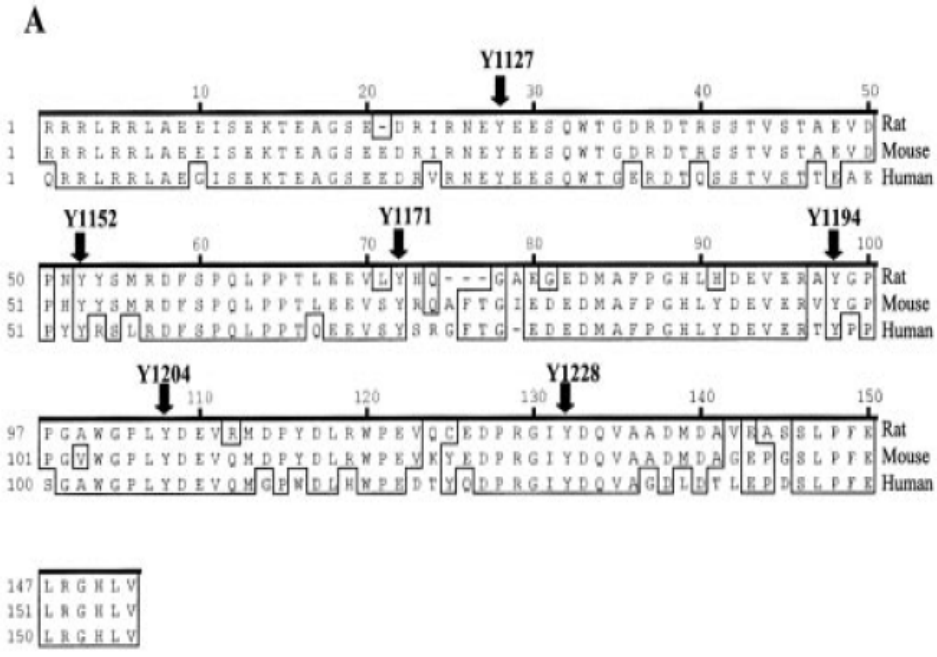

Figure 4. Alignment of the cytoplasmic domains of human, rat, and mouse nephrin. Six conserved tyrosine residues are indicated by arrows. Numbers are according to the rat sequence (accession no. NM022628). (Li H. et al. J. Am. Soc. Nephrol. 2004;15:3006-3015)

1.5. Nephrin phosphorylation and downstream of Nephrin signaling

Nephrin was believed to serve structural function and participate in common signaling pathways for establishing and maintaining an intact glomerular filter at the filtration slit [39]. Activation of nephrin is probably triggered either by homophilic binding of the two nephrin molecules and/or by heterophilic interactions of nephrin and Neph proteins [3]. Clustering of the ectodomain of nephrin with a nephrin antibody can induce tyrosine phosphorylation of nephrin in the cytoplasmic domain [38], which is dependent of the activities of Src-family kinases, Fyn and Yes [40].

Fyn is localized in the podocyte lipid raft, and interacts with nephrin through its SH3 domains [41]. Several tyrosine residues of nephrin (Y1153, Y1154, Y1198, Y1208, and Y1225 in mice) have been shown to be phosphorylated by Fyn [40]. Phosphorylation of nephrin by Fyn might lead to the conformational changes that modulate the interaction of nephrin with podocin [42]. Fyn null mice exhibit mild proteinuria accompanied by subtle foot process effacement and attenuated nephrin phosphorylation [41, 43] . The phenotype of Fyn and Yes double knockout mice (fyn-/-, yes-/-) is further enhanced and presents decreased nephrin phosphorylation [41], which indicates that Fyn and Yes are responsible for the phosphorylation of nephrin.

The tyrosine phosphorylation of nephrin could provide a docking site for SH2 domain containing kinases and adaptor proteins [10]. PI3-kinase and Nck have been identified as associating with nephrin via its tyrosine phosphorylation [27, 40, 44]. Other proteins regulating the actin cytoskeleton, including IQ motif-containing GTPase-activating protein 1(IQGAP1) and membrane-associated guanylate cyclase inverted 2 (MAGI2) have also been identified as associating with nephrin [45]. In the current study, we focus on the PI3-kinase and Nck pathways.

1.6. Introduction to PI3-kinase pathway

PI3-kinase family is classified into three distinct classes based on their substrate specificity and sequence homology. In mammals, the class I PI3-kinase is divided into two subclasses, class 1A and class 1B, based on structural and

functional differences [46]. Both the current review and our studies focus on the class 1A PI3-kinase.

Class 1A PI3-kinase is an enzyme that specifically phosphorylates the D-3 position of the inositol ring of the phosphatidylinositol [PI], phosphatidylinositol 4-phosphate [PI(4)] and phosphatidylinositol 4,5-biphosphate [PI(4,5)P2] to produce phosphatidylinositol 3-monophosphate [PI(3)P]), phosphatidylinositol 3,4-biphosphate [PI(3,4)P2] and phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P3] [46]. Several reports have shown that PI(4,5)P2 is the preferred substrate in vivo for this class 1 PI3-kinase [46-48]. PI3-kinase is a heterodimer, consisting of an 110kD (p110) catalytic subunit, and an 85kD (p85) regulatory subunit [49-51]. The p85 subunit contains an N-terminal Src homology 3 (SH3) domain that binds proline-rich sequences, and two Src homology 2 (SH2) domains that bind to tyrosine phosphorylated sites on receptor or docking proteins [52, 53]. The binding of the SH2 domains of p85 with tyrosine phosphorylated receptors or docking proteins is required to activate the catalytic subunit and to allow the catalytic subunit accessibility to lipid substrates [54, 55]. It has been identified that p*YXXM* (using the one-letter amino-acid code, where X represents any amino acid) is the consensus sequence for protein tyrosine kinase binding to SH2 domains of the p85 subunit [56].

Activation of PI3-kinase triggers a rapid increase of cellular phospholipids, PI(3,4)P2 and PI(3,4,5)P3. This could recruit target proteins through binding their pleckstrin homology (PH) domain, including serine/threonine kinase PKB/AKT and RacGEF such as Tiam and Vav [46, 57], which in turn regulate diverse cellular programs such as cell survival, proliferation/migration, polarity, phagocytosis and glucose homeostasis [58]. In this thesis, we focus on Akt and Rac1.

1.7. PI3-kinase downstream signaling –Akt

The serine/threonine kinase Akt, also known as protein kinase B (PKB), play a central role in cell signaling downstream of growth factors, cytokines, and other cellular stimuli. The three Akt isoforms (Akt1/PKBα, Akt2/PKBβ, Akt3/PKBγ) have extensive homology to protein kinases A, G, and C within their kinase

domains and are, therefore, members of the AGC kinase family [57]. All Akt isoforms share similar structures, namely an amino-terminal PH domain, a hinge region connecting the PH domain and a kinase domain, and a carboxyl-terminal region containing critical serine and threonine phosphoacceptor sites [59]. The PH domain of Akt promotes translocation to the plasma membrane by binding to the phospholipid, altering its conformation and allowing subsequent phosphorylation at Thr308 and Ser473 [60], which is required for full activation of this kinase. Further studies identified that the phosphoinositide-dependent kinase-1 (PDK-1) and PDK-2 are responsible for phosphorylation of Akt at Thr308 and Ser473 respectively [61-64].

Akt1 is ubiquitously expressed at high levels. In contrast, Akt2 is highly expressed in insulin-sensitive tissues including the liver, skeletal muscle and adipose tissue. The expression of Akt2 is drastically increased during the differentiation of adipose tissue and skeletal muscle. Akt3 is expressed most highly in the brain and testis and exhibits lower levels of expression in intestinal organs and muscle tissue [65]. Selective disruption of *Akt* genes in the mouse germ line results in isoform-specific knockout mice with specific phenotypes: Akt1-deficient mice display retardation of growth and reduction of body weight, Akt2-deficient mice have metabolic defects resembling diabetes mellitus, and Akt3-deficient mice show defects in brain development [66]. Studies on cell migration using mouse embryonic fibroblasts deficient of specific Akt isoforms have suggested that Akt1 promotes migration and Akt2 inhibits it. Akt1 knockout cells are larger and show more stress fiber, corresponding to the activation of p21-activated kinase 1(Pak1) by Akt1. Akt2 knockout cells are small and show more lamellipodia which correspond to the elevated Pak1 and Rac activities, suggesting Akt2 inhibits Rac and Pak1 [67].

Akt regulates a wide range of biological responses that include cell motility, growth, proliferation, and survival, etc [68]. It is obvious that Akt phosphorylates many different targets involved in these diverse cellular processes [68]. More than 100 Akt substrates have been reported [68]. The minimal consensus site for Akt phosphorylation- as defined by comparison of its known substrate

phosphorylation sites—consists of RXRXXS/T-B (B represents bulky hydrophobic residues) [69]. However, some Akt substrates do not contain the minimal requirement. Moreover, isoform specific substrates or isoform differentially regulated substrates of Akt are more common [68]. As mentioned above, mouse knockouts have revealed distinct physiological functions for the three Akt isoforms and Akt1 and Akt2 showed differential regulation of Pak1.

1.8. Introduction to RhoGTPases

Mammalian Rho GTPases consist of a family of 20 intracellular signaling molecules: Rho, Rac and Cdc42 are the three best-characterized members of the family [70]. In this thesis, we focus on RhoA and Rac.

Most Rho GTPases switch between an active GTP-bound form and an inactive GDP-bound form. Their activity is regulated by guanine nucleotide-exchange factors (GEFs), which stimulate the release of GDP, allowing GTP to bind. They are downregulated by GTPase- activating proteins (GAPs), which catalyse GTP hydrolysis, converting the proteins to the GDP-bound inactive conformation. Guanine nucleotide-dissociation inhibitors (GDIs) bind to RhoGTPases and prevent their interaction with membranes by masking the prenyl group, which sequester the RhoGTPases in a GDP-bound state in the cytosol and inhibit them from binding to downstream targets [71].

Rho proteins interact with and activate downstream effector proteins when bound to GTP. GEFs can be subdivided into two main subfamilies: the classical Dbl homology-pleckstrin homology domain (DH-PH)-containing family and Dock180-related proteins. First, the DH-PH-containing family consists of 69 members in mammalian genomes. DH domains are responsible for catalysing the exchange of GDP for GTP within Rho GTPases. DH-associated PH domains that bind phosphoinositides are responsible for localizing the proteins to plasma membranes and regulating their GEF activity through allosteric mechanisms. Second, Dock180-related proteins contain the Dock homology region (DHR)-1 and DHR-2 domains. This subfamily represents by 11 mammalian members. The DHR-1 domain interact with PI(3,4,5)P3. The DHR-2 domains contribute to the

binding of RhoGTPases and catalyzing nucleotide exchange within RhoGTPases [72].

1.9. Rho proteins and their functions

Rho proteins include three isoforms: RhoA, RhoB, and RhoC. They are highly homologous, and all induce stress fiber formation when overexpressed in fibroblast [73]. There are few differences in binding to either effector or upstream regulatory proteins, however, they have clearly distinguishable effects in cells [74]. RhoA and RhoC are growth-promoting, while RhoB is in many cases growth-inhibiting [74]. RhoA–knockout mice have not been reported. RhoB-null mice and RhoC-null mice are viable and have no major developmental defects [75, 76]. Nonetheless, RhoB-null mice have increased tendency to induce skin tumor formation, while RhoC-null mice exhibit a much reduced number and size of metastases.

Stress fibers are formed as a result of actin polymerization that was nucleated in focal adhesions and/or myosin-based contractility [74]. Active Rho proteins could activate Rho-kinase (ROCK, ROK) which in turn elevate myosin light chain (MLC) phosphorylation by phosphorylating and inhibiting the MLC phosphatase and/or direct phosphorylating MLC [74]. MLC phosphorylation increases MLC contractility resulting tension for the formation of stress fibers [74]. Other cytoskeletal targets in downstream of Rho-kinase include LIM kinase [74]. LIM kinase has been shown to phosphorylate the actin depolymerizing protein, cofilin/ADF and inhibit its function , which in turn stabilize the actin filament array such as stress fiber [74].

1.10. Rac proteins and their functions

Rac1, Rac2, Rac3 and RhoG form the Rac subfamily based on sequence similarity. The Rac subfamily all stimulate the formation of lamellipodia and membrane ruffles [74]. Despite their high sequence similarity, the Rac isoforms have different expression patterns and studies from knockout mice indicate that they have non-redundant functions [70]. Rac1 and RhoG are ubiquitously expressed, whereas Rac2 and 3 are mostly restricted to hematopoietic and neural tissues [74]. Rac1-knockout mice are embryonic lethal and exhibit a range of

defects in germ-layer formation [77]. Therefore, tissue-specific knockouts were used to study Rac1 function. Rac2, Rac3 and RhoG-knockout mice have cell-type-specific functional defects [70]. (Table 1).

Dominant-negative Rac1 and knockout of Rac1 have been shown to inhibit lamellipodium extension in multiple cell types, including epithelial cells, endothelial cells, fibroblasts and Schwann cells, etc [78-80]. Lamellipodia are the surface-attached sheet-like membrane protrusions observed in migrating cell. Lamellipodia are composed of dendritically branched actin filaments driving leading-edge protrusion [81]. Rac proteins regulate actin polymerization during lamellipodial extension in several ways. First, they can activate actin-nucleating proteins, including the Arp2/3 complex (through Wiskott-Aldrich syndrome protein (WASP) family verprolin-homologous protein (WAVE) proteins) and mDia formins [71]. Second, Rac proteins can affect the availability of free actin barbed ends through the removal of barbed-end capping proteins or the severing of actin filaments and increase the availability of actin monomers for incorporation into actin filaments through Pak-LIM kinase-cofilin pathway [82]. Pak is a downstream effector of Rac that has been implicated in cytoskeleton rearrangements and membrane ruffling, which will be reviewed below. Several Pak substrates or binding partners have been implicated in cytoskeletal organization, including LIM kinase and the adaptor protein Nck.

1.11. Crosstalk between Rho and Rac proteins

PI3-kinase activity is essential to Rac activation by growth factors, like PDGF, EGF, and insulin [74]. Many Rac GEFs, including Tiam1, Vav, PIX (PAK interacting exchange factor) are activated by the products of PI3-kinase, PIP2 or PIP3 [83]. In addition, Rac can bind to p85 regulatory subunit of class IA PI3-kinases, which might recruit PI 3-kinase to the leading edge to produce PI(3,4,5)P3, thereby making positive feedback loops between Rac activity and PI3-kinase [84]. Conversely, RhoA and its effector kinase ROCK can phosphorylate PTEN and stimulate its activity, leading to decreased PI(3,4,5)P3 in areas of RhoA [85]. Furthermore, one family member of RhoGTPases can depress the activity of another by stimulating a GAP, or elevate activity by stimulating a

Table 1 Rho GTPase knockout mouse models
(Heasman SJ. et al. Nature Reviews 2008; 9: 690-701)

Gene	Knockout strategy	Phenotype	Reference
RAC1	Global knockout	Embryonic lethal before E9.5; germ-layer formation defects	Sugihara, K. et al. 1998.
RAC1	LysM-Cre myeloid cells	Defects in neutrophil recruitment, chemotaxis and actin polymerization.	Glogauer, M. et al. 2003.
RAC1	Mx1-Cre haematopoietic cells	Haematopoietic stem/progenitor cell engraftment defect in the bone marrow.	Gu, Y. et al. 2003.
RAC1	K14-CreER epidermal cells	Loss of epidermal stem cells, defects in hair follicles.	Benitah, S.A. et al. 2005
RAC1	Cell permeable Cre Embryonic fibroblasts	Reduced lamellipodia and decreased proliferation.	Vidali, L. et al, 2006
RAC1	Foxg1-Cre forebrain	Defects in brain development and axon guidance.	Chen, L. et al. 2007
RAC1	Tie2-Cre endothelial cells	Embryonic lethal around E9.5; defects in vascular development.	Tan, W. et al. 2008
RAC2	Global knockout	Defects in neutrophil function; for example, chemotaxis and superoxide production.	Roberts, A.W. et al. 1999
RAC3	Global knockout	Developmentally normal and viable; behavioural differences with improved motor skills.	Corbetta, S. et al. 2005. Cho, Y.J. et al. 2005.
RhoG	Global knockout	Developmentally normal and viable; mild hyper-responsiveness of B and T lymphocytes to antigen	Vincent, S., et al. 1992. Vigorito, E. et al. 2004

GEF [74] . It has been identified that the activation of Rac leads to the inhibition of the low molecular weight protein tyrosine phosphatase (LMW-PTP) through production of reactive oxygen species, in turn increasing activation of p190RhoGAP and downregulating of Rho activity [86].

1.12. Role of RhoGTPase in podocytes

RhoGTPases regulate variety of processes, including morphogenesis, migration, neuronal development, cell division and adhesion, etc [70]. However, the role of RhoGTPases in podocytes has not been clarified. On one hand, RhoA might be necessary for maintaining the structural function of podocytes via actin filament stabilization [87, 88]. On the other hand, inhibition of RhoA and Rho-kinase by a Rho-kinase inhibitor (Y-27632) was shown to promote process elongation via regulating actin filament and microtubules assembly [89]. In addition, Y-27632 has also been shown to attenuate glomerular nephrin loss, effacement of podocyte foot processes and proteinuria in murine pyromycin aminonucleoside nephrosis [90]. Furthermore, RhoGDI (which binds to and inhibits the activity of RhoA) knockout mice exhibit foot process effacement and proteinuria at birth [91, 92]. These data suggest that excess Rho-Rho kinase signaling might disrupt normal podocyte structural function. Rac1 activity was shown to be increased in RhoGDI knockout mice, inhibition of Rac1 activity by NSC23766 (a Rac1 inhibitor) exhibit beneficial effect in preserving kidney function. However, NSC23766 could not completely inhibit Rac1 activity (~50% inhibiton), which could not exclude the role of Rac1 in maintaining normal kidney function [92]. Increased Rac1 activity was also observed in HIV protein Nef-induced and angitensin II stimulated signalings in podocytes, leading to lamellipodia formation, and cell migration [93, 94]. However, whether RhoGTPases are regulated by nephrin in podocytes has not been reported. The precise role and regulation of RhoGTPases in podocytes require further investigation.

1.13. Adaptor protein Nck

Nck belongs to SH2/SH3 domain-containing adaptor proteins. It has two isoforms in a human (Nck-1/Nckα and Nck-2/Nckβ), and two in a mouse (mNckα

and mNckβ/Grb4). Nck-1 shows 68% amino acid identity to Nck-2 [95, 96]. Both human Nck proteins are widely expressed [97].

SH2 and SH3 domains are protein modules of 100 and 50 amino acids, respectively. SH2 domains bind to distinct phosphotyrosine residues on various phosphotyrosine proteins [98]. Based on studies using degenerated phosphopeptide libraries, the SH2 domain of Nck prefers to bind to the **pY**DE(P/D/V) consensus motif in tyrosine-phosphorylated proteins [99]. Interestingly, neither of the known Nck binding sites on the PDGF RTK(SVD**pY**VPMLD) or IRS-1(SpYDTPG) contains this consensus recognition sequence [98], suggesting that some flexibility exists in the interaction between the SH2 domain and the phosphotyrosine motif. SH3 domains bind to proline-rich regions on various proteins with a minimum consensus of P-x-x-P [100] and are believed to regulate signaling pathways involving cytoskeletal change and cell morphology [101]. A number of ligands for the SH3 domain of Nck have been identified, including N-WASP, which stimulates the Arp2/3 complex to initiate actin polymerization, and the protein kinase Pak [97, 102, 103]. PAKs, implicated in the regulation of cytoskeletal organization, were shown to bind to the second SH3 domains of Nck [104]. PAKs could be recruited to the plasma membrane via Nck in response to growth factors, such as EGF and PDGF [104, 105].

Studies in both invertebrates and mammals have indicated that the major cellular function of Nck is to link cell surface receptors to the actin cytoskeleton, which is the prerequisite for various biological responses such as axon pathfinding, migration, chemotaxis, etc [97]. Genetic analyses of dreadlocks (Dock), the *Drosophila melanogaster* homolog of Nck, suggested that *dock* was an essential gene for proper photoreceptor axon guidance and targeting [106]. *Drosophila* Pak and Dock colocalize to axons and growth cones, and physically interact with each other via the middle SH3 domain of Dock and the N-terminal PxxP site of Pak. Furthermore, Pak protein kinase activity is essential to rescue the *dock* phenotype [107].

Mice lacking either Nck1 or Nck2 are viable and show no apparent renal defects, suggesting that Nck1 and Nck2 might have overlapping functions. Mice homozygous for both Nck1- and Nck2-null alleles die at embryonic day 9.5 [108]. Conditional knockout of Nck2 in the podocyte of Nck1 null mice showed nephritic-range proteinuria and foot processes effacement [44]. Further investigation identified that the tyrosine phosphorylation motif for Nck SH2 binding in human nephrin are Y1176, Y1193, Y1217 (mouse nephrin Y1191, 1208, 1232, rat nephrin Y1204, Y1228). Mutations in these sites prevent nephrin from interacting with Nck and, subsequently, disturbing nephrin signaling-mediated podocyte cytoskeleton assembly [44]. These data indicate that the Nck adaptor protein is involved in the nephrin regulated actin-cytoskeletal dynamics in podocytes.

1.14. Introduction to P21-activated kinases (PAKs)

The p21-activated kinases (PAKs) comprise a family of serine/threonine kinases, which were the first Rho family GTPase-regulated kinases to be identified [109]. In humans, six PAK isoforms have been identified and they are classified into two subfamilies based on biochemical and structural features. PAK1, PAK2 and PAK3 belong to to Group I, whereas PAK4, PAK5 and PAK6 belong to Group II [110, 111]. Group I PAKs (PAK1-3) are activated upon binding the Rho GTPases, Cdc42 and Rac1, whereas Group II PAKs (PAK4-6) bind Cdc42 and Rac1, but are not activated through the binding of GTPases [110]. Our current study only focuses on the Group I PAKs.

Many studies have shown Group I PAKs' tissue distribution on RNA and protein levels. PAK1 is highly expressed in brain, muscle, spleen and kidney, PAK2 is ubiquitously expressed and PAK3 is predominantly expressed in the brain [109, 112].

1.15. Structure of PAKs

The Group I PAKs are characterized by an N-terminal region that includes a conserved p21-binding domain (PBD), which overlaps with an autoinhibitory domain (AID) and a C-terminal kinase domain [111] (Figure 5). The N-terminal regions of Group I PAKs include two PXXP Src-homology 3 (SH3)-binding

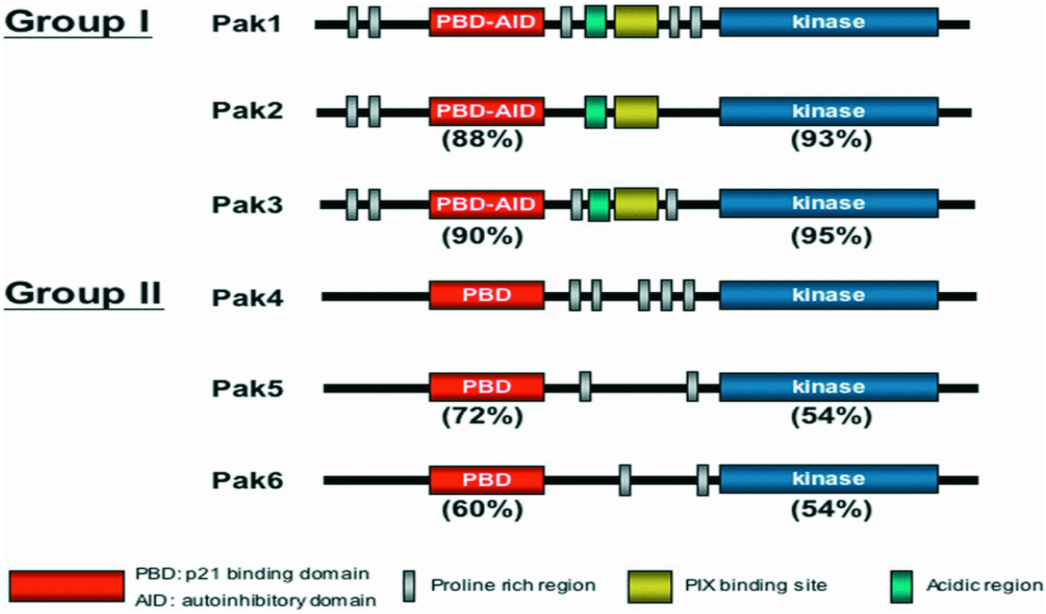

Figure 5: Domain structure of the group I and II Paks
The group I Paks contain a conserved PBD that overlaps with an AID. Cdc42/Rac1 binding to the PBD rearranges the AID and releases it from the catalytic domain. The group II Paks contain a PBD sequence that binds GTPases, but lacks the AID, although unrelated conserved sequences are present. All six Paks have conserved proline-rich motifs. In the case of group I Paks, interactions with the SH3-domain-containing proteins Nck, Grb2 and PIX have been demonstrated. For the group II Paks, no such binding partners have been identified. Percentage identities for PBDs and kinase domains are indicated, relative to Pak1 for group I and relative to Pak4 for group II. (Arias-Romero LE., et al Biol. Cell 2008; 100:97-108)

motifs and a conserved non-classical (PXP) SH3 –binding site for the guanine-nucleotide-exchange factor PIX (Pak-interacting exchange factor) [111, 113]. The first (most N-terminal) conserved SH3-binding site binds the adaptor protein Nck [104], whereas the second site binds to Grb2 (growth-factor-receptor bound protein 2) [114]. The overlap between PBD and AID functions as an inhibitory switch that controls the basal kinase activity of PAKs 1-3 [111]. Due to the similar structure, PAK1 is the prototype and most extensively studied.

1.16. Activation of PAKs

It was demonstrated that PAK1 form a trans-inhibited homodimer under basal condition in cells [115, 116], in which the N-terminal regulatory domain of one PAK1 molecule in the dimer binds and inhibits the C-terminal catalytic domain of the other [111]. The regulation of PAK1 activity is complex and is governed by protein-protein interactions, phosphorylation/dephosphorylation and lipid binding [117, 118]. GTP-bound Rac or Cdc42 GTPases and sphingosine-related lipids bind to the PAK1 amino (N)-terminal regulatory domain. This causes conformational changes that result in a relief of PAK1 autoinhibition and the phosphorylation, both by PAK1 itself and/or by exogenous kinase [111]. The active PAK1 is thought to be phosphorylated at seven residues (mouse amino acids 21, 57, 144, 149, 198, 203 and 422). All group I PAKs contains a threonine residue at the position corresponding to human Threonine 423 (mouse Thr 422) in the activation loop of PAK1 [111]. The phosphorylation at this residue is important for maintaining relief from auto-inhibition and for full catalytic function towards its substrates [119]. The amino acid sequence immediately upstream of Thr 423 was found to form a consensus PAK1 phosphorylation sequence of Lys-Arg-Ser-Thr [120]. This suggests that Thr 423 could be phosphorylated by another PAK1 molecule (transphosphorylation) [111]. PDK1 (phosphoinositide-dependent kinase-1) has also been shown to phosphorylate this site in vivo [121]. Autophosphorylation residues, including Ser 21, Ser144, Ser199 and Ser204 (human), also contribute to kinase activation and/or maintenance of kinase activity [118, 119, 122]. Sphingosine-mediated PAK1 activation is independent of Ser144, suggesting that lipid binding activates PAK1 via a different mechanism

[118] compared to activation by GTPases. Ser 199/204 of Pak1 are located in the inhibitory domain and autophosphorylation of these residues is thought to prevent the kinase from reverting to an inactive conformation [115]. Ser 21, which mediates Nck binding, could be phosphorylated by Akt1 and PKG [122, 123].

As mentioned previously, Group I PAKs can interact with SH3-containing adaptor proteins (Nck and Grb2) via their N-terminal PXXP motifs. These interactions can recruit PAKs to activated tyrosine kinase receptors at the plasma membrane and stimulate PAKs kinase activity [104, 105, 114, 124-126]. The addition of membrane–targeting sequences to PAK1 can also stimulate its kinase activity [103].

In addition to the activation mechanisms mentioned above, PAK2 is also activated through proteolytic cleavage by caspases or caspase-like proteases to release an amino(N)-terminal fragment (Pak2p27) and a pro-apoptotic catalytic fragment (Pak2p34). In response to stress stimulants, such as tumor necrosis factor (TNF) or growth-factor withdrawal, PAK2 is activated as a full-length enzyme and as the Pak2p34 fragment. Activation of full-length PAK2 stimulates cell survival, whereas proteolytic activation of Pak2p34 is involved in programmed cell death [127, 128].

Dephosphorylation of PAKs is important in shutting off PAK activity. Though the interaction of PAK with PP2A (protein-serine/threonine phosphatase 2A) has been reported, its physiological significance is unclear [129]. In brain extracts, PP2Cα has been demonstrated to dephosphorylate PAK1 and inhibit its activity [130]. Other two phosphatase POPX1 and POPX2 (partner of Pix-1 and Pix-2) bind to Pak1 via Pix (PAK- interacting exchangers) and dephosphorylate Thr423 of PAK1 [131] . The physiological role and regulation of the phosphatases in the context of PAK signaling remain undefined.

1.17. Biological function of PAKs

The PAK family of kinases plays an important role in diverse cellular processes, including cell morphology, motility, survival, gene transcription, apoptosis and cell transformation. The most well-characterized function of PAKs is the regulation of cytoskeletal organization, cell morphology and motility [132].

These multiple actions are executed by many interacting proteins and substrates for Paks [132]. The kinase domains of PAK1, PAK2 and PAK3 share 93% identity, suggesting that Group I PAKs may phosphorylate common substrates. More than 30 direct substrates of Group I PAKs have been identified so far, MLCK (myosin light chain kinase), LIMK (LIM domain kinase), and BAD (Bcl-2/Bcl-X_L-antagonist, causing cell death) are most extensively characterized substrates of PAK1 and PAK2 [132] .

PAK1 function has been shown to be particularly important in cytoskeletal regulation, especially in relation to neuronal function, and the development of cancer [111]. Microinjection of activated PAK1 into Swiss 3T3 cells induced the rapid formation of lamellipodia and filopodia [133]. Expression of various constitutively active forms of PAK1 induced disassembly of stress fibers and focal adhesion complexes [133, 134]. The mechanism for these effects is not completely understood, but involves the phosphorylation of multiple substrates, including LIMK, MLCK, stathmin, merlin, and filamin [135]. PAK1 phosphorylates MLCK and inhibit its activity to phosphorylate regulatory sites on the myosin regulatory light chain, thereby inhibiting contractility [136]. LIMK was shown to be activated by PAK1-mediated phosphorylation of Thr508, in turn phosphorylate and inactivate cofilin [137]. PAK1 also can modulate the microtubule cytoskeleton through the phosphorylation of stathmin/Op18 [138, 139], leading to inhibition of the binding of stathmin to microtubules and stabilization of microtubule assembly.

The specific functions of PAK family members have been studied using gene knockout mice and loss of function experiments. *PAK1-/-* mice are viable, but have immune defects; *PAK2-/-* mice are embryonic lethal; *PAK3-/-* mice show mental retardation, which resemble human X-linked, non-syndromic mental retardation (in which *PAK3* is specifically mutated) [132]. Using RNAi strategy in tumor cells, depletion of PAK1 increased cofilin phosphorylation, whereas depletion of PAK2 increased MLC phosphorylation; in addition, the depletion of PAK1 has stronger inhibition of lamellipodia protrusion than does the depletion of

PAK2 [140], which indicate that PAK1 and PAK2 might have differential role on actin organization.

1.18. Cofilin, LIM kinase and slingshot

The actin-depolymerisation factor (ADF)/cofilin family in mammalian systems consists of three highly similar paralogs: cofilin-1 (non-muscle cofilin, n-cofilin), cofilin-2 (muscle cofilin, m-cofilin) and ADF (actin-depolymerization factor or destrin) [141]. Among them, cofilin-1 is the predominant isoform and it is ubiquitously expressed [141]. The cofilin-1 knockout mice are not viable. Brain-specific knockout of cofilin-1 indicated that cofilin-1 is important for the cell migration and cell cycle progression in the cerebral cortex [141].

ADF/cofilins bind actin monomers and filaments. Cofilin has been shown to shift from nucleating new filament to severing actin filament with a gradient of high to low cofilin activity [141]. Nucleating and severing filaments contribute to dynamic turnover of actin filaments, which is the basis of lamellipodia formation in migrating cells [141]. Cofilin is inactivated by phosphorylation on Ser3, leading to inhibition of G-and F-actin binding [142]. Two families of kinases, LIM kinases (LIMK) and testicular kinases (TESK), are responsible for the phosphorylation of cofilin. The phosphatases of the slingshot (SSH) family and the haloacid dehalogenase phosphatase chronophin (CIN) contribute to the reactivation of phosphorylated ADF/cofilin [141]. In addition, cofilin has been shown to interact with phosphoinositides, in particular PI(3,4,5)P3 and PI(4,5)P2 [143, 144]. The Cofilin–PI(4,5)P2 interaction decreases the actin-binding activity by competitive binding, since binding sites of F-actin and PI(4,5)P2 on cofilin are overlapping [145]. Consistently, cofilin, but not p-cofilin, has been observed at the membrane in many type of cells, strengthening the idea about the role of PI(4,5)P2 in sequestering unphosphorylated cofilin [146-148].

LIM kinases-1 and -2 belong to a family of serine/threonine kinases [149]. LIM kinases are characterized by the presence of two-N-terminal LIM domains followed by a PDZ domain and a C-terminal kinase domain. LIM kinase-1 and -2 share 50% identity overall, with 70% identity in the kinase domain [150-153]. The two LIMK proteins have an identical overall structure and a similar effect on actin

polymerization. However, LIMK1 is the only isoform that is expressed in kidney glomeruli [141].

LIMK1 is ubiquitously expressed with the highest levels in brain, kidney, lung, stomach and testis in mouse tissues [154]. LIMK1 mainly remains in the cytoplasm, but it is also found in the nucleus [154]. In the cytoplasm, it was found to colocalize with actin stress fiber and focal adhesion [154].

The major function of LIMK1 is to regulate the organization of the actin cytoskeleton through the phosphorylation and inactivation of ADF/cofilins. Phosphorylation of cofilin on Ser3 blocks its ability to bind to actin, therefore altering the rate of actin depolymerization [155, 156]. *LIMK1*-null mice are normal and show mild defects in central nervous system, including abnormal spine morphology, reduced dendritic branch size, and absence of growth cone [157]. These phenotypes are further enhanced in the double *LIMK1/2*-knockout mice [158].

Similar to PAK, Rho effector kinases, Rho-associated kinase (ROCK) can also phosphorylate LIMK1 on Thr508 in the activation loop of LIMK1 kinase domain [137, 155, 159, 160]. In addition, the binding of heat shock protein 90 (Hsp90) to the kinase domain of LIMK1 promotes its homodimerization and transphosphorylation, leading to increased stability and activity [161]. There are several proteins that downregulate the activity of LIMK1. The only phosphatase known to dephosporylate and inactivate LIMK1 is slingshot phosphatase (SSH), which also dephosphorylate cofilin [162]. Moreover, nischarin and the large tumor suppressor 1(Lats1) have been shown to interact with LIMK1 and inhibit its activity [163, 164].

Slingshot phosphatases are represented by three genes (SSH-1,-2 and -3). In kidneys, SSH1 is dominantly expressed [165]. SSH phosphatases contain a protein phosphatase domain and a C-terminal F-actin binding region. In mammalian cells, SSH dephosphorylate cofilin at Ser3 residue [165].

The regulation of SSH activity is not very clear. SSH has F-actin binding region, 14-3-3 binding motifs and Akt consensus phosphorylation motif [165]. F-actin binding appears to stimulate SSH phosphatase activity [162]. Binding of 14-

3-3 has been shown to sequester SSH away from actin filament [166]. It has been reported that PI3-kinase activated SSH, probably through its release from 14-3-3 in the cytoplasm, consequently translocating to the F-actin-rich lamellipodia [167]. However, the mechanism of activation of SSH by PI3K has not been clarified.

Chronophin has been identified as a second cofilin phosphatase, which belongs to the haloacid dehalogenase (HAD) family of phosphatases. It contains a highly conserved catalytic domain and three conserved sequence motifs characteristic of the HAD hydrolases. The regulation of its activity is poorly characterized [165]. There is no sequence similarity between SSH and chronophin, which suggests that the two proteins might be regulated differently. The data from siRNA-mediated depletion of chronophin or SSH in cells also suggest that the two phosphatases are not functionally redundant [165].

The spatial and temporal segregation of the activation of cofilin is important in the context of a dynamic cellular environment. The coordinated function of both actin-polymerizing and actin-depolymerizing/severing factors is fundamental to the actin organization at the leading edge of eukaryotic cells. It has been proposed that branched actin filaments are generated at the leading edge through the action of the Arp2/3 complex and /or filamin A, and active cofilins depolymerize/sever F-actin from the rear of the actin network to recycle actin monomers to the leading edge for further rounds of polymerization [141]. Therefore, future research requires a comprehensive approach in which cofilin activity is spatio-temporally studied.

Objective of the thesis:

The actin-based podocyte foot processes contribute to the selective filtration barrier of the renal glomerulus. The dynamic regulation of the podocyte actin cytoskeleton is vital to kidney function. Nephrin has been identified as a signaling protein that is important to maintain integrity of the podocyte foot process. It has been reported that tyrosine phosphorylation of nephrin was responsible for its interaction with PI3-kinase and Nck. PI3-kinase and Nck-Pak signalings are well known regulators of actin dynamics. The role of PI3K-Akt and Nck-PAK in nephrin signaling in podocytes has not been characterized. The aim of the project was to study the role of PI3K and Nck-Pak signalings activated via the tyrosine phosphorylation of nephrin in actin cytoskeleton organization of podocytes.

Chapter 2

Nephrin mediates actin reorganization via phosphoinositide 3-kinase in podocytes

Running title: Actin reorganization by nephrin in podocytes.

2.1. Abstract

Nephrin is a slit diaphragm protein critical for structural and functional integrity of visceral glomerular epithelial cells (podocytes) and is known to be tyrosine phosphorylated by Src family kinases. We studied the role of phosphoinositide 3-kinase (PI3K), activated via the phosphorylation of nephrin, in actin cytoskeletal reorganization of cultured rat podocytes. Phosphorylation of rat nephrin by the Fyn kinase markedly increased its interaction with a regulatory subunit of PI3K. Stable transfection of rat nephrin in the podocytes with podocin led to nephrin tyrosine phosphorylation, PI3K-dependent phosphorylation of Akt, increased Rac1 activity, and an altered actin cytoskeleton with decreased stress fibers and increased lamellipodia. These changes were reversed with an inhibitor of PI3K and not seen when the nephrin-mutant Y1152F replaced wild-type nephrin. Rac1 and Akt1 contributed to lamellipodia formation and decreased stress fibers, respectively. Finally, in the rat model of puromycin aminonucleoside nephrosis, nephrin tyrosine phosphorylation, nephrin-PI3K association, and glomerular Akt phosphorylation were all decreased. Our results suggest that PI3K is involved in nephrin-mediated actin reorganization in podocytes. Disturbed nephrin–PI3K interactions may contribute to abnormal podocyte morphology and proteinuria.

2.2. Introduction

Visceral glomerular epithelial cells (GEC, also known as podocytes) play a central role in maintaining the selective filtration barrier of the renal glomerulus. Podocytes project numerous actin-rich processes called 'foot process.' Foot processes from adjacent podocytes form tight interdigitation and surround and support glomerular capillaries. Nephrin is a transmembrane protein, which belongs to the Ig superfamily and is localized at the slit diaphragm, which connects foot processes from adjacent podocytes [32]. Mutations of nephrin cause congenital nephrotic syndrome of the Finnish type [7]; thus nephrin has a pivotal role in glomerular permselectivity. Nephrin molecules from adjacent foot processes interact with each other in an antiparallel, homophilic manner, serving as a structural backbone of the slit diaphragm [168]. In addition to its structural role, research efforts in the recent years unraveled the importance of nephrin as a component of the slit diaphragm protein complex, which transmits signals into the cells [39]. The cytoplasmic domain of nephrin consists of approximately 150 amino acids and contains several tyrosine residues, six of which are conserved among human, mouse, and rat [37]. We and others have reported that the cytoplasmic domain of nephrin is tyrosine phosphorylated by the Src family kinase Fyn [37, 38, 41]. Tyrosine phosphorylation modulates the interaction of nephrin with other proteins such as another slit diaphragm protein, podocin [37], adaptor protein, Nck [40, 44, 169], and phosphoinositide 3-kinase (PI3K) [27]. It is noteworthy that many of the nephrin-interacting proteins are known for their roles in actin regulation, suggesting the important role of nephrin in regulating the actin cytoskeleton and podocyte morphology [1].

PI3K phosphorylates phosphatidylinositol lipids at the D-3 position of the inositol ring and converts the plasma membrane lipid phosphatidylinositol-4,5-bisphosphate PI(4,5)P_2 into phosphatidylinositol-3,4,5-trisphosphate PI(3,4,5)P_3 [170]. PI3K consists of the two subunits, that is the regulatory p85 subunit (PI3K-p85) and the catalytic p110 subunit (PI3K-p110). The p85 interacts with phosphotyrosine containing motifs of activated growth factor receptors or adaptor proteins, bringing the p110 to the plasma membrane, the site of its enzymatic

action [170]. PI(3,4,5)P_3 generated by PI3K recruits pleckstrin homology containing proteins such as Akt and Rac guanine nucleotide exchange factors (GEF) to the plasma membrane, initiating downstream signaling cascades [170]. Akt, generally considered as one of the main effectors of PI3K, is best known for its antiapoptotic/prosurvival actions [171]. In addition, a growing body of evidence indicates that Akt regulates the actin cytoskeleton and cell motility; Akt promotes the formation of lamellipodia and cell migration via its binding partner, Girdin, in Vero fibroblasts [172]. Similarly, activation of PI3K leads to decreased stress fibers and increased lamellipodia/filopodia via Akt and p70S6K in chicken embryo fibroblasts [173]. Akt also stimulates cell migration via modulating the interaction between Pak1 and Nck in HeLa cells [122]. A recent proteomics approach confirmed the direct interaction of Akt and actin in MCF-7 breast cancer cells [174]. In this study, the authors also demonstrated that the cortical remodeling of actin associated with cell migration was reversed by small interfering RNA directed against Akt. Furthermore, recent studies suggest the isoform-specific actions of Akt; Akt1-deficient cells showed increased stress fibers and decreased cell migration, whereas Akt2-deficient cells showed increased membrane ruffling and migration. Akt2 appeared to contribute to the inhibition of Pak1 and Rac1 as well [67]. Rac1 belongs to the Rho family of small GTPases. As many Rac GEFs (for example, Tiam1) are activated by PI(4,5)P_2 or PI(3,4,5)P3 [74], Rac1 can also be activated by PI3K. Rac1 is one of the key regulators of the actin cytoskeleton in mammalian cells. In particular, Rac1 has been shown to mediate lamellipodia formation and membrane ruffling in response to growth factor stimulation [74]. In addition, Rac1 activity appears to be essential in regulating cell–extracellular matrix interaction and cell migration [74].

A previous study by Huber *et al.* showed that nephrin interacts with PI3K in a tyrosine phosphorylation manner, leading to the activation of Akt and increased cell survival [27]. However, in this study, the precise mapping of phosphotyrosine-containing motifs in nephrin responsible for its interaction with PI3K was not reported. Also, potential consequences of PI3K activation other than cell survival were not addressed. In this study, we characterized a tyrosine

phosphorylation-dependent interaction between rat nephrin and PI3K-p85 and focused on the impact of PI3K activation on the actin cytoskeleton. We demonstrated that nephrin–PI3K interaction leads to the activation of the Akt and Rac1 pathways, resulting in the remodeling of the actin cytoskeleton in cultured rat GEC.

2.3. Materials and methods

Materials

Tissue culture media and Lipofectamine 2000 were from Invitrogen Life Technologies (Burlington, ON, Canada). Electrophoresis reagents were from Bio-Rad Laboratories (Mississauga, ON, Canada). Anti-nephrin antibody was described previously [37]. Anti-p85, anti-RhoA, anti-Rac1 antibodies, and rhotekin Rho-binding domain fused to GST (GST-RBD) were from Upstate Biotechnology (Lake Placid, NY, USA). Antibodies for phospho-Akt (Ser473), Akt, Akt1, Akt2, phospho-cofilin (Ser3), and cofilin were from Cell Signaling (Beverly, MA, USA). Anti-HA antibody was from Zymed Laboratories (South San Francisco, CA, USA). Anti-Nck antibody and anti-phosphotyrosine antibody (PY69) were from BD Biosciences. Enhanced chemiluminescence (ECL) detection reagents and glutathione–Sepharose beads were from Amersham Bioscience (Baie d'Urfé, QC, Canada). TRITC-phalloidin was from Molecular Probes (Eugene, OR, USA). Male Sprague–Dawley rats were from Charles River Canada (St Constant, QC, Canada). Hydrogen peroxide was from Fisher Scientific (Lawn, NJ, USA). PP2, LY294002, puromycin aminonucleoside, and other chemicals were from Sigma-Aldrich (Mississauga, ON, Canada).

Plasmids

Plasmids encoding wild-type, full-length rat nephrin, rat nephrin tyrosine mutants, and Fyn were described previously [37]. pcAGGS-p85α encoding HA-tagged mouse p85 subunit was a gift from Dr Asano (Tokyo University, Tokyo, Japan). Mouse podocin cDNA was from Dr Benzing (University Hospital Freiburg, Freiburg, Germany). The plasmids pRK5-Rac1(L61)-Myc (constitutively active, CA), pRK5-Rac1(N17)-Myc (dominant negative, DN), and Cdc42/Rac interactive binding domain fused to GST (GST-CRIB) were from Dr Lamarche-Vane (McGill University, Montreal, QC, Canada) [175, 176]. Plasmid encoding CA-Akt1 (human) was from Dr Gotoh (University of Tokyo, Tokyo, Japan).

Cell culture

Rat GEC culture and characterization were described previously [177, 178]. Briefly, GEC were cultured in K1 medium (50% DMEM (Dulbecco's modified Eagle's medium), 50% Ham F-12, 10% NuSerum, hormone mix) and studies were carried out between passages 10 and 60. GEC were stably transfected with pcDNA3.1 (GEC-neo), mouse podocin (GEC-P), mouse podocin, rat nephrin (GEC-P/N), mouse podocin and rat nephrin-mutant Y1152F (GEC-Y1152F). Nck-null (Nck1-/-, Nck2-/-) and Nck1+/-, Nck2+/+ MEFs were obtained from Dr Pawson (Samuel Lunenfeld Research Institute, Toronto, Ontario, Canada) [108]. Transient transfection of Cos-1 cells and GEC was performed using Lipofectamine 2000 (Invitrogen Life Technologies).

Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting were performed as reported previously [37]. Protein content was quantified using scanning densitometry (ImageJ software).

Induction of PAN and isolation of rat glomeruli

PAN was induced with a single intravenous injection of puromycin aminonucleoside (50 mg kg^{-1} body weight) in male Sprague–Dawley rats (150–175 g body weight) as described previously [179]. Rats were killed on day 7, when significant proteinuria was observed. Isolation of rat glomeruli was performed as described previously [179]. Studies were approved by the Animal Care Committee at McGill University.

Pull-down assays for active RhoA and Rac1

Preparation of GST-CRIB was described previously [120]. Cells or glomeruli were lysed in lysis buffer (25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (pH 7.5), 1% NP-40, 10 mM $MgCl_2$, 100 mM NaCl, 5% glycerol, 5 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin). Equal amounts of protein (250–1000 μg) were incubated for 1 h at 4 °C with purified GST-CRIB or GST-RBD (10–15 μg) bound to glutathione–Sepharose beads. The beads and proteins bound to the fusion protein were washed twice and were subjected to sodium

dodecyl sulfate-polyacrylamide gel electrophoresis (12%) and immunoblotting with anti-RhoA or anti-Rac1 antibody.

Immunofluorescence staining

Cells were plated on glass coverslips, fixed in paraformaldehyde (4%, 10 min, room temperature), permeabilized with 0.1%. Triton X-100, and blocked with 3% bovine serum albumin. F-actin was visualized by TRITC-phalloidin. Cells were examined by a confocal laser scanning microscope (Fluoview FV1000, OLYMPUS).

Quantification of lamellipodia and stress fibers

For the quantification of lamellipodia (membrane ruffles) in un-transfected GEC (Figure 3), at least 100 cells located at the periphery of the colony were counted per sample from randomly chosen fields. When >50% of the free margin of the cell was covered with lamellipodia, cells were considered positive for lamellipodium. For the quantification of stress fibers, at least 100 cells were studied per sample from randomly chosen fields, regardless of their location within the colonies, for the presence of clearly defined stress fibers. The percentages of lamellipodium- or stress fiber-positive cells were calculated for 5–7 samples and were averaged. For quantification of lamellipodia in GEC transfected with GFP-CA-Rac1, GFP-DN-Rac1, or GFP (Figure 4), only the successfully transfected cells, identified by green fluorescence, were counted. The other criteria were the same as in un-transfected GEC. For this series of experiments, at least 50 cells were counted per sample.

Data analysis

Data are presented as mean±s.d. The *t*-statistic was used to determine significant differences between two groups. One-way analysis of variance was used to determine significant differences among groups. Where significant differences were found, individual comparisons were made between groups using the *t*-statistic and adjusting the critical value according to the Bonferroni method.

2.4. Results

Tyrosine 1152 is responsible for interaction of rat nephrin and PI3K-p85

We and others have reported that the cytoplasmic domain of nephrin is tyrosine phosphorylated by Src family kinases [37, 38, 41]. It was also reported that human nephrin interacts with PI3K in a tyrosine phosphorylation-dependent manner [27]. We first confirmed these results with rat nephrin. Wild-type, full-length rat nephrin and PI3K-p85 were transiently expressed in Cos-1 cells with or without the Src family kinase Fyn. Nephrin was strongly tyrosine phosphorylated only in the presence of Fyn (Figure 1a), consistent with our previous results [37]. Nephrin co-immunoprecipitated with PI3K-p85 in the presence but not in the absence of Fyn (Figure 1a). We also obtained similar results using the chimeric construct, in which the extracellular domain of the human interleukin-2 receptor is connected to the transmembrane/cytoplamic domain of rat nephrin (Tac/nephrin) [37], in the place of wild-type nephrin (data not shown). These results indicate that the cytoplasmic domain, but not the extracellular domain of nephrin, interacts with PI3K-p85 in a tyrosine phosphorylation-dependent manner.

To identify the binding site(s) for PI3K-p85, we analyzed the protein sequence of the cytoplasmic domain of nephrin by Motif scan analysis (http://scansite.mit.edu). This program identifies potential interacting proteins for various protein motifs in a given molecule. The results suggested that a motif containing Y1152 of rat nephrin (corresponding to Y1153 in mouse and Y1139 in human) is a likely binding site for PI3K-p85. Thus, we generated a nephrin Y1152F mutant and expressed it in Cos-1 cells with p85 and Fyn. In contrast to wild-type nephrin, the Y1152F mutant failed to co-immunoprecipitate with PI3K-p85 even in the presence of Fyn (Figure 1b). We showed previously that the Y1204F mutant of nephrin is tyrosine phosphorylated by Fyn significantly less than wild type and that its ability to interact with podocin is also significantly reduced [37]. Thus, for comparison, we studied the Y1204F mutant for its ability to interact with PI3K-p85. The Y1204F mutant co-immunoprecipitated with p85 in a Fyn-dependent manner similar to wild-type nephrin (Figure 1b). We have also tested other nephrin mutants including Y1127F (Figure 1b), Y1171F, and

Y1194F (data not shown). All the mutants tested other than Y1152F co-immunoprecipitated with p85 in a Fyn-dependent manner. These results indicate that tyrosine phosphorylation of Y1152, but not the other tyrosine residues, plays a critical role in the nephrin (rat)–p85 interaction.

Nephrin activates Akt via PI3K

Nephrin is a transmembrane protein. Thus, by analogy to growth factor receptors, its interaction with PI3K-p85 is likely to activate the catalytic activity of p110, leading to the increased local concentration of $PI(3,4,5)P_3$ and the activation of downstream signaling cascades such as the Akt pathway. To study whether nephrin activates Akt, we used cultured rat GEC and studied the activity of Akt using the antibody specific to the active form of Akt, which is phosphorylated at Ser473. In a subclone of GEC, which stably overexpress podocin and nephrin (GEC-P/N), nephrin was clearly tyrosine phosphorylated without any stimulation (Figure 2a). In these cells, phosphorylation of Akt was increased, as compared with GEC-neo (vector-transfected control; Figure 2a) or with GEC-P (overexpressing podocin alone; Figure 2b). Akt phosphorylation was not different between GEC-neo and GEC-P, suggesting that overexpression of podocin alone does not have an impact on Akt phosphorylation/activation. Akt phosphorylation was completely inhibited in GEC-P/N by the PI3K inhibitor LY294002 and the Src kinase inhibitor PP2 (Figure 2a), suggesting that nephrin phosphorylates/activates Akt in a manner dependent on PI3K and Src family kinase(s). Stable expression of nephrin-mutant Y1152F, which lacks the ability to interact with PI3K-p85 in a tyrosine phosphorylation-dependent manner (Figure 1b), did not increase the phosphorylation of Akt (Figure 2a), supporting the role of PI3K in nephrin-mediated Akt activation. Additional clones of GEC-P/N and GEC-Y1152F were studied for Akt phosphorylation with similar results (Figure S1A). In addition, antibody-mediated crosslinking of the extracellular domain of nephrin, a technique known to induce tyrosine phosphorylation of nephrin [180], induced Akt phosphorylation (Figure S1B). These results indicate that tyrosine phosphorylation of nephrin, most likely by Src family kinases such as Fyn [37, 41], activates the Akt pathway via PI3K.

Nephrin activates Rac1 in GEC

In addition to the Akt pathway, PI3K is known to activate the Rho-GTPase, Rac1 [170]. Thus, we next studied Rac1 activity in various lines of GEC using a pull-down assay. The amount of active Rac1 was significantly increased in GEC-P/N, as compared with GEC-neo (Figure 2d). Rac1 activity was not different between GEC-neo and GEC-P, indicating that overexpression of podocin alone does not contribute to Rac1 activation (Figure 2d).

Similar to Akt phosphorylation, the increased Rac1 activity in GEC-P/N was inhibited by LY294002 and PP2 (Figure 2d). Furthermore, nephrin-mutant Y1152F failed to activate Rac1 (Figure 2d). Additional clones of GEC-P/N and GEC-Y1152F were studied with similar results (Figure S1C). These data suggest that, in addition to the Akt pathway, nephrin activates Rac1 in a manner dependent on PI3K and Src family kinase(s).

Nephrin expression modulates the actin organization in GEC

The above results indicate that nephrin activates both Akt and Rac1 via PI3K. It was reported previously that nephrin-mediated PI3K/Akt activation leads to increased cell survival in GEC [27]. However, Akt is also known to regulate the actin cytoskeleton, which plays a central role in the morphology and function of GEC. In addition, Rac1 is a well-known regulator of the actin cytoskeleton. Therefore, we hypothesized that nephrin may modulate the actin cytoskeleton in GEC via PI3K-mediated Akt/Rac1 activation. First, we examined the pattern of F-actin in the cells by phalloidin staining (Figure 3). Control GEC (GEC-neo) showed well-defined cortical F-actin. Twenty-one percent of the cells demonstrated stress fibers, whereas 45% of the cells with free cell margin demonstrated membrane ruffles (or lamellipodia. These two terms are usually used interchangeably [74]). In contrast, GEC-P/N showed cytosolic F-actin aggregates, whereas the cortical F-actin was less prominent. Stress fibers were observed only in ~4% of the cells, whereas a higher percentage of cells with free margin (~90%) demonstrated membrane ruffles. Decreased stress fibers and increased membrane ruffles observed in GEC-P/N were abolished by incubating the cells with LY294002 overnight. In striking contrast to wild-type nephrin, the

Y1152F mutant of nephrin did not increase membrane ruffles nor did it decrease stress fibers. Rather, the number of stress fiber-positive cells increased significantly to 70% in GEC-Y1152F. The pattern of F-actin in GEC-P was not different from GEC-neo (data not shown). Additional clones of GEC-P/N and GEC-Y1152F were studied with similar results (Figure S2). Taken together, these results suggest that nephrin increases membrane ruffles/lamellipodia and decreases stress fibers in cultured rat GEC, and that these changes depend on PI3K.

Rac1 contributes to membrane ruffles in GEC

We next studied the role of Rac1 in the cytoskeletal changes observed in GEC-P/N. It is well known that Rac1 induces lamellipodia/membrane ruffles in fibroblasts [74]. Therefore, we hypothesized that Rac1 activated by PI3K contributes to increased membrane ruffles in GEC-P/N. First, we transfected CA-Rac1 in GEC-Y1152F, which had the lowest level of Rac1 activity (Figure 2d) and membrane ruffles (Figure 3) among various cells we established. Only 19% of the cells transfected with GFP alone showed membrane ruffles (Figure 4a), similar to untransfected GEC-Y1152F (23%, Figure 3). In contrast, most of the cells transfected with CA-Rac1 (82%) showed membrane ruffles. We next transfected GEC-P/N, which has the highest level of Rac1 activity (Figure 2d) and membrane ruffles (Figure 3), with DN-Rac1. Eighty-five percent of GEC-P/N transfected with GFP alone showed membrane ruffles (Figure 4b), similar to untransfected GEC-P/N (90%, Figure 3), whereas significantly less cells transfected with DN-Rac1 (61%) showed membrane ruffles (Figure 4b). Taken together, we concluded that Rac1 contributes, at least in part, to nephrin-mediated increase in membrane ruffles in GEC-P/N.

Akt1 decreases stress fibers in GEC

We next addressed the role of Akt activation in the cytoskeletal organization of GEC. It was reported previously that Akt1 decreases stress fibers in breast cancer cells [181], whereas Akt1-deficient mouse embryonic fibroblast (MEF) shows increased stress fibers. To study if Akt1 also decreases stress fibers in GEC, we studied the impact of CA-Akt1 in cultured rat GEC. We chose to use GEC-

Y1152F because these cells had low basal level of Akt activity (Figure 2a) and most cells had prominent stress fibers (Figure 3). Most GEC-Y1152F transfected with CA-Akt1 lost stress fibers, although such effects were not observed with GFP alone (Figure 5a). Similar results were obtained with GEC-neo, although the loss of stress fibers was less striking owing to the limited amounts of stress fibers in GEC-neo (Figure S3). We also confirmed these results using MEF deficient in Nck [108], which have very low levels of Akt activity (Figure 2c) and prominent stress fibers [44]. Transient transfection of CA-Akt1 in Nck-null MEF decreased stress fibers significantly, as compared with cells transfected with GFP alone (Figure 5b). Taken together, these results support that Akt1 activation leads to decreased stress fibers both in GEC and in MEF. We also transfected GEC-P/N with DN-Akt1 to study if DN-Akt1 could increase stress fibers in these cells. However, we were unable to observe a clear increase of stress fibers by DN-Akt1 in these cells (data not shown, see Discussion).

Mechanisms of decreased stress fibers by nephrin

We further addressed the mechanism by which nephrin decreases stress fibers in GEC, focusing on the role of cofilin. Cofilin is an actin filament-severing protein and is inactive when phosphorylated. It is known that PI3K activates the cofilin phosphatase slingshot, leading to dephosphorylation and activation of cofilin [167]. Slingshot was colocalized with p-Akt in membrane ruffles, supporting the role of Akt in the activation of slingshot [167]. Thus, we reasoned that cofilin may be activated by nephrin via PI3K and Akt. We first compared cofilin phosphorylation in various GEC lines; cofilin was clearly phosphorylated (inactive) in GEC-neo, but its phosphorylation was decreased (more active) in GEC-P/N (Figure 6a). This decrease of cofilin phosphorylation was not observed in GEC-Y1152F (Figure 6a). We also showed that transient transfection of CA-Akt1 further decreases cofilin phosphorylation (activates cofilin) in GEC-P/N (Figure 6b). These results are compatible with the notion that PI3K activates cofilin via Akt, which may contribute to decreased stress fibers in GEC.

Another well-known regulator of stress fibers is the small GTPase, RhoA. When RhoA is activated, activation of LIM kinase via Rho kinase leads to

phosphorylation and inactivation of cofilin, contributing to the stabilization of actin filaments [74]. RhoA also stimulates stress fiber formation via modulating the activity of a myosin light chain [74]. Therefore, we studied whether RhoA activity is regulated by nephrin. RhoA activity, quantified by a pull-down assay, was not different between GEC-neo, GEC-P, and GEC-Y1152F, but was consistently lower in GEC-P/N (Figure 7), corresponding to the level of cofilin phosphorylation and reciprocal to the changes in Rac1 activity. Of interest, when CA-Rac1 expression was induced in GEC, tyrosine phosphorylation of p190RhoGAP (GTPase-activating protein) was increased (data not shown), consistent with the previous report [86]. As the activity of p190RhoGAP corresponds to the level of its tyrosine phosphorylation, these results suggest that Rac1 activates p190RhoGAP, leading to inactivation of RhoA. This pathway may, at least in part, contribute to reduced RhoA activity in GEC-P/N, which, in turn, would contribute to decreased stress fibers in GEC-P/N.

Akt activity is decreased in the rat model of PAN

We next used puromycin aminonucleoside nephrosis (PAN), a well-established rat model of podocyte injury and proteinuria [182], to study the nephrin-mediated activation of the PI3K pathway *in vivo*. Nephrin was tyrosine phosphorylated in normal rat glomeruli and this phosphorylation was reduced in rats with PAN (Figure 8a), consistent with our previous reports [37, 44]. In rats with PAN, there was a small but significant decrease in the amount of nephrin protein in total glomerular lysates (Control: 100±1 (5 rats), PAN: 88±7 (3 rats), arbitrary units, $P<0.05$). The amount of PI3K-p85 was not different between control and PAN. The amount of nephrin co-immunoprecipitated with p85 was decreased in PAN, as compared with control, and even after being normalized to the amount of nephrin in total lysates, this decrease was statistically significant (Figure 8b), indicating that the nephrin–p85 interaction is diminished in PAN. Akt phosphorylation was also decreased in glomeruli of rat with PAN, as compared with control rats (Figure 8c). As GEC is the major site of cell injury in PAN, it is reasonable to assume that this change in Akt phosphorylation occurs in GEC. Interestingly, similar changes were already observed at day 3.5, when proteinuria

was either minor or negligible (Figure S4). These results indicate that in the PAN model of rat GEC injury, tyrosine phosphorylation of nephrin and nephrin–p85 interaction are reduced, likely contributing to decreased Akt activity in GEC.

2.5. Discussion:

Although it is widely acknowledged that the actin cytoskeleton is central to the normal morphology and function of podocytes, there is limited knowledge regarding how the actin cytoskeleton is regulated in podocytes. Recent report by Mundel and co-workers highlighted the importance of synaptopodin in podocyte migration via indirectly regulating the stability of RhoA protein and modulating actin polymerization [87]. These findings underscore the importance of the temporally/spatially coordinated collaboration of various cytoskeletal regulators for podocytes to migrate to the appropriate location in the glomerulus, extend primary, secondary, and tertiary processes, eventually forming and maintaining the intricate morphology of the mature podocytes. Rac1 is a regulator of the actin cytoskeleton and is best known for its ability to stimulate lamellipodia formation in fibroblasts [74]. The lamellipodium is a membrane ruffling usually observed at the leading edge of motile cells. It is also observed at the site of directed outgrowth of the actin cytoskeleton, such as growth cones of axons, and the role of Rac1, in collaboration with the other Rho-GTPases, in neurite (including axon) outgrowth is well established [183]. In this study, we showed that nephrin-mediated Rac1 activation led to increased membrane ruffles in cultured rat GEC, accompanied by decreased cortical F-actin and stress fibers, most likely mediated by Akt activation (Figure 3). Proposed signaling pathways are summarized in Figure 9. Extrapolation of these findings to the *in vivo* setting requires caution, but we propose that disassembly of stress fibers/cortical F-actin by Akt activation liberates actin monomers necessary for actin remodeling and, combined with Rac1 activation, may lead to membrane protrusions, leading to cell migration and/or directional outgrowth, such as process formation. Conversely, decreased activities of Akt/Rac1 may contribute to foot process effacement. Notably, we did not observe obvious outgrowth of cellular processes in nephrin-expressing GEC. It is possible that diffuse expression of nephrin, as opposed to very specialized location of nephrin at the slit diaphragm *in vivo*, was not sufficient to stimulate directional cellular process formation. Alternatively, some key elements for process formation may be missing in cultured rat GEC used in this study.

Obviously, the results obtained using the overexpression system need to be interpreted with caution. Unfortunately, however, there are no cultured podocyte lines to date, which reproduce genuine foot processes or the slit diaphragm seen *in vivo*.

Significance of stress fibers in cultured podocyte lines are still under debate. Podocyte foot processes *in vivo* are rich in actin filaments and foot process effacement, the hallmark of podocyte injury and proteinuric kidney disease, is often accompanied by the disappearance of these well-aligned filaments [184]. Mundel *et al.* established conditionally immortalized mouse podocytes, which could be differentiated, and showed that in differentiated cells, the actin cytoskeleton was rearranged into fibroblast-like stress fibers extending into the processes [185]. Some investigators have assumed that stress fibers in cultured podocytes correspond to the filamentous actin in podocyte foot processes *in vivo* and as such represent differentiation of podocytes. However, stress fibers are generally observed only in cultured cells when cells make stable connections to substrates via focal adhesions and in this sense could be considered as an artifact of the cell culture system. Therefore, we suggest a view that stress fibers reflect various changes in intracellular signaling pathways and actin dynamics and are one of the useful tools in analyzing the behaviors of cultured podocytes, especially when combined with other markers. Nonetheless, by analogy to neurite outgrowth, it is possible that disassembly of stress fibers and formation of lamellipodia contribute to foot process formation in podocytes (as discussed above).

Nephrin-expressing GEC showed decreased stress fibers and increased membrane ruffles, as compared with control cells. In contrast, GEC overexpressing the Y1152F mutant of nephrin (which lacks binding to PI3K) showed significantly more stress fibers and less membrane ruffles, as compared with control cells (Figure 3). Within the scope of this study, we could not identify the cause(s) for the effects of nephrin Y1152F. We speculate that signaling pathways activated by this mutant nephrin are in favor of stress fiber formation, which becomes more visible when the counterbalancing force (PI3K/Akt1

activation via Y1152) is absent. Also, it was shown previously that effective membrane targeting of active Rac1 requires PI(3,4,5)P_3[186]; thus in the cells expressing nephrin-Y1152F, Rac1 may not be properly targeted at the plasma membrane because of the lower activity of PI3K, leading to less membrane ruffles. These hypotheses need further verification.

On the basis of our hypothesis that nephrin-mediated Akt activation is a major contributor of stress fiber disassembly, we expected that inactivation of Akt would increase stress fibers in GEC-P/N. However, expression of DN-Akt1 in GEC-P/N did not have consistent effects on stress fibers. Recent studies have established isoform-specific actions of Akt on the actin cytoskeleton. Rat GEC used in this study and rat glomerulus express both Akt1 and Akt2 (Figure 6c). In MEF, Akt1 and Akt2 had opposing impacts on stress fiber formation [67]. As DN-Akt1 used in this study blocks both Akt1 and Akt2 by sequestering upstream stimulators, it is possible that the effect of Akt1 inhibition was masked by the simultaneous inhibition of Akt2. The phosphor-Akt (Ser473) antibody used in this study reacts with both isoforms; thus we were not able to discern which isoform(s) is/are activated by nephrin in GEC. Alternatively, there might be Akt-independent signaling pathways, which lead to stress fiber disassembly (see Figure 9).

In the rat model of podocyte injury and proteinuria (PAN), we observed that tyrosine phosphorylation of nephrin, as well as nephrin–p85 interaction and Akt phosphorylation, was decreased significantly (Figure 8). Although a causal relationship cannot be established in this model, it is reasonable to assume that impaired nephrin–p85 interaction contributes, at least in part, to decreased Akt phosphorylation. Because Akt is a well-known antiapoptotic molecule, these results are in line with the previous observations that podocyte apoptosis is often seen in PAN [182]. In addition, impaired nephrin–p85 activation may also contribute to deranged podocyte morphologies, such as foot process effacement.

2.6. ACKNOWLEDGEMENTS

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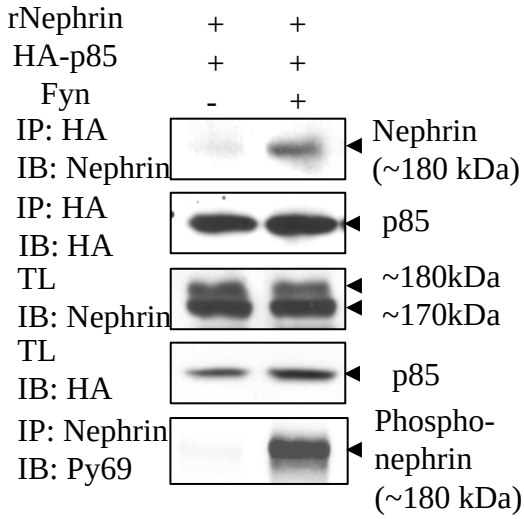
2.7. Figures

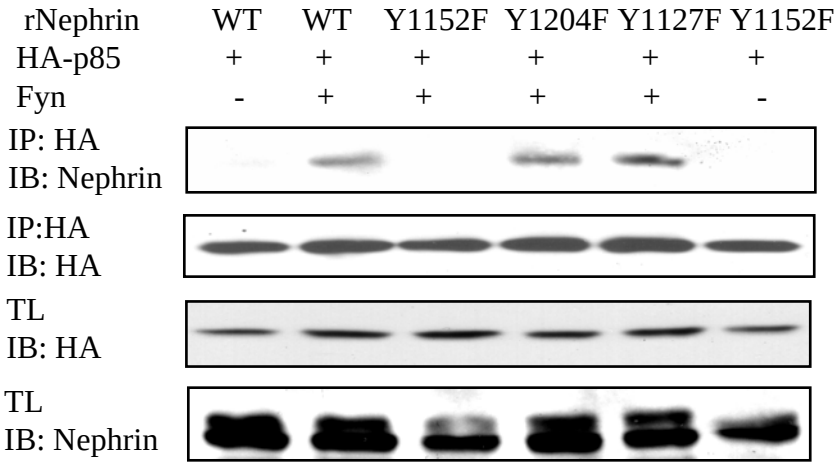
Figure 1. Y1152 is responsible for tyrosine phosphorylation-dependent nephrin (rat)-p85 interaction. A. Cos-1 cells were transient transfected with the indicated plasmids. After 24 h, cells were lysed and subjected to immunoprecipitation (IP). Precipitates were analyzed by immunoblotting (IB). TL: total cell lysates. Nephrin co-immunoprecipiated with PI3K-p85 only in the presence of Fyn. B. Cos-1 cells were transiently transfected with the indicated plasmids and analyzed as in A. Top: representative blots, bottom: densitometry. * $p<0.05$ vs WT with Fyn, n = 3. Y1152F mutation, but not the other mutations of nephrin, abolished the interaction with PI3K-p85.

Figure 1

A


rNephrin + +
HA-p85 + +
Fyn - +
IP: HA
IB: Nephrin
Nephrin
(~180 kDa)
IP: HA
IB: HA
p85
TL
IB: Nephrin
~180kDa
~170kDa
TL
IB: HA
p85
IP: Nephrin
IB: Py69
Phospho-
nephrin
(~180 kDa)

B


rNephrin WT WT Y1152F Y1204F Y1127F Y1152F
HA-p85 + + + + + +
Fyn - + + + + -
IP: HA
IB: Nephrin
IP:HA
IB: HA
TL
IB: HA
TL
IB: Nephrin

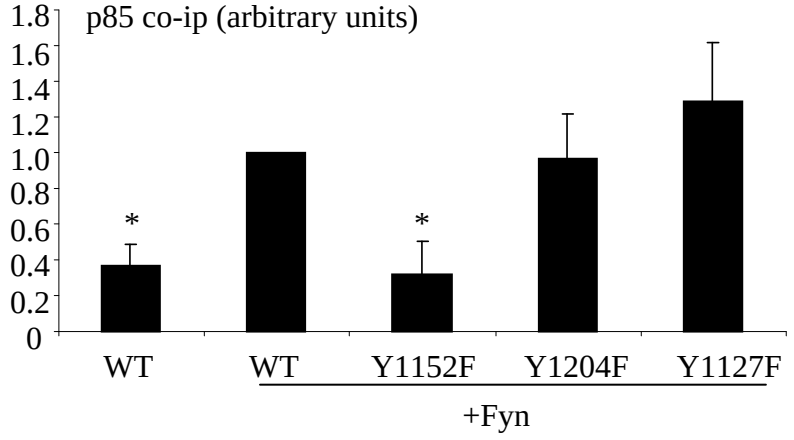

p85 co-ip (arbitrary units)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
*
WT
WT
Y1152F
Y1204F
Y1127F
+Fyn

Figure 2. Nephrin activates Akt and Rac1 via PI3K. A. GEC were stably co-transfected with podocin and wild type rat nephrin (GEC-P/N) or podocin and nephrin mutant Y1152F (GEC-Y1152F). After 16 h of serum starvation, cells were treated with H_2O_2 (1 μM), LY294002 (PI3K inhibitor, 10 μM), or PP2 (Src-family kinase inhibitor, 10 μM) for 30min and were lysed and subjected to immunoprecipitation/immunoblotting. Akt phosphorylation was increased in GEC-P/N, as compared with GEC-Neo (vector-transfected control cells) or GEC-Y1152F, which was abolished by LY294002 and PP2. B. GEC were stably transfected with podocin alone (GEC-P). Akt phosphorylation in GEC-P was not different from GEC-Neo, and was less than in GEC-P/N. C. Akt phosphorylation was diminished in mouse embryonic fibroblasts (MEF) deficient in Nck1 and Nck2, as compared with Nck1+/-, Nck2+/+ MEF, under serum-starved conditions. D. GEC-neo, GEC-P/N, GEC-Y1152F were serum starved for 16 h and LY294002 and PP2 were added 30 min before cells were lysed. Cell lysates were subjected to GST-Crib (Cdc42/Rac1 interaction binding) pull down (PD) followed by immunoblotting with anti-Rac1. Normalized to total Rac 1 and GEC-Neo. Similar to Akt phosphorylation, Rac1 activity was increased in GEC-P/N. * $p<0.05$ vs GEC-Neo, n=3-5. Rac1 activity was not different between GEC-Neo and GEC-P (top right panel).

Figure 2

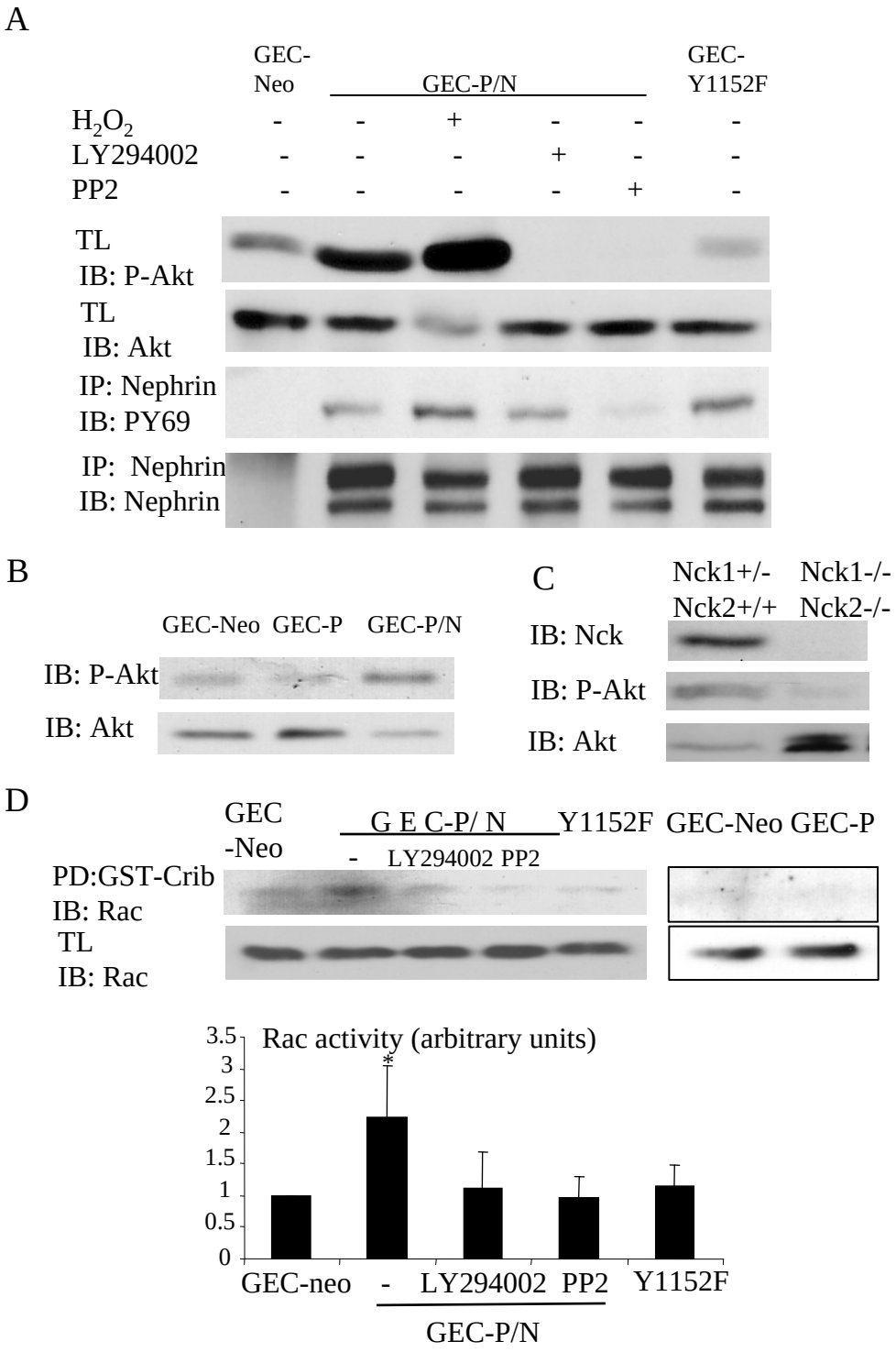
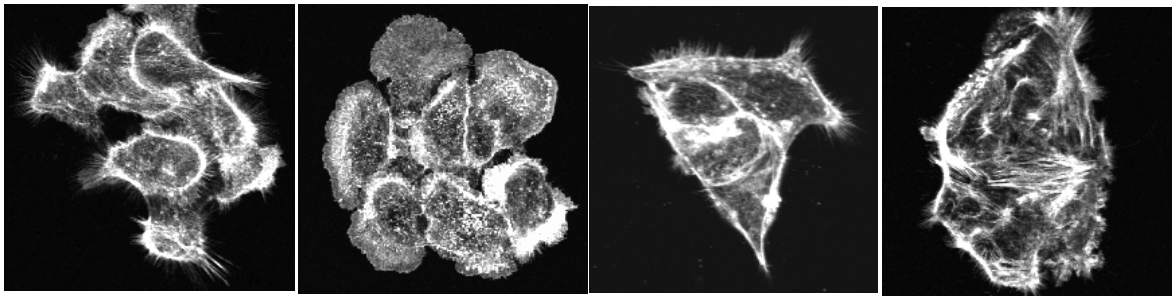
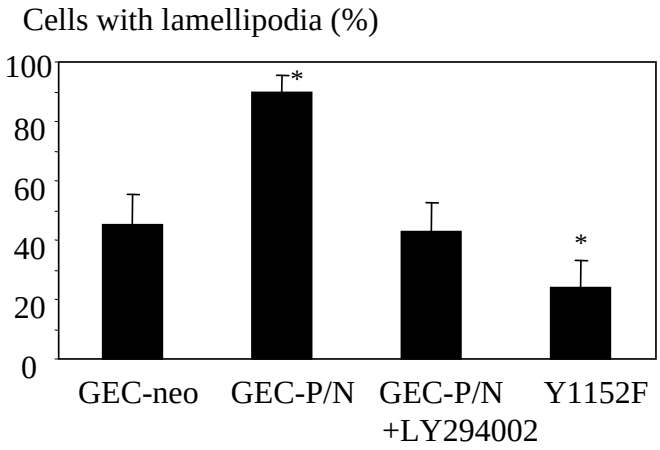

A
GEC-Neo
GEC-P/N
GEC-Y1152F
H2O2
LY294002
PP2
TL
IB: P-Akt
TL
IB: Akt
IP: Nephrin
IB: PY69
IP: Nephrin
IB: Nephrin
B
GEC-Neo GEC-P GEC-P/N
IB: P-Akt
IB: Akt
C
Nck1+/- Nck1-/-
Nck2+/+ Nck2-/-
IB: Nck
IB: P-Akt
IB: Akt
D
GEC-Neo
G E C-P/ N
Y1152F
- LY294002 PP2
PD:GST-Crib
IB: Rac
TL
IB: Rac
GEC-Neo GEC-P
Rac activity (arbitrary units)
3.5
3
2.5
2
1.5
1
0.5
0
*
GEC-neo - LY294002 PP2 Y1152F
GEC-P/N

Figure 3. Nephrin expression modulates the actin cytoskeleton in GEC. Serum starved GEC-neo, GEC-P/N, GEC-Y1152F were stained with TRITC-phalloidin. LY294002 (10 μM) was added overnight. Cells with membrane ruffles and stress fibers were quantified as in Methods. * $p<0.05$ vs GEC-neo, n=5. GEC-P/N showed more membrane ruffles and less stress fibers, as compared with GEC-Neo, and these changes were abolished by LY294002. Mutant nephrin (Y1152F) failed to induce these changes. F-actin pattern was not different between GEC-neo and GEC-P (not shown).

Figure 3



GEC-Neo GEC-P/N GEC-P/N + LY294002 Y1152F



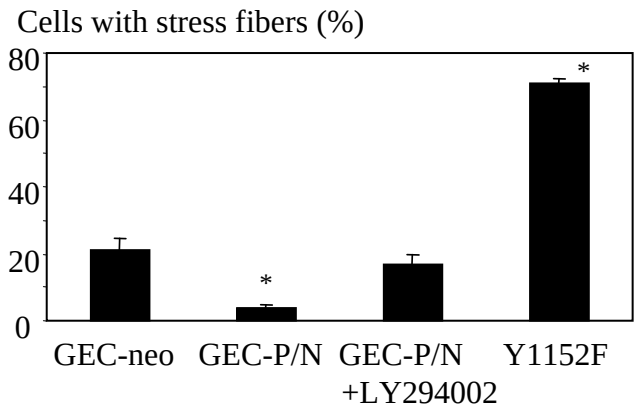


Figure 4. Rac1 activation contributes to membrane raffling in GEC. A. GEC-Y1152F were transiently transfected with CA-Rac1 (GFP-tagged) or GFP alone. Cells transfected with CA-Rac1 showed prominent membrane ruffles at the periphery of the colonies (arrowheads). B. GEC-P/N cells were transiently transfected with DN-Rac1 (GFP-tagged) or GFP alone. Cells transfected with DN-Rac1 were mostly devoid of membrane ruffles (arrow). Cells with lamellipodia were quantified as in Methods. * $p<0.01$ vs GFP transfected cells, n=3.

Figure 4

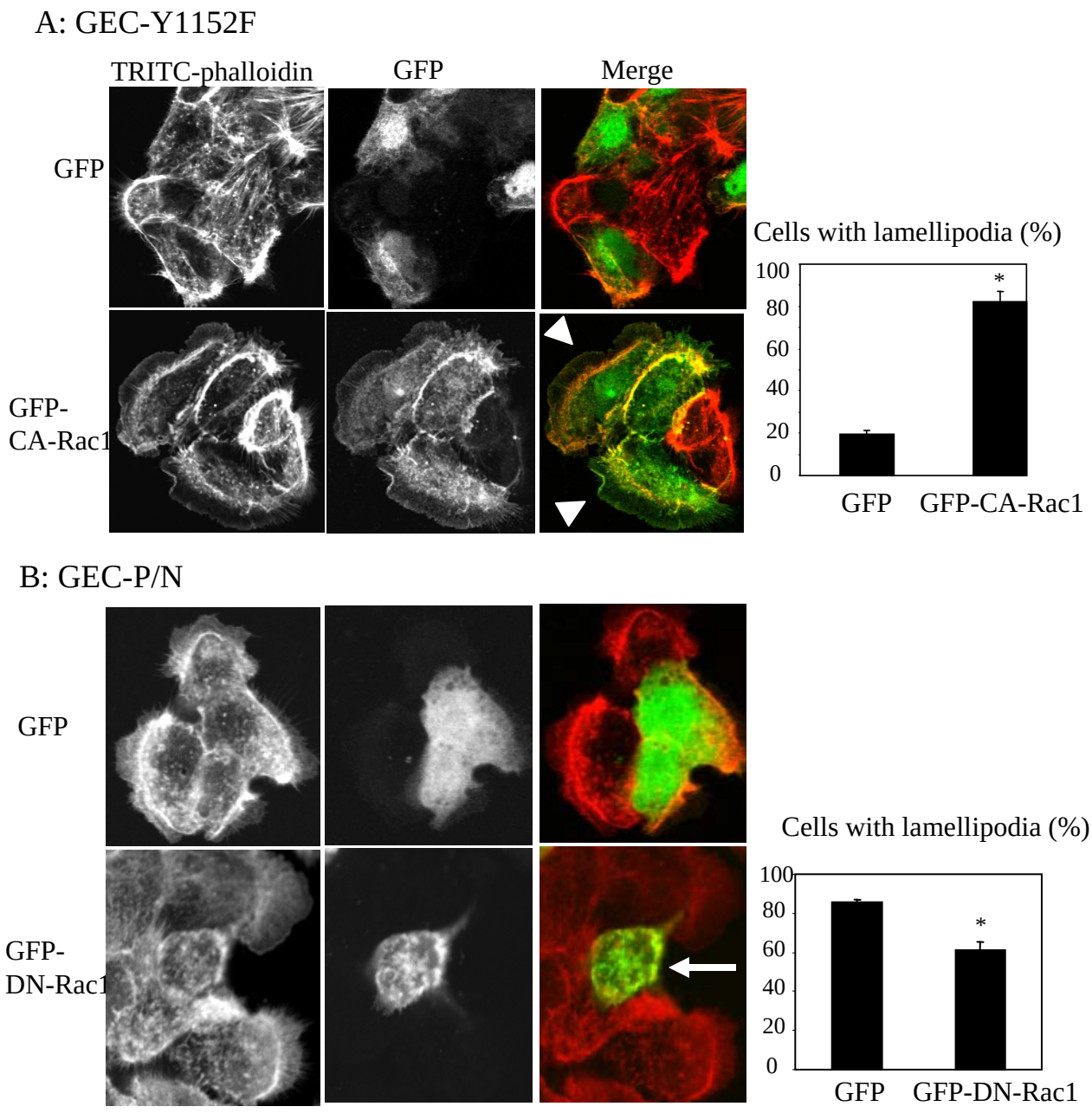

A: GEC-Y1152F
TRITC-phalloidin
GFP
Merge
GFP
GFP-CA-Rac1
Cells with lamellipodia (%)
100
80
60
40
20
0
*
GFP
GFP-CA-Rac1
B: GEC-P/N
GFP
GFP-DN-Rac1
Cells with lamellipodia (%)
100
80
60
40
20
0
*
GFP
GFP-DN-Rac1

Figure 5. Active Akt1 decreases stress fibers in GEC and MEF. A. GEC-Y1152F, which demonstrate prominent stress fibers in ~70% of the cells (see Fig. 3) were transfected with CA-Akt1 (GFP-tagged) or GFP alone. Cells transfected with CA-Akt1 generally did not show prominent stress fibers (arrowheads). B. For comparison, Nck deficient MEF (Nck1-/-, Nck2-/-), which have prominent stress fibers in ~100% of the cells and very low level of Akt phosphorylation (Fig. 2C), were transiently transfected with CA-Akt1 (GFP-tagged) or GFP alone. Cells transfected with CA-Akt1 completely lost stress fibers and well-spread morphology (arrows).

Figure 5

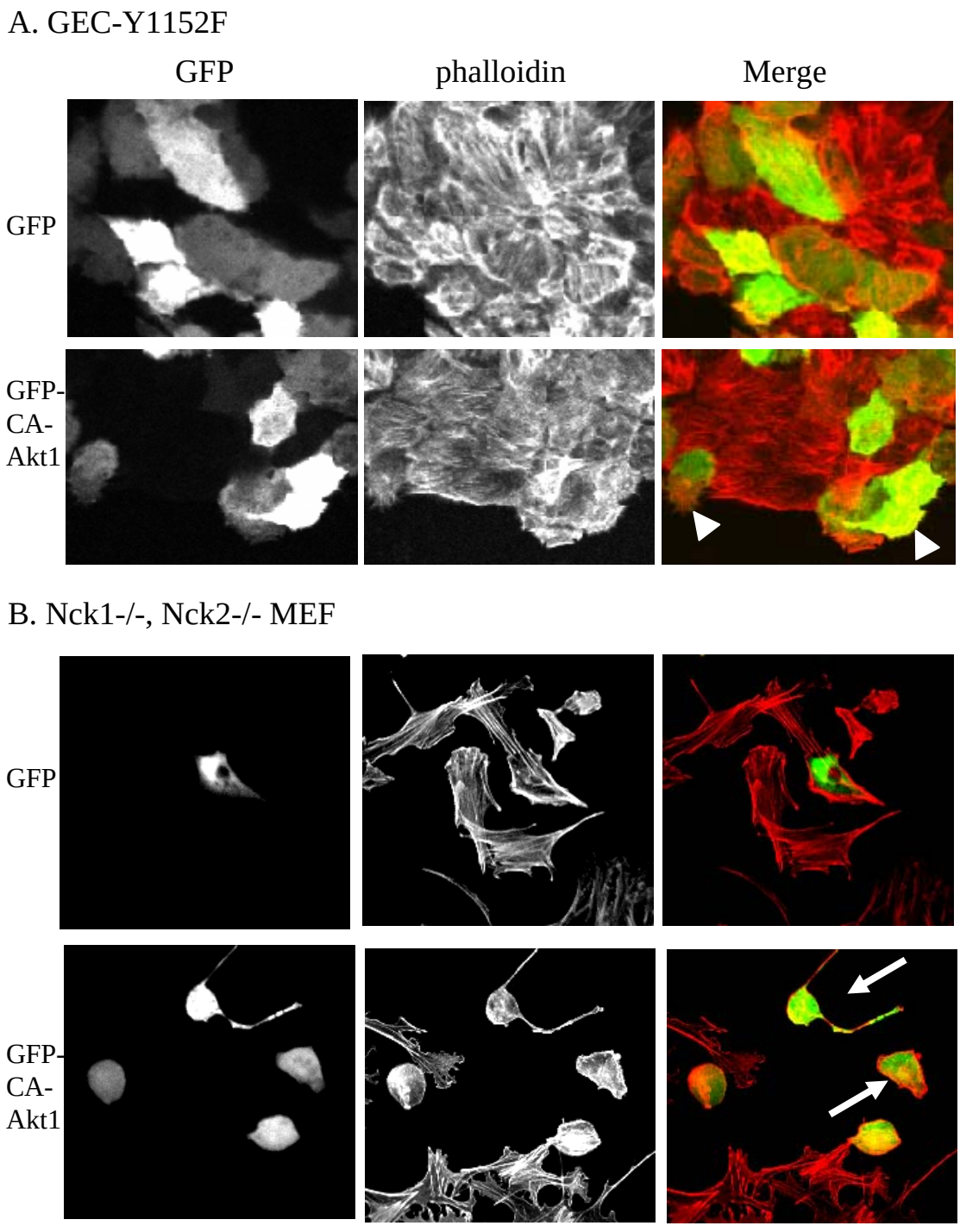

A. GEC-Y1152F
GFP
phalloidin
Merge
GFP
GFP-CA-Akt1
B. Nck1-/-, Nck2-/- MEF
GFP
GFP-CA-Akt1

Figure 6. Akt1 activates cofilin in GEC. A. Serum starved GEC-neo, GEC-P/N and GEC-Y1152F were lysed and subjected to immunoblotting. GEC-P/N showed decreased phosphorylation (i.e. increased activity) of cofilin, as compared with GEC-neo or GEC-Y1152F. B. GEC-P/N were transiently transfected with CA-Akt1 or vector. Top: representative blot, bottom: densitometric analysis. CA-Akt1 decreased phosphorylation (i.e. increased activity) of cofilin. * $p<0.05$ vs vector, n = 3. C. Akt1 and Akt2 expression in GEC and rat glomerulus. Lysate from GEC-neo, GEC-P/N, rat glomerulus and HEK293T cells transfected with Akt1 were subjected to immublotting. Both Akt1 and Akt2 are expressed in GEC and rat glomerulus.

Figure 6

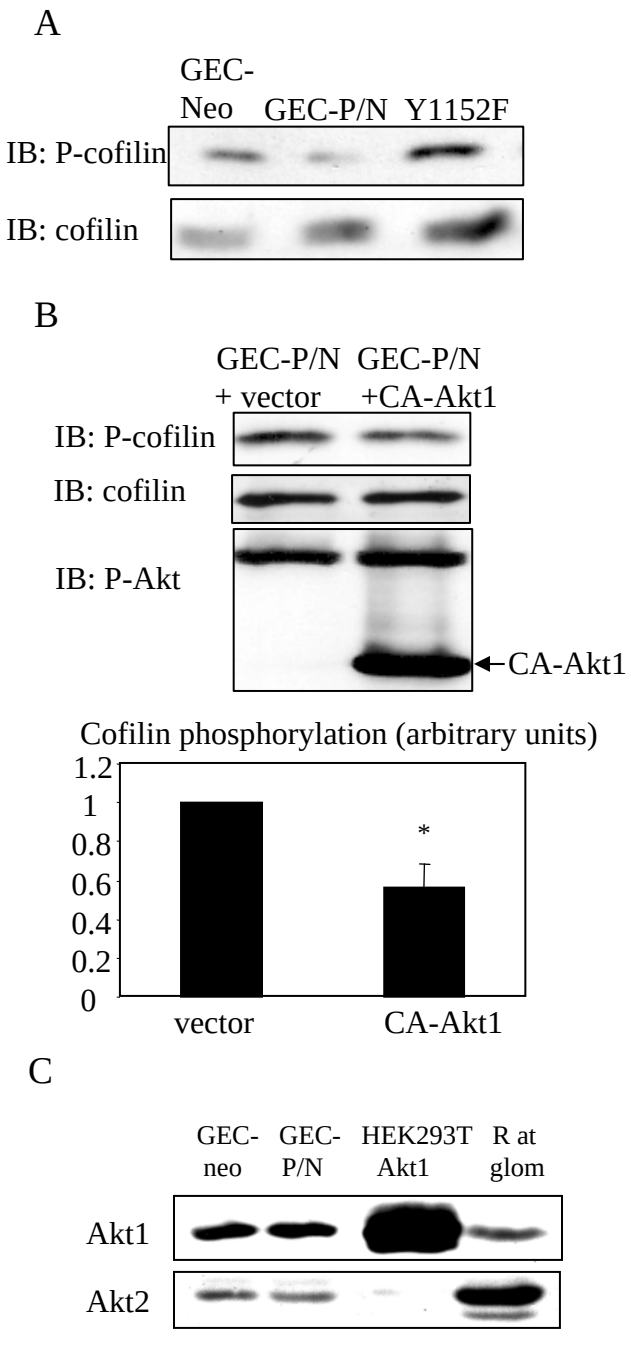

A
GEC-Neo
GEC-P/N
Y1152F
IB: P-cofilin
IB: cofilin
B
GEC-P/N + vector
GEC-P/N +CA-Akt1
IB: P-cofilin
IB: cofilin
IB: P-Akt
CA-Akt1
Cofilin phosphorylation (arbitrary units)
1.2
1
0.8
0.6
0.4
0.2
0
*
vector
CA-Akt1
C
GEC-neo
GEC-P/N
HEK293T Akt1
R at glom
Akt1
Akt2

Figure 7. RhoA activity is decreased in nephrin-expressing GEC. RhoA activity was compared by pull-down assay in GEC-neo, GEC-P/N, and GEC-Y1152F. RhoA activity was lower in GEC-P/N, but not in Y1152F, as compared with GEC-neo (left panel). RhoA activity was not different between GEC-neo and GEC-P (right panel).

Figure 7

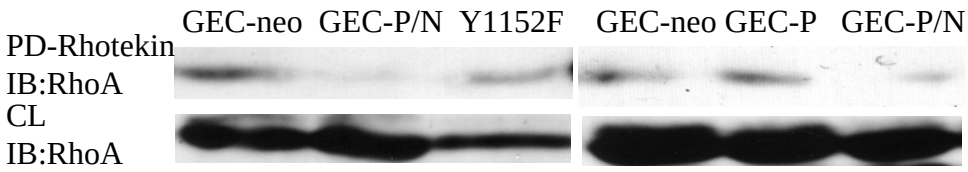
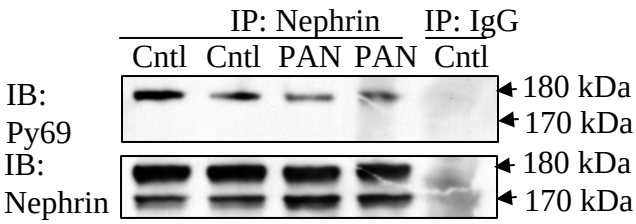

GEC-neo
GEC-P/N
Y1152F
GEC-neo
GEC-P
GEC-P/N
PD-Rhotekin
IB:RhoA
CL
IB:RhoA

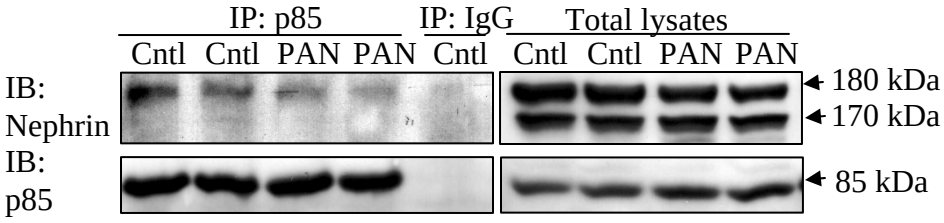
Figure 8. Nephrin-p85 interaction and Akt phosphorylation are decreased in PAN. PAN was induced as in Methods. Glomerular lysates from rats with PAN (day 7) and control rats were immunoprecipitated for nephrin (A) or p85 (B) and precipitates were blotted for phosphotyrosine (A) or nephrin (B). A. Tyrosine phosphorylation of nephrin was decreased in PAN. B. The amount of nephrin co-immunoprecipitated with p85 was quantified by densitometry and normalized to the amount of nephrin in total lysates. *$p<0.05$, n=5 rats for control, 3 rats for PAN. There was a small decrease (~12 %) in the amount of nephrin in PAN (see Text). The amounts of p85 were not different between control and PAN. C. Akt phosphorylation was decreased in PAN.

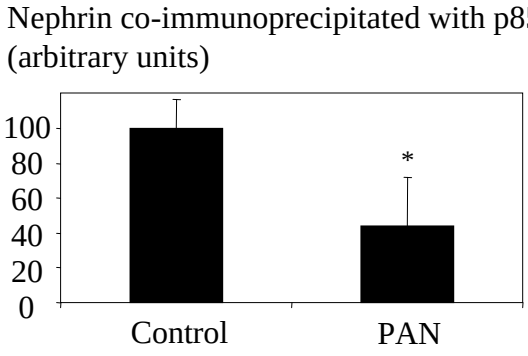
Figure 8

A


IP: Nephrin
IP: IgG
Cntl Cntl PAN PAN Cntl
IB:
Py69
180 kDa
170 kDa
IB:
Nephrin
180 kDa
170 kDa

B


IP: p85
IP: IgG
Total lysates
Cntl Cntl PAN PAN Cntl Cntl Cntl PAN PAN
IB:
Nephrin
180 kDa
170 kDa
IB:
p85
85 kDa


Nephrin co-immunoprecipitated with p85
(arbitrary units)
100
80
60
40
20
0
*
Control
PAN

C

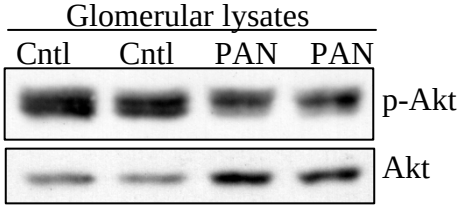
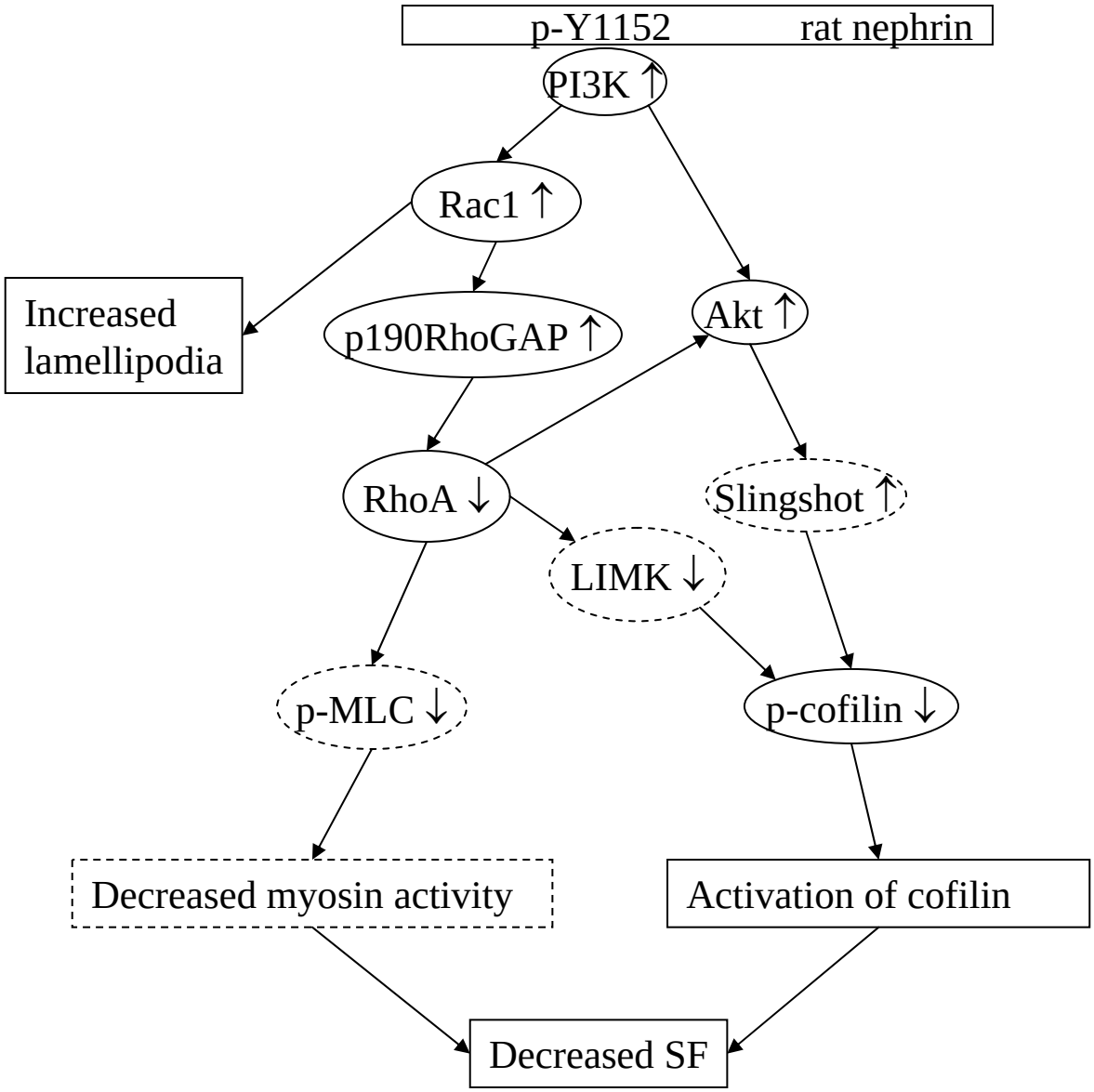

Glomerular lysates
Cntl Cntl PAN PAN
p-Akt
Akt

Figure 9. Hypothesis for nephrin-mediated activation of the PI3K pathway and reorganization of the actin cytoskeleton. Dotted line represents the points not directly addressed in the current study.

Figure 9


p-Y1152 rat nephrin
PI3K ↑
Rac1 ↑
Increased lamellipodia
p190RhoGAP ↑
Akt ↑
RhoA ↓
Slingshot ↑
LIMK ↓
p-MLC ↓
p-cofilin ↓
Decreased myosin activity
Activation of cofilin
Decreased SF

Connecting text between Chapter 2 and Chapter 3

In Chapter 2, we characterized a tyrosine phosphorylation-dependent interaction between rat nephrin and PI3K-p85 and focused on the impact of PI3K activation on the actin cytoskeleton rearrangement in podocytes. We demonstrated that nephrin–PI3K interaction leads to the activation of Akt1 and Rac1 pathways, resulting in the remolding of the actin cytoskeleton in podocytes. It was reported that nephrin-Nck association is essential to maintaining podocyte structure and function. Pak is a well-known downstream effector of Nck in regulating cytoskeletal dynamics. However, the role of Nck-Pak signaling in podocytes has not been elucidated. In Chapter 3, we characterized the role of Nck-Pak signaling mediated by the tyrosine phosphorylation of nephrin in actin cytoskeleton organization of podocytes.

Chapter 3.

p21-activated kinases regulate actin remodeling in glomerular podocytes

Running title: Pak modulates actin assembly in podocytes

3.1. ABSTRACT

The tyrosine phosphorylation of nephrin is reported to regulate podocyte morphology via the Nck adaptor proteins. The Pak family of kinases are regulators of the actin cytoskeleton and are recruited to the plasma membrane via Nck. Here, we investigated the role of Pak in podocyte morphology. Pak1/2 were expressed in cultured podocytes. In differentiated mouse podocytes, Pak2 was predominant, concentrated at the tips of the cellular processes, and its expression/phosphorylation were further increased when differentiated. Overexpression of rat nephrin in podocytes increased Pak1/2 phosphorylation, which was abolished when the Nck binding sites were mutated. Furthermore, dominant-negative Nck constructs blocked the Pak1 phosphorylation induced by antibody-mediated cross-linking of nephrin. Transient transfection of constitutively kinase active Pak1 into differentiated mouse podocytes decreased stress fibers, increased cortical F-actin, and extended the cellular processes, while kinase-dead mutant, kinase inhibitory construct, and Pak2 knockdown by shRNA had the opposite effect. In a rat model of puromycin aminonucleoside nephrosis, Pak1/2 phosphorylation was decreased in glomeruli, concomitantly with a decrease of nephrin tyrosine phosphorylation. These results suggest that Pak contributes to re-modeling of the actin cytoskeleton in podocytes. Disturbed nephrin-Nck-Pak interaction may contribute to abnormal morphology of podocytes and proteinuria.

3.2. INTRODUCTION

Podocytes surround glomerular capillary loops and are critical determinants of glomerular permselectivity. Their function is tightly associated with their intricate morphology, which features interdigitating foot processes from adjacent podocytes. The actin cytoskeleton, an integral component of podocyte foot processes, is regulated by a number of proteins expressed in podocytes including nephrin, NEPH1, FAT1, podocin, CD2AP, α-actinin-4 etc [187]. However, the means by which these proteins regulate actin dynamics in podocyte foot processes is only partially understood. On tyrosine phosphorylation, for example, the slit diaphragm transmembrane proteins nephrin and NEPH1 recruit the adaptor molecule Nck and Grb2, respectively, and work in concert to facilitate local actin polymerization [44, 188]. Similarly, the proto-cadherin FAT1 binds to Ena/VASP, which regulates actin polymerization [20].

Members of the Pak family of Ser/Thr kinases have been identified as targets of Rho-family of small GTPases, Rac1 and Cdc42. These Pak kinases play important roles in a wide range of cellular events, including cytoskeletal regulation, mitogen-activated protein kinase activation, and gene regulation [189]. Three isoforms of Pak (Pak1-3) share the same structural organization and regulation and have similar biological actions [111]. Pak consists of an N-terminal regulatory domain, which includes the binding domain for GTP-Rac and GTP-Cdc42, and a C-terminal kinase domain. Among Pak1, 2, and 3, which have the molecular size of 68, 62, and 65 kDa, respectively, Pak1 is the prototype and most extensively studied [111]. Although Pak has versatile biological actions, its main action appears to be the regulation of cytoskeletal dynamics [111]. Pak1 is known to cause the disassembly of focal adhesions, and promote lamellipodia formation and membrane ruffling [190]. Of particular interest, Pak1 caused neurite outgrowth in PC12 cells [191]. However, the mechanisms of Pak-mediated cytoskeletal changes are yet to be clearly elucidated. Information from genetic studies is limited: gene knockout of Pak1 in mouse resulted in immune defects, while Pak2 knockout was embryonic lethal [135]. Pak3 is relatively specific to the

brain and its mutation in human is known to cause X-linked, nonsyndromic mental retardation [135].

In addition to Rac1/Cdc42, the adaptor molecule Nck is known as an upstream regulator of Pak [104]. Here, we investigated the role of the Pak family of kinases in the cytoskeletal regulation of glomerular podocytes. Results showed that Pak1 and 2 are expressed in podocytes. Pak is recruited to nephrin via Nck, a process which leads to increased Pak phosphorylation. This increased phosphorylation in turn is likely to contribute to actin remodeling and potentially to the formation/extension of cellular processes in podocytes.

3.3. MATERIALS AND METHODS

Materials. Tissue culture media, hygromycin and lipofectamine 2000 were from Invitrogen-Life Technologies (Burlington, ON). Interferon-γ was from Medicorp (Montreal, QC). Electrophoresis reagents were from Bio-Rad Laboratories (Mississauga, ON). Anti-nephrin antibody was described previously [37]. Other antibodies were obtained from the following sources; Pak1 (Zymed Laboratories, South San Francisco, CA), Pak1, (2, 3), Pak2, GFP, Myc (FITC-labeled), and Myc (TRITC-labeled)(Santa Cruz Biotechnology, Santa Cruz, CA), phospho-Pak for Ser199/204 of Pak1 and Ser192/197 of Pak2 (Cell Signaling, Beverly, MA), phosphotyrosine (PY69) and Nck (BD Biosciences, Mississauga, ON), synaptopodin (Progen Biotechnik, Heidelberg, Germany), Alexa Fluoro 633-phalloidin (Molecular Probes, Eugene, OR), tubulin, TRITC-phalloidin (Sigma-Aldrich, Missisauga, ON). NSC23766 was from CalBiochem (San Diego, CA). Enhanced chemiluminescence (ECL) detection reagents were from Amersham Bioscience (Baie d'Urfé, PQ). Puromycin aminonucleoside and other chemicals were from Sigma-Aldrich.

Plasmids. Plasmids encoding wild type full-length rat nephrin and rat nephrin tyrosine mutants were described previously [37]. Mouse podocin cDNA was a gift from Dr. T. Benzing (University Hospital Freiburg, Freiburg, Germany). Myc-Pak1, GFP-Pak1(WT), GFP-Pak1T423E, GFP-Pak1K299R, GFP-Pak1PID were gifts from Drs. Bokoch and DerMardirossian (Scripps Institute, La Jolla, CA). GFP-Nck, GFP-Nck-SH2, GFP-Nck-W143R were gifts from Dr. Larose (McGill University, Montreal, QC).

Cell culture. Rat GEC culture and characterization were previously described [192]. GEC stably transfected with mouse podocin alone (GEC-P) or mouse podocin and rat nephrin (GEC-P/N) were described previously [192]. GEC stably transfected with mouse podocin and rat nephrin mutant Y1127F (GEC- Y1127F) or mouse podocin and rat nephrin mutant Y1204/1228F (GEC-Y2F) were also established. Conditionally immortalized mouse podocytes (MP: gift from Dr. K. Endlich, University of Heidelberg) were maintained or differentiated as described previously [193]. MP-nephrin was derived from MP by stably expressing rat

nephrin cDNA using retrovirus transduction. HEK-nephrin was described previously [194]. Transient transfection of HEK293T cells was performed using lipofectamine 2000 following the manufacturer's instructions. Cells were used 18 h after transfection.

Antibody-mediated cross-linking of nephrin was performed as described previously with minor modification [194]. Briefly, cells were incubated with 10 μg/mL of mAb-5-1-6 diluted in serum-free medium supplemented with buffer (0.5 M HEPES, pH 7.5) for 30 min at 4ºC. Medium was discarded and cells were cross-linked with 10 μg/mL of goat anti-mouse IgG, also diluted in serum-free medium supplemented with buffer at 37ºC for the indicated times.

Immunoprecipitation and immunoblotting. Cells or glomeruli were lysed and were subjected to immunoprecipitation and/or immunoblotting as described previously [37]. Protein content was quantified using scanning densitometry (ImageJ software).

Nucleofection was performed using Amaxa Basic Nucleofector Kit for Primary Mammalian Epithelial Cells (ESBE Scientific, Markham, ON) according to the manufacturer's instructions using the program T-23, which we found to generate the highest transfection efficiency in MP.

qPCR and PCR Total RNA was prepared from cells using Trizol Reagent (Invitrogen). cDNA synthesis was performed using a QuantiTect Reverse Transcription kit (Qiagen, Mississauga, ON). The PCR primer set used for Pak2 were fwd:aacctttgccttctgttcca, rev:aacatggatggtgtgctcaa. qPCR was carried out with iTaq SYBR Green supermix with ROX (Biorad) and an ABI 7300 using the delta Ct method with GAPDH as an endogenous control. For conventional PCR, the same primer sets were used for Pak2. Primers for Pak1 were fwd: ggctccttgacagatgtggt, rev: tgtccctgtgaatgacttgg; for Pak3 were fwd: tcattgcaccaagaccagag, rev: gtggtggaattggcattctc.

Induction of puromycin aminonucleoside nephrosis (PAN) and isolation of rat glomeruli. PAN was induced and rat glomeruli were isolated as described previously [179]. Studies were approved by the Animal Care Committee at

McGill University. Urine was collected for 24 hrs in metabolic cages and urine protein concentration was determined by BioRad Protein Assay Kit.

Immunohistochemistry was performed as we described previously [179] using rabbit anti–Pak (SC-881, Santa Cruz, 1:100 dilution). Normal rabbit IgG (negative control) showed no staining and the Pak staining was blocked by the specific peptide antigen (not shown).

Rac activation assay Rac1 activity was measured using a G-LISA kit (BK125, Cytoskeleton, Denver, CO) according to the manufacturer's instructions.

Immunofluorescent staining and quantification of actin assembly

Immunostaining was performed as described previously [195] except that cover slips were coated with type I collagen for mouse podocytes. Images were obtained with a confocal microscope (Fluoview FV1000, OLYMPUS). To quantify the actin assembly of transfected cells, transfected cells were randomly selected and F-actin fluorescence images were acquired with the confocal microscope. Cell membranes and membrane protrusions were outlined on the fluorescence image with a width of 2 μm. The average fluorescence intensity (mean pixel density) in the membrane region and remaining cytosol region were measured using Olympus Fluoview (Ver1.7a). The fluorescence intensities were collected from 20-22 different cells per group. Statistical analysis was performed using Student's *t* test.

Pak2 knockdown shRNAs for mouse Pak2 were designed using a web-based program (www.genscript.com). Three shRNAs were cloned into RNATin-H1.2/Neo (GenScript) and stably transfected into MP-nephrin using Nucleofection. Only one sequence (tentatively named : GATCCCGTGATTGGCTGATAAAGGATCTTTGATATCCGAGATCCTTTATCAGCCAATCATTTTTTCCAAAAGCT) gave effective knockdown of Pak2.

Data analysis Data are presented as the mean ± sem. The *t* statistic was used to determine significant differences between two groups. One-way analysis of variance (ANOVA) was used to determine significant differences among groups. When significance was found, the Tukey HSD Test was used for post-ANOVA pair-wise comparisons.

3.4. RESULTS

Expression of Pak isoforms in podocytes. To test the hypothesis that the Pak family of kinases contributes to the regulation of the actin cytoskeleton in glomerular podocytes, we first studied the expression of Pak isoforms in podocytes in culture. By immunoblotting, undifferentiated mouse podocytes showed a clear band corresponding to the reported molecular size of Pak2 (62 kDa) and doublets of approximately the reported size of Pak1 (68 kDa) (Fig 1A). Similarly, cultured rat glomerular epithelial cells (GEC) and normal rat glomerular lysates both showed bands corresponding to Pak1 and Pak2. Antibody specific to Pak1 recognized the doublets of ~68 kDa, indicating that both bands represent Pak1. Expression of Pak 1/2 in mouse podocytes, rat GEC, and rat glomeruli was also confirmed by RT-PCR (36 cycles for Pak1, 32 cycles for Pak2, Fig. 1B). Pak3 mRNA was not detected at 36 cycles, but was detected at 41 cycles in rat GEC and mouse podocytes but not in rat glomerulus (Fig. 1B). Taken together, these findings indicated that Pak1 and Pak2 are expressed in cultured podocytes and rat glomerulus, but that the level of Pak3 expression is markedly low in cultured cells and negligible in rat glomerulus.

We next studied the expression of Pak in the rat kidney by immuno-histochemistry using the same antibody as used in the immunoblot, which recognizes all three isoforms (Fig. 1A). Pak staining was observed both in the tubules and glomerulus. Some of the staining in the glomerulus was seen at the periphery of the glomerular tufts, consistent with the podocyte distribution (Fig. 1C).

Expression and phosphorylation of Pak2 is increased with differentiation in mouse podocytes. It is well established that cultured mouse podocytes can be induced into a more differentiated phenotype [185]. This phenotypic change typically includes the expression of differentiation markers such as synaptopodin and nephrin, as well as reorganization of the actin cytoskeleton [185]. We next studied the temporal expression profiles of the Pak isoforms in differentiating mouse podocytes. By immunoblotting, Pak2 expression was significantly increased when podocytes were differentiated for 1-2 wks (Fig. 2A). In contrast,

Pak1 expression decreased significantly after 2 wks of differentiation (Fig. 2A), thus rendering Pak2 predominant in differentiated mouse podocytes.

Activation of Pak kinase is known to induce autophosphorylation [196]. Ser 199/204 of Pak1 and Ser 192/197 of Pak2 are located in the amino-terminal inhibitory domain and autophosphorylation of these residues is thought to prevent the kinase from reverting to an inactive conformation [115]. We found that phospho-Pak2 (Ser 192/197) was consistently increased when mouse podocytes were differentiated (Fig. 2B), suggesting that Pak2 is not only upregulated in differentiated podocytes but also activated.

We next studied the intracellular distribution of Pak2 in differentiating mouse podocytes. In undifferentiated podocytes, Pak2 was localized at the leading edge of cellular processes. In differentiated podocytes, in contrast, cellular processes were further extended and Pak2 staining was localized at the tips of cellular processes and at the edges of lamellipodia (Fig. 2C).

Pak is recruited to nephrin. It was shown previously that the cytoplasmic domain of nephrin becomes tyrosine phosphorylated and recruits the adaptor protein Nck [40, 44, 194]. Given that Nck binds to the Pak family of kinases via its second SH3 domain [104], we next investigated whether Pak occurs in the same protein complex as nephrin. In the subsequent series of experiments, which required Pak transfection, we utilized the Pak1 plasmid instead of the Pak2 plasmid owing to the latter's markedly low expression, which made the experiments technically difficult. When HEK-nephrin cells (HEK293T cells stably transfected with rat nephrin, [194]) were transfected with Pak1 (GFP-tagged), nephrin co-immunoprecipitated with Pak1 only when the extracellular domain of nephrin was cross-linked with antibodies, a maneuver known to induce the tyrosine phosphorylation of nephrin [194]. This cross-linking-dependent association of nephrin and Pak1 was markedly attenuated when the two tyrosine residues in nephrin responsible for Nck binding were mutated to phenylalanine (Y1204/1228F or Y2F, [194]) (Fig 3A). We also verified whether Pak is recruited to nephrin using immunofluorescence staining. When HEK-nephrin were transfected with Pak1, Pak1 was distributed diffusely in the cytoplasm. When

cells were subjected to antibody-mediated nephrin cross-linking, in contrast, Pak1 formed aggregates, which co-localized with the site of nephrin cross-linking (Fig 3B). This effect was abolished when the nephrin mutant Y2F was used (Fig. 3B). These results suggest that Pak1 is recruited into the protein complex, which also includes nephrin, in a tyrosine phosphorylation-dependent manner, likely via Nck.

We next investigated whether Pak also forms a molecular complex with nephrin in vivo, using normal rat glomerular lysates. When Nck was immunoprecipitated with anti-Nck antibody, both nephrin and Pak were co-immunoprecipitated (Fig. 3C), supporting the idea that nephrin-Nck-Pak form a protein complex in vivo. While the Pak1 bands were consistently recognized in the precipitates, the precipitation of Pak2 could not be consistently confirmed (Fig. 3C). These findings support the idea that the intracellular domain of nephrin recruits Pak in a tyrosine phosphorylation- and Nck- dependent manner.

Nephrin increases Pak phosphorylation. We next asked whether the recruitment of Pak to nephrin leads to the phsphorylation of Pak. Using our previously established subclones of rat GEC, which stably overexpress wild-type rat nephrin together with podocin (GEC-P/N)[192], we found that nephrin was tyrosine phosphorylated without exogenous stimuli, albeit weakly [192]. We then compared these subclones to control GEC, which overexpress only podocin (GEC-P, [192]), to see if the expression of tyrosine-phosphorylated nephrin contributed to the phosphorylation of Pak, utilizing the phospho-specific Pak antibody. After being normalized to their expression levels, both Pak1 and Pak2 were more strongly phosphorylated in GEC-P/N than GEC-P (Fig. 4A). Rac1 is a known activator of Pak [111] and is known to be activated by nephrin [192]. When GEC-P/N were incubated with NSC23766 (200 μM) for 16 hrs, as in Fig. 4A, Rac1 activity was inhibited by 43±12 % (N=5), but the increased Pak activity in GEC-P/N was not affected (Fig. 4A). These results suggest that Rac1 may not be a major pathway of Pak phosphorylation via nephrin. However, since the Rac1 inhibition was only partial, further studies, such as Rac1 knockdown, are required to determine the role of Rac1 definitively.

Of interest, when the Y2F mutant of nephrin was expressed in the place of wild-type nephrin (GEC-Y2F), Pak1/2 phosphorylation was markedly reduced, as compared with GEC-P/N (Fig 4A). These results were confirmed with two additional clones each of GEC-P/N and GEC-Y2F, excluding the possibilities of clonal variations (not shown). In contrast, another nephrin mutant, Y1127F, did not have significant impact on Pak1/2 phosphorylation (Fig. 4A). These results suggest that tyrosine-phosphorylated nephrin contributes to Pak phosphorylation, likely via Nck.

In addition, antibody-mediated cross-linking of the extracellular domain of nephrin in mouse podocytes expressing nephrin (MP-nephrin) led to increased phosphorylation of Pak1/2 (Fig. 4B). Taken together, nephrin increases Pak phosphorylation both in rat GEC and mouse podocytes, likely in a tyrosine phosphorylation-dependent manner.

Pak phosphorylation induced by nephrin cross-linking is blocked by dominant-negative Nck mutants. Given that Fig. 4A argues for a role of Nck in nephrin-mediated Pak activation, we next addressed the role of Nck in Pak phosphorylation induced by nephrin. We co-transfected Pak1 and two dominant negative mutants of Nck, GFP-NckSH2 (containing only the SH2 domain of Nck, which fails to bind to any of the effectors) or GFP-NckW143R (with a mutation in the second SH3 domain, which fails to bind to Pak) [197, 198] into HEK-nephrin and induced nephrin tyrosine phosphorylation by antibody-mediated cross-linking. Cross-linking markedly increased Pak1 phosphorylation in the absence of the Nck dominant-negatives (Fig. 5), consistent with the previous results (Fig.4B). Pak1 phosphorylation was markedly blunted by GFP-NckSH2 and GFP-NckW143R (Fig. 5), supporting the idea that Nck is essential to Pak phosphorylation induced by nephrin.

Pak modulates F-actin assembly in podocytes. The Pak family of kinases is a known regulator of the actin cytoskeleton [111]. To test the role of Pak in the cytoskeletal regulation in podocytes, we first utilized transient transfection of Pak plasmids. Transient transfection of constitutively kinase active Pak1 (PakT423E)[133] into differentiated podocytes decreased transcellular stress

fibers and increased cortical F-actin (Fig. 6). In contrast, kinase-dead mutant (K299R)[199], which acts as a dominant-negative of Pak kinases, increased transcellular stress fibers and decreased cortical F-actin (Fig. 6). A kinase inhibitory construct (PID)[196] had a similar impact as the kinase-dead mutant (Fig. 6). To quantify the above changes, we measured the ratio of F-actin fluorescence intensities in the membrane (subcortical) and cytosol regions (Methods). The ratio was increased significantly in PakT423E-transfected cells, but was decreased in cells transfected with K299R or PID (Fig. 6). In addition, we observed that the processes in PakT423E-transfected cells were more prominent than in the control, because their length after leaving the cell body tended to be long, while those in DN/PID transfected cells tended to be short. These results suggest that Pak contributes to actin reorganization in podocytes, and that this effect is, at least in part, dependent on kinase activity.

Pak is required for normal morphology of differentiated podocytes. As shown above, the kinase-dead (dominant negative) mutant of Pak caused aberrant morphology of mouse podocytes. To further confirm the role of Pak in podocyte morphology and cytoskeleton, we generated subclones of mouse podocytes which stably expressed Pak2 shRNA. Of the three shRNAs tested, one significantly reduced the level of Pak2 mRNA by around 60% (Fig. 7A). The protein level was also reduced (Fig. 7A). Cells morphology was examined at 2 wk of differentiation: in control cells stably transfected with vector alone (also encoding GFP), Pak2 localized along the cellular processes (Fig. 7B), similar to untransfected cells (Fig. 2), whereas in Pak2-knockdown cells, Pak2 expression was markedly diminished and cells showed more intense trans-cellular stress fibers and shorter cell processes. The ratio of F-actin fluorescence intensities in the membrane (subcortical) and cytosol regions was decreased in Pak2-knockdown cells (Fig. 7C). Thus, the effects of Pak2 knockdown were similar to those of the DN/PID of Pak (Fig. 6), supporting the role of Pak in actin reorganization in podocytes.

Pak phosphorylation is decreased in rats with PAN. We previously reported that in the rat model of puromycin aminonucleoside nephrosis (PAN), in which podocyte injury leads to foot process effacement and heavy proteinuria [182],

tyrosine phosphorylation of nephrin is decreased, accompanied by the dissociation of nephrin and its molecular partners such as Nck and the regulatory unit of PI3-K [192, 194]. Since nephrin-mediated Pak phosphorylation appears to be dependent on Nck, at least in part, we next tested if Pak phosphorylation is altered in rats with PAN. After a single intravenous injection of puromycin aminunucleoside, heavy proteinuria was observed on Day 7 (control, 9±3 mg/d; PAN, 299±45 mg/d; $p < 0.01$, N = 8 rats each). On Day 14, proteinuria started to decline (160±47 mg/d; $p < 0.05$ vs Day 7, N = 8 rats each). In the lysates of normal rat glomeruli, Pak1/2 were clearly phosphorylated, albeit weakly, suggesting that Pak1/2 are weakly active in normal glomeruli. Pak1/2 phosphorylation was decreased on Days 4 and 7 and returned to normal on Day14, while the expression level of Pak1/2 was unchanged (Fig. 8). The timing of the decrease in Pak1/2 phosphorylation corresponded to decreased tyrosine phosphorylation of nephrin (Fig. 8).

3.5. DISCUSSION

Nephrin is a slit diaphragm protein which is critical to the structural and functional integrity of podocytes. It was previously shown that tyrosine phosphorylated nephrin recruits the Nck adaptor proteins [40, 44, 194], and that myristylated Nck-SH3-II domain (which binds to Pak) can activate Pak1 in the absence of GTP-Rac and Cdc42, while unmyristylated Nck-SH3-II or myristylated but Pak1-binding-defective Nck-SH3-II did not activate Pak1 [200]. By analogy, it is likely that tyrosine phosphorylated nephrin recruits Pak to the plasma membrane via Nck, leading to its activation. In rat, we showed that Y1204 and Y1228 are responsible for Nck binding [194], and that these two residues are therefore likely to contribute to Pak recruitment. In support of this, the Y2F mutant of nephrin failed to recruit (Fig. 3A, B) or to increase phosphorylation of Pak (Fig. 4A). Although we confirmed that Y1127 did not contribute to Pak phosphorylation (Fig. 4A), the role of other tyrosine residues in Pak activation is yet to be investigated. We and others previously reported that nephrin is tyrosine-phosphorylated under normal conditions [44, 194]. This finding was recently confirmed using phospho-specific antibodies of nephrin, which recognize the Nck binding sites of nephrin (Y1204 and Y1228 in rat nephrin) [201, 202]. It is therefore likely that signal transduction via nephrin-Nck-Pak is relevant in normal podocyte morphology and function.

Regulation of Pak1 is complex and multiple mediators have been shown to activate the protein. The serine/threonine kinase PDK1 was shown to phosphorylate human Pak1 at Thr423, which increases Pak1 activity [121]. We previously reported that nephrin recruits PI3-kinase upon tyrosine phosphorylation [192], which would likely increase the local concentration of PI(3,4,5)P3 (product of PI3-kinase), leading to the recruitment of the PH domain of PDK1 and its activation [63]. On this basis, one possible scenario is that Pak is recruited to nephrin via Nck, bringing Pak to the proximity to active PDK1, which phosphorylates and activates Pak. Alternatively, Pak may be activated by lipids such as sphingosine in a GTPase independent manner, as reported previously [117, 203].

Transfection of active Pak in podocytes resulted in an increase in subcortical F-actin and decrease in cytosolic stress fibers, accompanied by longer cellular processes, whereas the inhibition/knockdown of Pak had the opposite effects (Fig. 6, 7). The significance of these changes in cultured podocytes in the context of podocyte physiology and pathology in vivo can only be speculated. In the neurite growth cone, increased F-actin assembly at the tip has been found to be important for neurite growth [204]. Similarly, F-actin assembly in the subcortical areas of podocytes might be important for process extension. In fact, cells in which Pak activity was reduced generally showed shorter cellular processes (Fig. 6, 7).

Pak1 and Pak2 share 91% sequence identity within their kinase domains, suggesting their redundant functions [205]. Indeed, both Pak1 and Pak2 are expressed in many tissue types and phosphorylate a number of common substrates, including Bad, Raf, Mek and Merlin [205]. In some instances, however, Pak1 and Pak2 may have differential roles. For example, knockdown of Pak1 in tumor cells increased cofilin phosphorylation, whereas that of Pak2 increased myosin light chain phosphorylation [140]. Also, Pak2, but not Pak1, is essential for the viability of mice [135]. We acknowledge that overexpression of kinase-dead or other mutant Pak proteins might override the isoform specificities; nonetheless, the effects of Pak1 DN/PID were similar to those of Pak2 knockdown (Fig. 6, 7), suggesting that the two isoforms have similar effects on actin assembly in podocytes. Interestingly, increased cofilin phosphorylation and myosin light chain phosphorylation, caused by knockdown of Pak1 and Pak2, respectively [140], both contributed to the increase in stress fibers observed in the current study when Pak expression/activity was inhibited (Fig. 6, 7). It is therefore possible that Pak1 and Pak2 act in concert in podocytes. The differential roles of Pak1 and Pak2 in podocytes require further investigations.

The present study did not address the mechanisms by which Pak modulates the actin cytoskeleton in podocytes. It was previously reported that Pak1 is essential to lamellipodial stability [206]. Since the lamellipodium is a key structural feature of the motile tips of growing processes [207, 208], it is possible that Pak promotes

process extension via the stabilization of lamellipodia. LIM-kinase is a major substrate of Pak and is known to regulate the activity of the actin severing protein cofilin. Although complex, the regulation of LIM-kinase/cofilin activities is mediated by Pak and other signaling molecules [111]. Of particular interest, it was shown that both excessive or insufficient cofilin activity disturbs neurite extension in PC12 cells, suggesting that the tight regulation of cofilin activity is critical for neurite extension [204]. In the current study, we found that GEC expressing nephrin (GEC-P/N) has higher Pak activity than control cells (GEC-P) (Fig. 4). We previously reported that GEC-P/N have greater cofilin activity (lower cofilin phosphorylation) than GEC-P [192]. It is likely that increased cofilin activity in GEC-P/N promotes actin remodeling, facilitating process extension.

In the rat model of PAN, the decrease in Pak phosphorylation (Fig. 8) coincided with the decrease in nephrin tyrosine phosphorylation. Although the exact location where the change of Pak phosphorylation occurred was not determined, since podocyte is the major site of cell injury in PAN [182], it is reasonable to assume that the changes occurred mainly in podocytes. It is therefore likely that the dissociation of Nck from nephrin secondary to nephrin de-phosphorylation leads to the dissociation of Pak from nephrin and dephosphorylation of Pak. Decreased phosphorylation of Pak and nephrin were obvious on Day 4 before the proteinuria started, continued on Day 7 when the proteinuria reached the peak, and started to resolve on Day 14. Given this time course, it is tempting to speculate that decreased Pak phosphorylation /activity in podocytes may contribute to foot process effacement and proteinuria in PAN and that Pak phosphorylation/activity starts to increase as the podocytes start to recover from the injury. Since Pak1 knockout mice have no obvious renal phenotype and Pak2 knockout mice are embryonic lethal [135], a more sophisticated approach, such as podocyte-specific gene targeting of Pak2 combined with Pak1 knockout, will be needed to further define the role of Pak in podocyte morphology and function in vivo.

3.6. ACKNOWLEDGEMENTS

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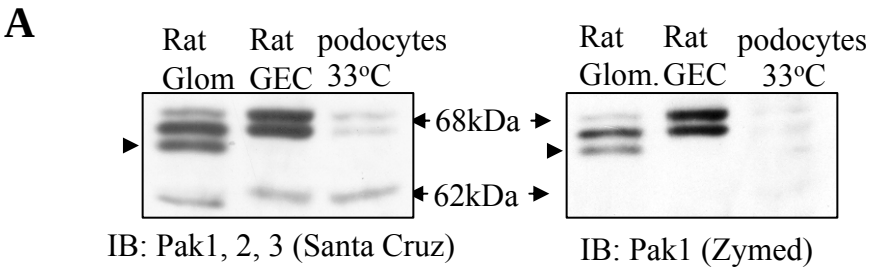
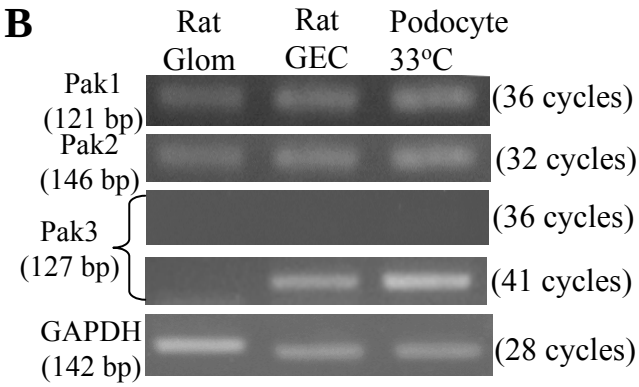
3.7. Figures

Figure 1. Pak isoforms are expressed in glomerular podocytes. A. Lysates of normal rat glomeruli, cultured rat glomerular epithelial cells (GEC) and cultured mouse podocytes (33^{o}C-undifferentiated) were analyzed by immunoblotting using antibodies from Santa Cruz (recognizing Pak 1, 2, 3, left) and Zymed (specific to Pak1, right). Doublets ~68 kDa are Pak1 and the band ~62 kDa is Pak2. The extra band in rat glomeruli (arrowhead) may be a degradation product of Pak1 or may reflect different phosphorylation status of Pak1 in vivo. Note that the Pak1 bands are barely visible in mouse podocytes in the right panel because of the short exposure time. B. RT-PCR. Conventional RT-PCR was performed. Pak1 and 2 mRNA were clearly detected after 36 and 32 cycles, respectively. Pak3 mRNA was not detected at 36 cycles but was visible at 41 cycles. C. Immuno-histochemistry of Pak in the rat kidney. Pak antibody used was the same as in Fig. 1A-left (Santa Cruz). Positive staining was observed both in the tubules (arrowhead) and glomerulus (arrows). Magnification: left and middle (60x), right (100x). IB: immunoblot.

Figure 1


A
Rat Glom
Rat GEC
podocytes 33°C
68kDa
62kDa
IB: Pak1, 2, 3 (Santa Cruz)
Rat Glom.
Rat GEC
podocytes 33°C
IB: Pak1 (Zymed)


B
Rat Glom
Rat GEC
Podocyte 33°C
Pak1 (121 bp)
(36 cycles)
Pak2 (146 bp)
(32 cycles)
Pak3 (127 bp)
(36 cycles)
(41 cycles)
GAPDH (142 bp)
(28 cycles)

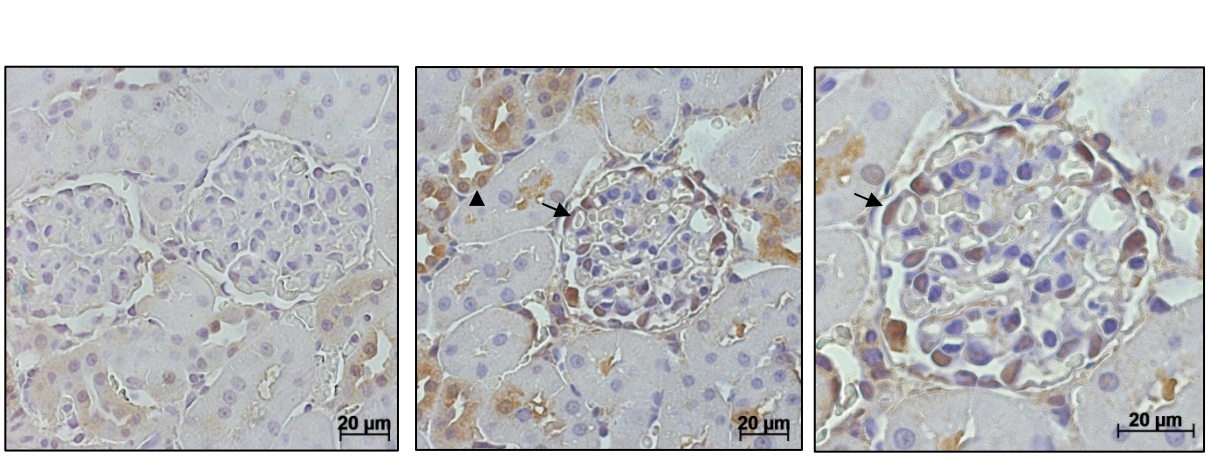

C
20 µm
20 µm
20 µm

Figure 2. Expression and phosphorylation of Pak2 is increased with differentiation in mouse podocytes. A. Lysates of mouse podocytes (undifferentiated or differentiated for 1 or 2 wk) were analyzed by immunoblotting (Santa Cruz, Pak 1, 2, 3 antibody). Pak1/2 protein expression was quantified by densitometry and normalized to α-tubulin and undifferentiated. * $p<0.01$ vs undifferentiated; **$p<0.05$ vs undifferentiated. N=5. B. Mouse podocytes lysates (undifferentiated or differentiated for 1 or 2 wk) were blotted with phospho-specific Pak antibody (recognizing Ser 199/204 of Pak1 and Ser 192/197 of Pak2) and Pak1,2,3 antibody (Santa Cruz). Differentiated cells showed increased phosphorylation of Pak2. Phosphorylated Pak tended to run at an apparently higher molecular weight than the total Pak. p-Pak1/2 was quantified by densitometry and normalized to Pak1/2 expression and to undifferentiated. + $p<0.05$ vs undifferentiated. N=5. C. Immunofluorescence staining of Pak2 in cultured mouse podocytes (undifferentiated or differentiated for 2 wks). Pak2 staining is concentrated at the tips of cellular processes and on the edges of membrane protrusion (arrows). Magnification: undifferentiated (100x), differentiated (60x).

Figure 2

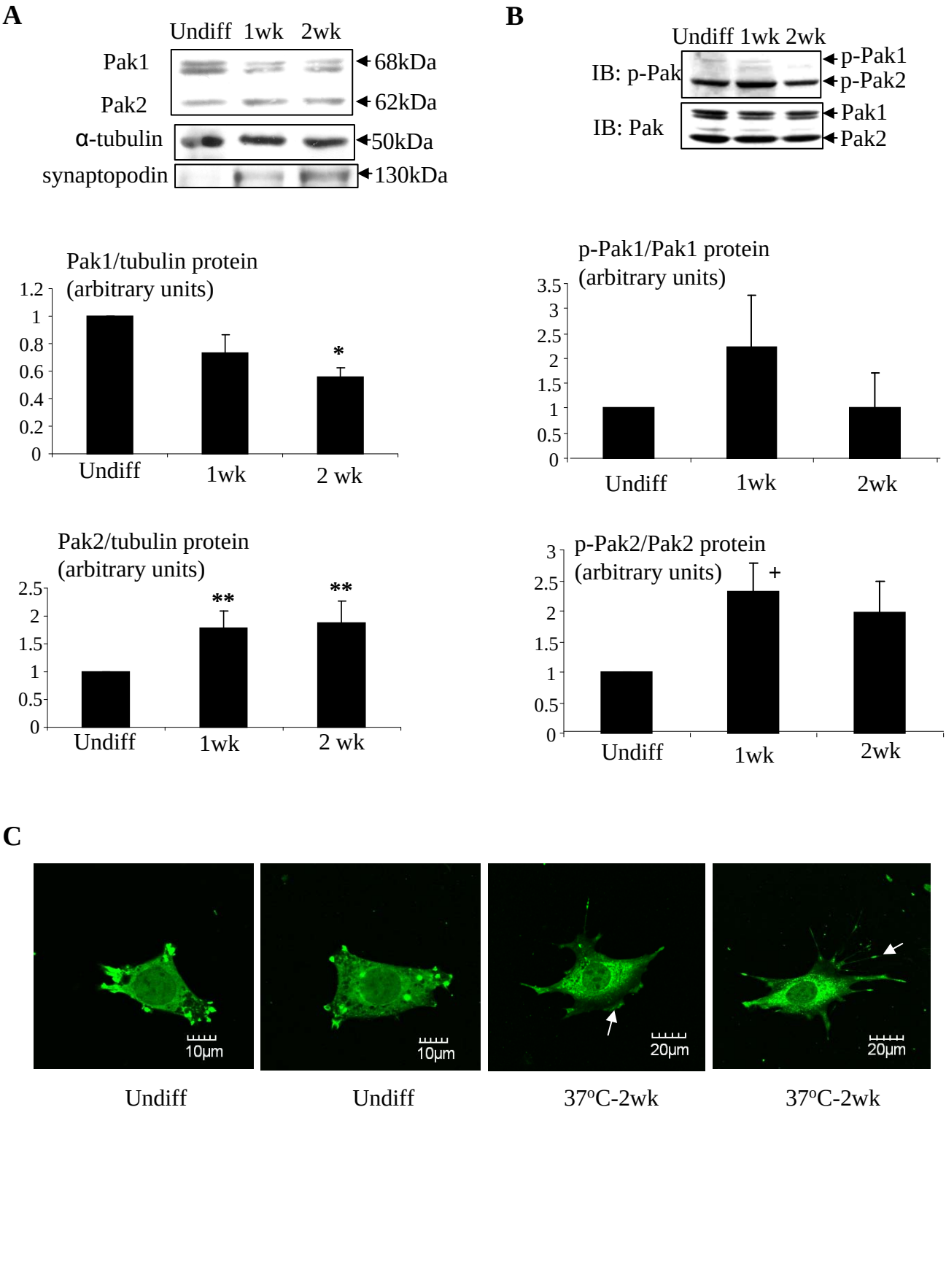
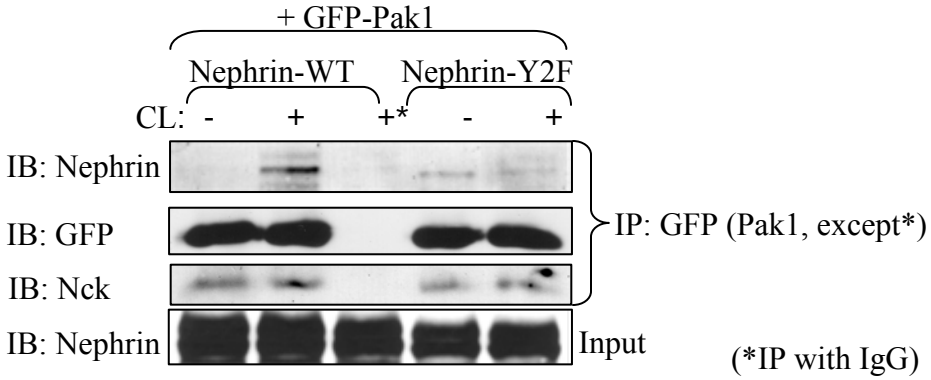

A
Undiff 1wk 2wk
Pak1
Pak2
α-tubulin
synaptopodin
68kDa
62kDa
50kDa
130kDa
B
Undiff 1wk 2wk
IB: p-Pak
IB: Pak
p-Pak1
p-Pak2
Pak1
Pak2
Pak1/tubulin protein
(arbitrary units)
Undiff
1wk
2 wk
p-Pak1/Pak1 protein
(arbitrary units)
Undiff
1wk
2wk
Pak2/tubulin protein
(arbitrary units)
Undiff
1wk
2 wk
p-Pak2/Pak2 protein
(arbitrary units)
Undiff
1wk
2wk
C
10μm
10μm
20μm
20μm
Undiff
Undiff
37ºC-2wk
37ºC-2wk

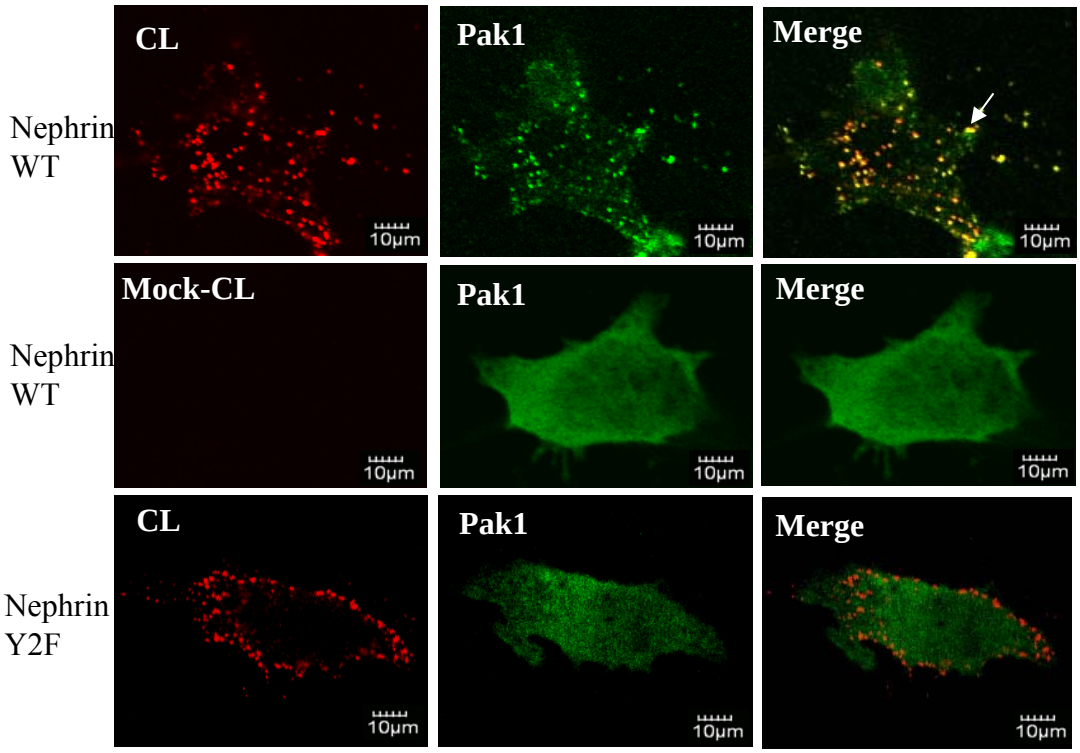
Figure 3. **Nephrin recruits Pak in an Nck and tyrosine phosphorylation-dependent manner**. A. HEK-293T cells were transfected with GFP-Pak1 and wild type nephrin or nephrin mutant (Y2F: Y1204/1228F, which fails to bind to Nck). After nephrin was cross-linked with mAb5-1-6 and secondary antibody, cells were lysed and subjected to immunoprecipitation and immunoblotting. Wild type nephrin precipitated with Pak1 after cross-linking (CL), while nephrin Y2F mutant did not. B. Top: HEK-nephrin cells were transfected with Myc-Pak1 and nephrin was cross-linked with mAb5-1-6 and rhodamine-anti-mouse IgG (CL: red). Pak1 was stained with FITC-anti-Myc. Pak1 aggregated at the sites of nephrin CL (arrow). Middle: Same cells were mock cross-linked. Pak1 was diffusely distributed in the cell. Bottom: HEK293T cells were transfected with Myc-Pak1 and nephrin mutant Y1204/1228F and nephrin was cross-linked as above. Pak1 was diffusely distributed in the cells. Magnification (100x). C. Normal rat glomerular lysates were immunoprecipitated with anti-Nck antibody or normal rabbit IgG, and nephrin and Pak were detected with antibodies for nephrin and Pak1,2,3 (Santa Cruz). IP: immunoprecipitation.

Figure 3

A


+ GFP-Pak1
Nephrin-WT
Nephrin-Y2F
CL: - + +* - +
IB: Nephrin
IB: GFP
IB: Nck
IB: Nephrin
IP: GFP (Pak1, except*)
Input
(*IP with IgG)

B


Nephrin WT
CL
Pak1
Merge
Nephrin WT
Mock-CL
Pak1
Merge
Nephrin Y2F
CL
Pak1
Merge
10μm

C

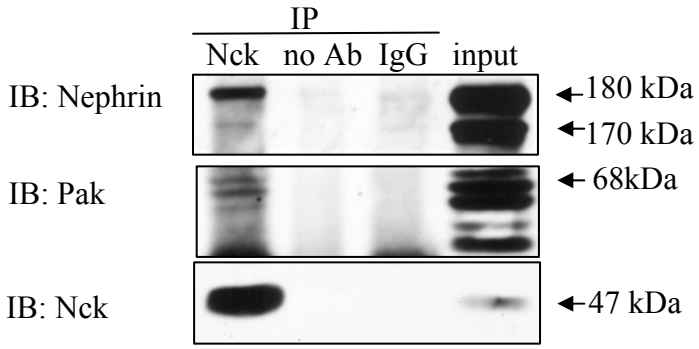

IP
Nck
no Ab
IgG
input
IB: Nephrin
IB: Pak
IB: Nck
180 kDa
170 kDa
68kDa
47 kDa

Figure 4. **Nephrin increases Pak phosphorylation.** A. GEC were stably transfected with podocin alone (GEC-P), podocin and wild type rat nephrin (GEC-P/N), podocin and nephrin mutant Y1127F (GEC-Y1127F), or podocin and nephrin mutant Y1204/1228F (GEC-Y2F). After 16 hrs of serum starvation, cells were treated or not treated with NSC23766 (Rac1 inhibitor, 200 μM) for 16 hrs, and then lysed and subjected to IB as in Fig. 2B. Densitometric analysis is shown at the bottom. Values were normalized to the protein expression and to GEC-P/N. *$p<0.01$ vs GEC-P/N, N=3. Pak1/2 phosphorylation was increased in GEC-P/N, as compared with GEC-P or GEC-Y2F. p-Pak in GEC-P/N was not affected by the Rac1 inhibitor. B. Mouse podocytes were stably transfected with rat nephrin (MP-N) and subjected to antibody-mediated cross-linking. Cells were lysed and subjected to IB as in Fig 2B. Pak2 phosphorylation was markedly increased after cross-linking, peaking at 2 min. Pak1 phosphorylation was also increased, albeit weakly. Densitometric analysis is shown at the bottom. Values were normalized to the protein expression and to control (0 min). +$p<0.05$ vs 0 min, N=3.

Figure 4

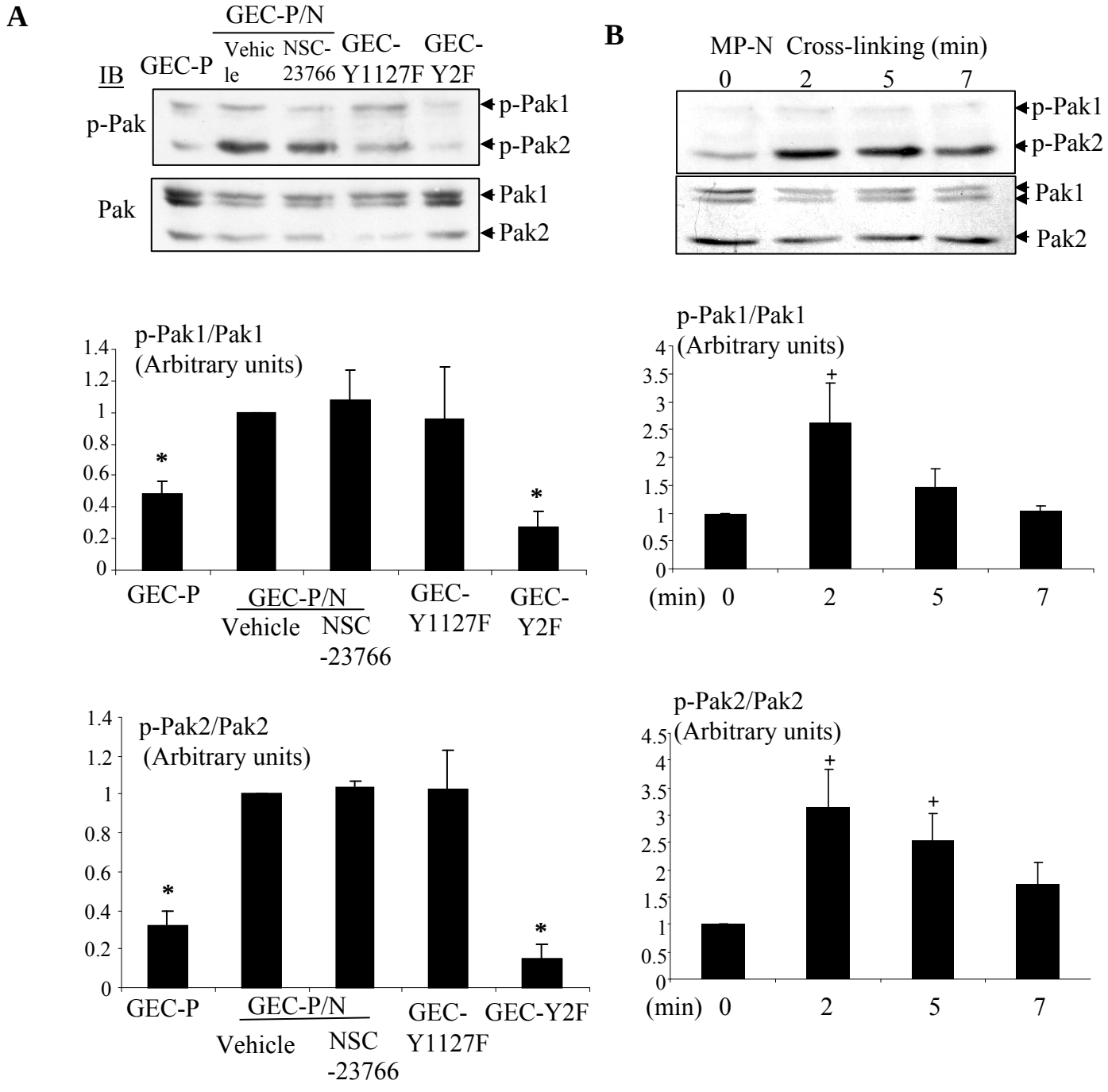

A
GEC-P/N
IB
GEC-P
Vehicle
NSC-23766
GEC-Y1127F
GEC-Y2F
p-Pak
p-Pak1
p-Pak2
Pak
Pak1
Pak2
B
MP-N
Cross-linking (min)
0
2
5
7
p-Pak1/Pak1 (Arbitrary units)
p-Pak2/Pak2 (Arbitrary units)
(min)
GEC-P/N
Vehicle
NSC-23766
GEC-Y1127F
GEC-Y2F

Figure 5. **Pak phosphorylation induced by nephrin cross-linking is blocked by dominant-negative Nck mutants.** HEK-nephrin cells were co-transfected with myc-Pak1 and GFP alone, GFP-NckSH2 (which fails to bind to any of the effectors) or GFP-NckW143R (which fails to bind to Pak). After cross-linking cells were lysed and subjected to IB as in Fig. 2B. Pak1 phosphorylation was increased on cross-linking of nephrin, however, this effect was abolished by GFP-NckSH2 and GFP-NckW143R. C: control (mock cross-linking). Note that the molecular size of Pak is ~70 kDa because of the myc-tag.

Figure 5

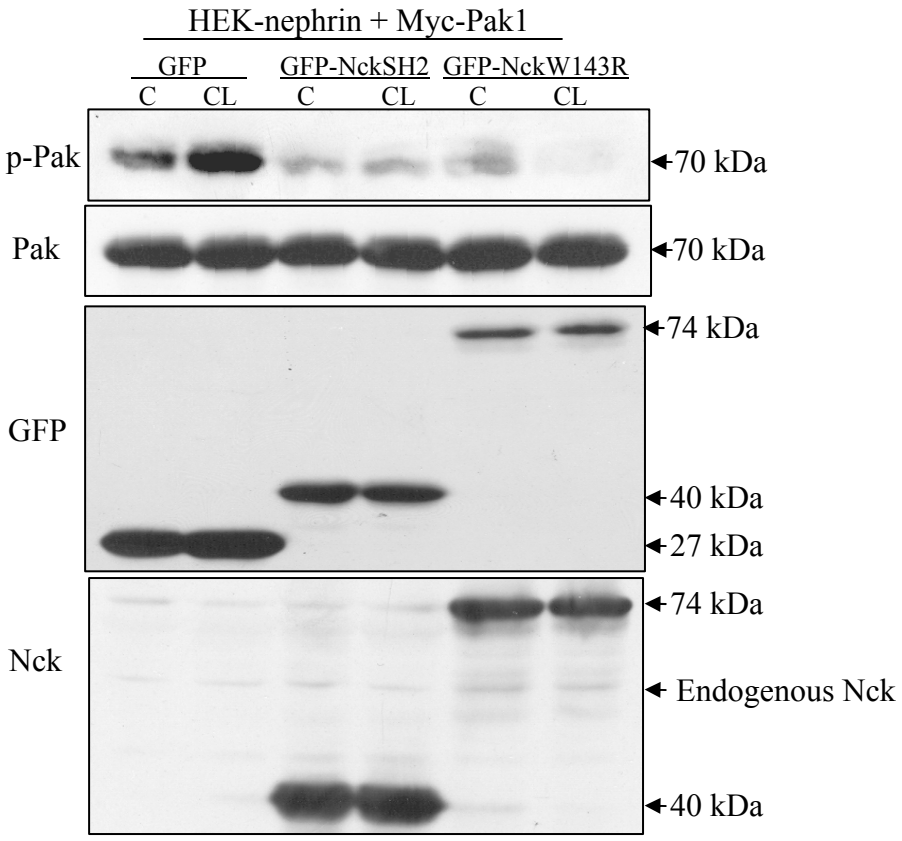
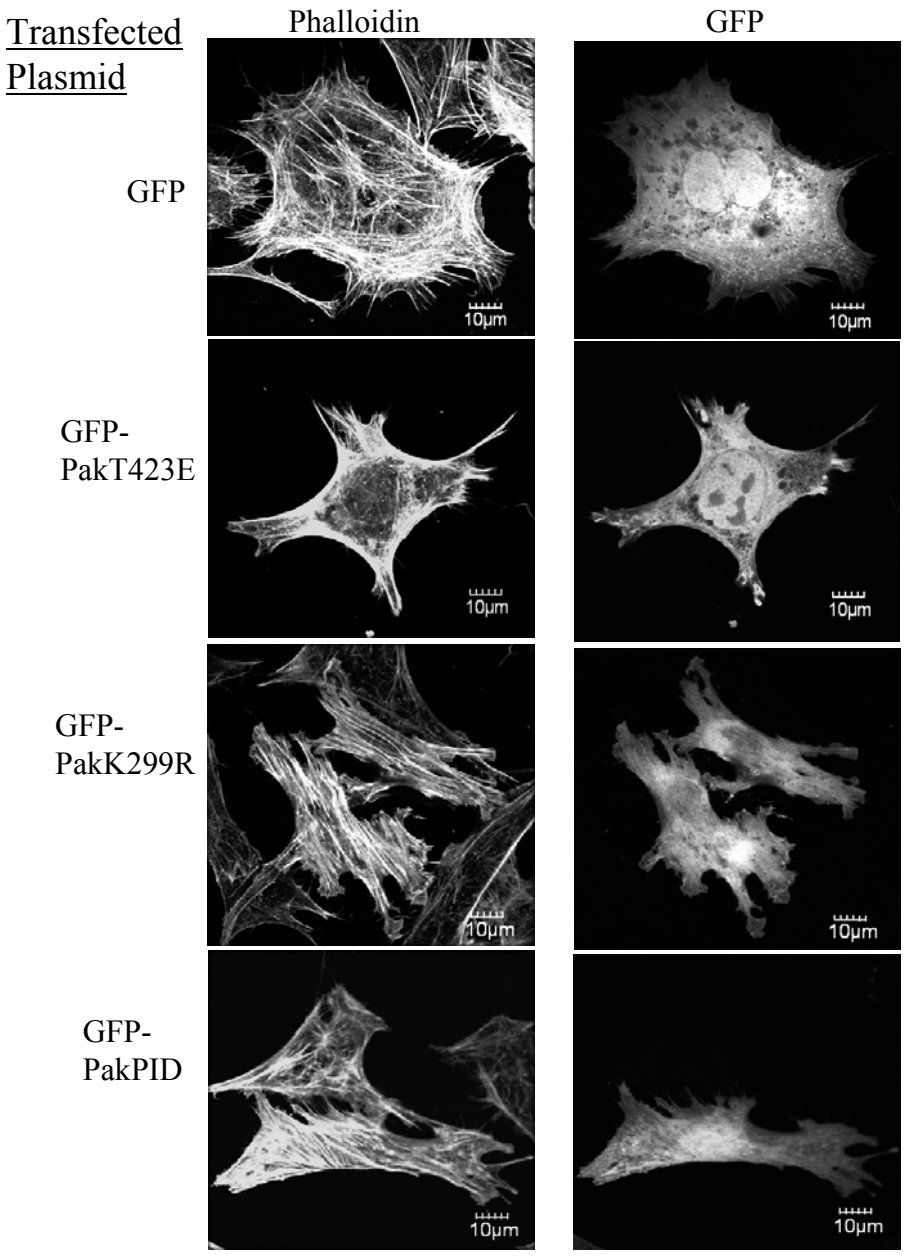

HEK-nephrin + Myc-Pak1
GFP
GFP-NckSH2
GFP-NckW143R
C
CL
C
CL
C
CL
p-Pak
70 kDa
Pak
70 kDa
74 kDa
GFP
40 kDa
27 kDa
74 kDa
Nck
Endogenous Nck
40 kDa

Figure 6. **Pak modulates F-actin assembly in podocytes**. Differentiated mouse podocytes were transfected with GFP alone, GFP-PakT423E (constitutively active), or GFP-PakK299R (kinase dead, dominant negative). After 24 hrs, cells were fixed and stained with TRITC-phalloidin. GFP-PakT423E transfected cells showed decreased stress fibers, increased cortical F-actin and elongated cellular processes. GFP-PakK299R transfected cells showed increased stress fibers and decreased cortical F-actin. GFP-PakPID (kinase inhibitory construct) showed similar effects as GFP-PakK299R. Magnification (100x). F-actin fluorescence intensity of the membrane (subcortical) region and the cytosol were quantified as described in "Methods". The data represent average ± S.E. from 20-21 cells. * $p < 0.05$ compared with GFP transfected cells.

Figure 6


Transfected Plasmid
Phalloidin
GFP
GFP
GFP-PakT423E
GFP-PakK299R
GFP-PakPID
10μm

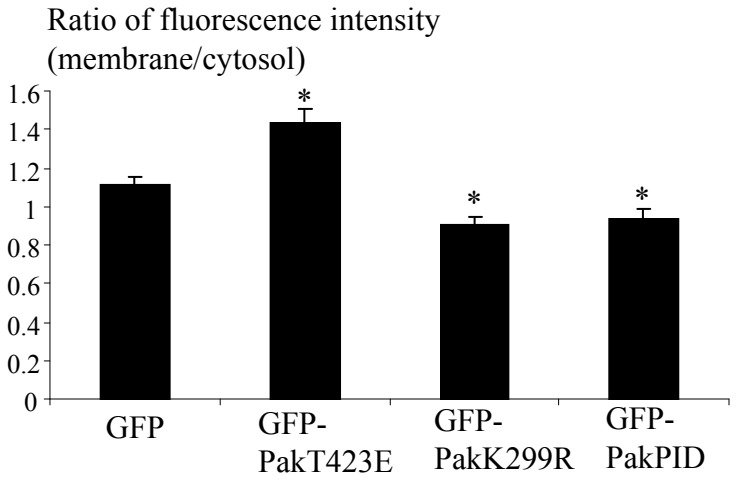

Ratio of fluorescence intensity (membrane/cytosol)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
GFP
GFP-PakT423E
GFP-PakK299R
GFP-PakPID

Figure 7. Pak2 is required for normal morphology of differentiated mouse podocytes. A. MP-nephrin were stably transfected with pRNATin-H1.2/Neo (empty vector with GFP) or a Pak2 shRNA cloned into pRNATin-H1.2/Neo, which demonstrated significant reduction of Pak2 mRNA (Top-left, qPCR, *p<0.05 **p<0.01 vs MP, N=3) and protein (Top-right, IB). C-2 and C-3 represent two independent clones expressing Pak2shRNA-C. B. Both vector expressing and shRNA expressing cells were differentiated for 2 wk, fixed and stained with anti-Pak2 antibody (TRITC) and phalloidin (far-red, all colors are demonstrated in white). shRNA-expressing cells show markedly reduced Pak2 staining, more intense stress fibers and shorter processes, as compared with vector-expressing cells. Magnification(100x). C. Quantitative analysis of actin assembly in the membrane (subcortical region) and cytosol as described in Fig. 6. The data represent average ± S.E. from 20-22 different cells. * $p < 0.05$ compared with GFP transfected cells.

Figure 7

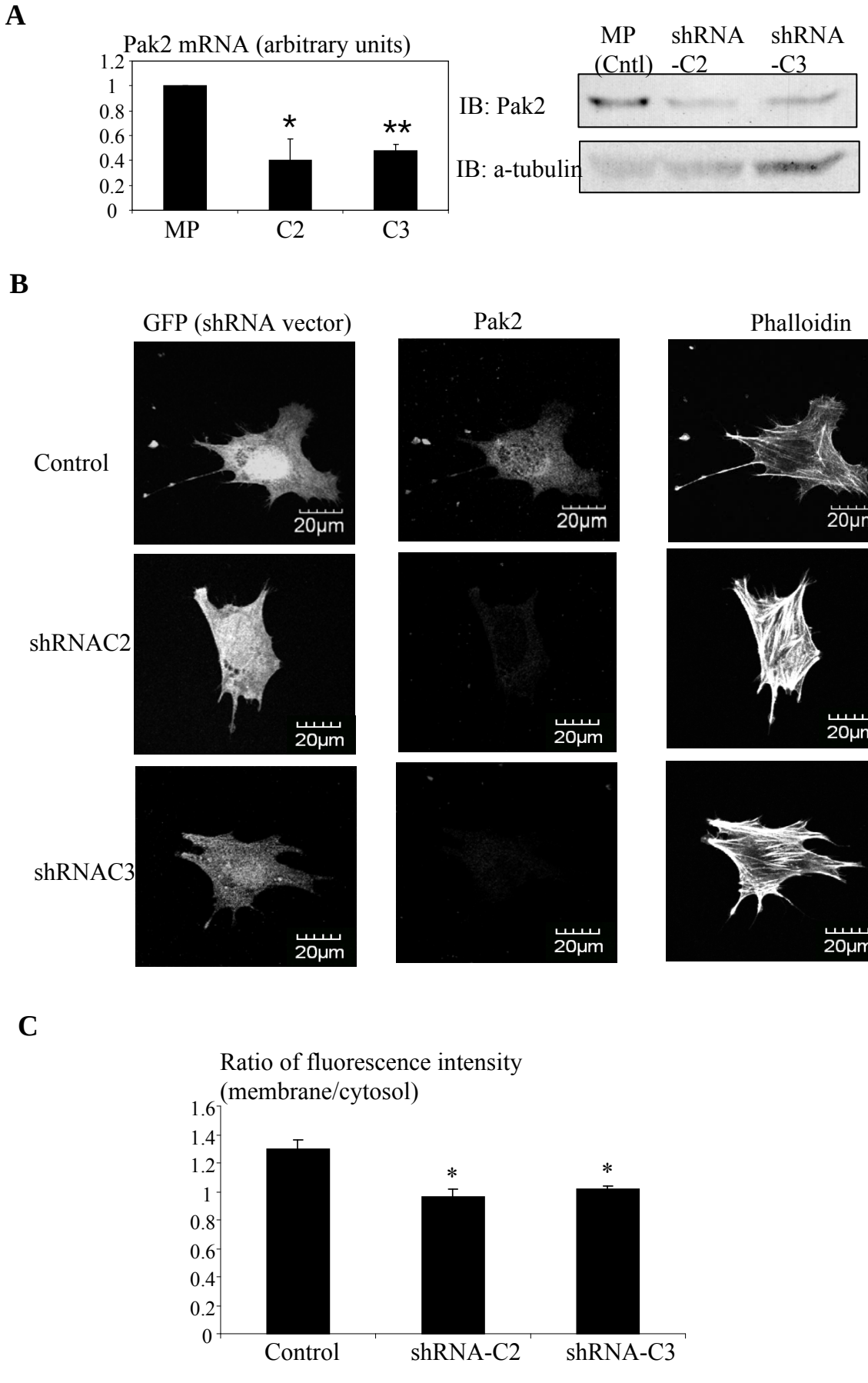
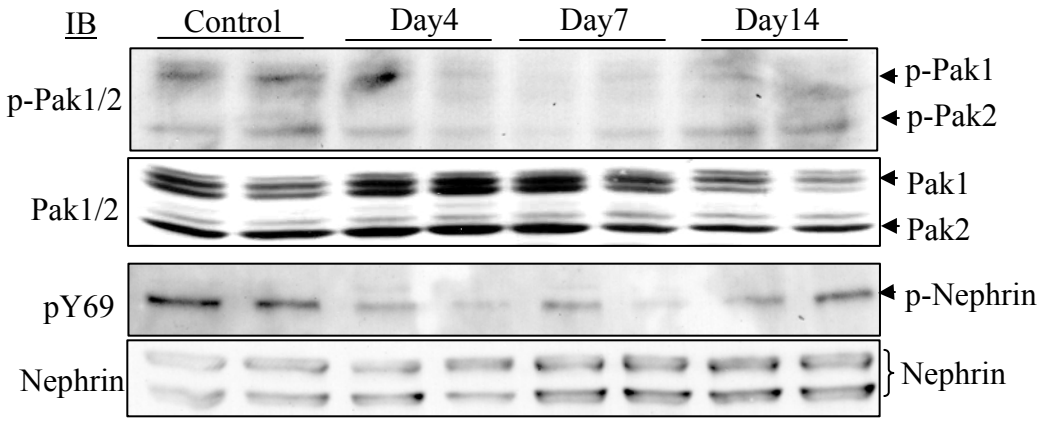
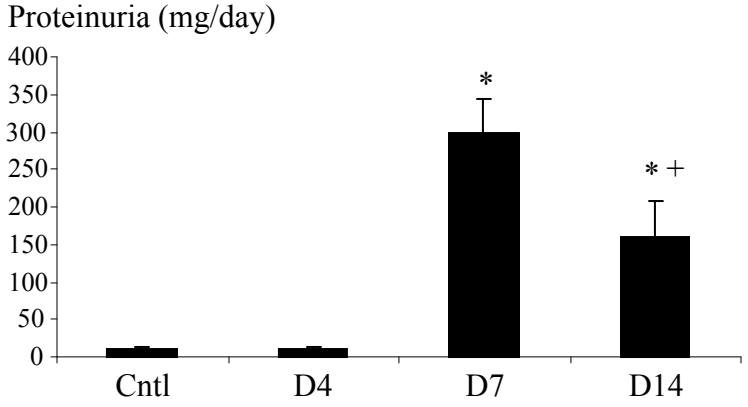

A
Pak2 mRNA (arbitrary units)
1.2
1
0.8
0.6
0.4
0.2
0
*
**
MP
C2
C3
MP (Cntl)
shRNA -C2
shRNA -C3
IB: Pak2
IB: a-tubulin
B
GFP (shRNA vector)
Pak2
Phalloidin
Control
shRNAC2
shRNAC3
20μm
C
Ratio of fluorescence intensity (membrane/cytosol)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Control
shRNA-C2
shRNA-C3

Figure 8. **Pak phosphorylation is altered in PAN.** PAN was induced by a single injection of puromycin aminonucleoside. Glomeruli were isolated on Day 4, 7, and 14 and lysates were subjected to immunoblotting for phospho-Pak1/2, Pak 1,2,3 or were immunoprecipitated by nephrin antibody and blotted for pY69 and nephrin. Note that nephrin runs as doublets likely because of differential glycosylation and pY-nephrin corresponds to the upper band [37]. Heavy proteinuria was observed on Day 7, but started to decline on Day 14. Pak 1/2 phosphorylation was decreased on Day 4 and Day 7, coinciding with a decrease in nephrin tyrosine phosphorylation but returned to normal on Day 14. *$p < 0.01$ vs control, +$p < 0.05$ vs D7, N=8.

Figure 8


IB
Control
Day4
Day7
Day14
p-Pak1/2
p-Pak1
p-Pak2
Pak1/2
Pak1
Pak2
pY69
p-Nephrin
Nephrin
Nephrin


Proteinuria (mg/day)
400
350
300
250
200
150
100
50
0
*
* +
Cntl
D4
D7
D14

General Discussion:

1. Y1152 of rat nephrin is responsible for PI3K-p85 association.

Accumulating evidence suggests that nephrin not only serves the structural function of podocytes, but also participates in common signaling pathways to maintain the integrity of podocytes [39]. Activation of nephrin is mediated by the phosphorylation of its several intracellular tyrosine residues [38], six of which are conserved among human, mouse and rat [37]. It has been reported that tyrosine phosphorylated nephrin associates with the p85 regulatory subunit of PI3K, leading to the Akt activation and increased cell survival [27]. However, the precise mapping of phosphotyrosine-containing motifs in nephrin responsible for its interaction with PI3K has not been reported.

It has been identified that SH2 domain mapped within the p85 subunit specifically recognizes the phosphotyrosine motif p*YXXM* [56]. Therefore, tyrosine residue Y1152 of rat nephrin Y^{1152}YSM motif is a likely binding site for p85. In Chapter 2, we demonstrated that, in contrast to wild-type nephrin and its other mutants, rat nephrin mutant Y1152F failed to coimmunoprecipitate with PI3K-p85. The result indicated that tyrosine phosphorylation of Y1152 in rat nephrin is critical to the nephrin-p85 interaction. Y1152 of rat nephrin is a binding site for PI3K-p85.

2. Nephrin mediates actin reorganization via PI3K in podocytes.

The actin cytoskeleton in podocytes have emerged as key contributors to the podocytes' function. Disorganization of actin cytoskeleton in podocytes lead to foot process effacement and proteinuria [4]. PI3K regulates a variety of cellular programs, e.g. cell survival, actin cytoskeletal dynamics, and metabolism [209]. However, the role of PI3K in regulating actin cytoskeleton in podocytes has not been addressed.

PI3K activation leads to production of phospholipid PIP3 (phosphatidylinositol-3,4,5-triphosphate) and PIP2(phosphatidylinositol-3,4-biphosphate), which recruit PH domain containing proteins, such as Akt and Rac GEF (Tiam1, Vav [209]), leading to activation of Akt and Rac1. These two molecules have been reported to regulate actin assembly in cell migration [209, 210]. In Chapter 2, we found that

Akt and Rac1 were activated via PI3K by nephrin tyrosine phosphorylation. Rac1 and Akt contributed to lamellipodia formation and decreased stress fibers, respectively. GEC-P/N showed decreased stress fibers and increased membrane ruffles, corresponding to higher Rac1 and Akt activities. In contrast, PI3K inhibitor treatment and GEC-Y1152F showed increased stress fibers and membrane ruffles, corresponding to lower Rac1 and Akt activities. Of interest, the dominant negative Akt mutant could not increase stress fiber in GEC-P/N as we expected. One possibility for this could be the isoform-specific role of Akt on actin cytoskeleton and that the DN-Akt mutant used in this study blocked both Akt1 and Akt2 by sequestering upstream activators. Alternatively, there could be Akt independent signaling pathways for these actin cytoskeleton changes in podocytes.

In Chaper 2, we also found that there is higher cofilin activity in GEC-P/N compared to GEC-P and GEC-Y1152F, which might account for the decreased stress fiber and increased lamellipodia in GEC-P/N. It has been reported that cofilin activity was regulated by PI3K via the activation of slingshot [167]. However, the mechanism of activation of slingshot by PI3K has not been elucidated. We found that Akt can activate cofilin. Slingshot has the Akt consensus phosphorylation sequence [165]; therefore, we proposed that Akt might regulate slingshot activity, which in turn can activate cofilin. How nephrin mediated signaling and PI3K-Akt pathway regulate cofilin activity in podocyte requires further investigation.

3. Nephrin mediates actin reorganization via Nck-Pak in podocytes.

It has been reported that tyrosine phosphorylation of nephrin recruits the adaptor protein Nck [44]. Several of the phosphorylated sites on nephrin (Human Y1176, Y1193, and Y1217, and Rat Y1204, Y1228) contribute to the binding of Nck [44, 180]. Conditional deletion of Nck2 in podocytes of Nck1-null mice presented with foot process effacement and proteinuria, similar to that obeserved in mice deficient in nephrin. This indicates that Nck represents a mediator between neprhin and the actin cytoskeleton in podocytes and is required for the

formation of foot process and the maintainence of proper podocyte morphology [44, 201].

The Pak family of kinases have been reported to associate with the second SH3 domain of Nck via their N-terminal PXXP motifs and be recruited to receptor tyrosine kinases via Nck [104, 105]. However, the role of Nck-Pak on actin assembly in podocytes has not been addressed. In Chapter 3, we demonstrated that Pak associates with nephrin via Nck in vitro and in vivo. Activation of nephrin by overexpression or antibody-mediated crosslinking in podocytes increased Pak phosphorylation, and Nck is indispensable to these processes. Inhibition of Rac1 activity by the Rac1 inhibitor could not block the nephrin-induced Pak phosphorylation, which suggests that Rac1 is not a major pathway of Pak phosphorylation via nephrin.

Pak1 and Pak2 belong to Group I Pak subfamily, which share the highly conserved N-terminal regulatory domain and C-terminal catalytic kinase domain [132]. It has been shown that Pak1 and Pak2 can activate LIMK leading to cofilin phosphorylation and inactivation [137, 211]. Interestingly, knockdown of Pak1 in tumor cells increased cofilin phosphorylation, in contrast, knockdown of Pak2 had no significant effect [140], suggesting that the regulation of cofilin activity by Pak is complex. In Chapter 3, we found that Pak1/2 activity contributed to decreased transcellular stress fiber, increased cortical F-actin, and extension of cell processes in podocytes. It has been reported previously that active cofilin depolymerizes F-actin and is required for lamellipodia extension [212]. Therefore, the actin cytoskeleton reorganization by Pak corresponds to the high cofilin activity. These data are consistent with the result of Chapter 2, where we showed that GEC-P/N have a higher cofilin activity than GEC-P. However, how Pak1/2 regulates cofilin phosphorylation spatio-temporally in podocytes need further investigation.

4. Cross-talk between PI3K-Akt and Pak signaling

It has been previously reported that Akt is activated downstream of Rac1 [210]. However, the mechanism by which Rac activates Akt has been unclear. Recent data has suggested that Pak is necessary for Akt phosphorylation by Rac1 [213]. It

was demonstrated that growth factor stimulation promotes the association of the C-terminal catalytic domain of Pak1 with both PDK1 and Akt, which facilitates translocation of Akt to the membrane fraction and PDK1-mediated phosphorylation of Akt. Interestingly, knockdown of Akt1, not Akt2, blocked the PDGF-induced, RacV12-induced and Pak mediated motility. In addition, Pak1 promoted Akt1 phosphorylation more efficiently than Akt2, which suggests that Pak1 may selectively associate with and activate Akt1, and facilitate Akt1-specific function [213]. Furthermore, it has been reported that PDK1 [121] and Akt1[122] phosphorylate Pak1 at Thr423 and Ser 21, respectively, which increase Pak1 activity. Therefore, there may be positive-feedback among PDK1, Pak and Akt1. In this thesis, we did not demonstrate the crosstalk among them. However, we found that activation of nephrin induced the activation of Akt and Pak, which led to similar actin reorganization in podocytes. These data suggest that there might be a crosstalk between them. Whether the activation of Akt and Pak by nephrin occur independently or synergistically, and how Akt and Pak are activated spatio-temporally in podocytes need further investigation.

5. Nephrin mediates PI3K-Akt and Nck-Pak signalling in vivo.

In this thesis, we analysed the nephrin signalling in cultured podocytes. Extrapolation of our findings to the in vivo setting requires caution. The activation of nephrin can be initiated by homophilic binding of two nephrin molecules and/or heterophilic interactions of nephrin and Neph proteins in vivo [14, 38], which suggests that activation (phosphorylation) of nephrin is probably necessary for the maintenance of the podocyte structural function.

The rat model of PAN is a well-established rodent model of acquired podocyte disease that resembles human minimal change disease. After a single tail vein injection of puromycin aminonucleoside, proteinuria develops between Day 4 and 6, peaks around Day 8, and normalizes at 4 wks. Actin cytoskeleton remodelling starts at Day 3, leading to foot processes effacement, followed by podocyte detachment and proteinuria. The changes of glomerular structural function in PAN accompany molecular reorganization of slit diaphragm proteins. Resolution of these changes parallels normalization of proteinuria [214]. Therefore, we used

this model to investigate the nephrin mediated PI3K-Akt and Nck-Pak signaling in rat glomeruli. In Chapter 2, our result indicated that nephrin tyrosine phosphorylation, nephrin-p85 association, and Akt phosphorylation were decreased at Day 3.5, when proteinuria was either minor or negligible. These changes continued on Day 7, when proteinuria was obvious. In Chapter 3, we extended the study for two weeks. We observed the same change of nephrin tyrosine phosphorylation at Day 4 and 7 as before. At two weeks, the proteinuria started to resolve, and the tyrosine phosphorylation of nephrin also started to increase. The change of phosphorylation of Pak1/2 paralleled that of nephrin tyrosine phosphorylation and neprhin-Nck association [180]. Although a causal relationship cannot be established in this model, it is tempting to speculate that decreased nephrin tyrosine phosphorylation mediated PI3K-Akt and Nck-Pak signalings in podocytes may contribute to foot process effacement and proteinuria in PAN.

It has also been reported that nephrin tyrosine phosphorylation is minimal in normal rodent but increase transiently in proteinuric models with foot process effacement, as well as during glomerular development when podocyte foot processes are formed [40, 188]. This suggests that nephrin tyrosine phosphorylation may occur transiently during development and injury repair. However, We and others reported that nephrin is tyrosine-phosphorylated under normal conditions [44, 194]. This finding was recently confirmed using phospho-specific antibodies of nephrin, which recognize the Nck binding sites of nephrin (Y1204 and Y1228 in rat nephrin) [201, 202]. Further, nephrin phosphorylation on these residues is decreased in PAN-induced podocyte damage and proteinuria. It is therefore likely that nephrin phosphorylation-mediated signaling is relevant in normal podocyte morphology and function. The above discrepancies may be explained by differences in the animal models and experimental protocols and antibodies used, but they may also reflect the dynamic nature of nephrin phosphorylation under changing circumstances.

Original contribution:

1. Y1152 of rat nephrin is responsible for the nephrin-p85 interaction.
2. Nephrin-PI3K interaction leads to the activation of Akt and Rac1, and altered actin cytoskeleton with decreased stress fibers and increased lamellipodia.
3. Activated Rac1 and Akt contribute to lamellipodia formation and decreased stress fibers respectively in cultured podocytes.
4. Nephrin–Nck interaction leads to the activation of Pak, which is Rac1 activity independent.
5. Activated Pak leads to decreased stress fibers, increased cortical actin and extended cellular processes in cultured podocytes.
6. In the rat model of PAN, nephrin tyrosine phosphorylation, nephrin-PI3K association, glomerular Akt and Pak phosphorylation are all decreased, which parallel occurrence of proteinuria in PAN.

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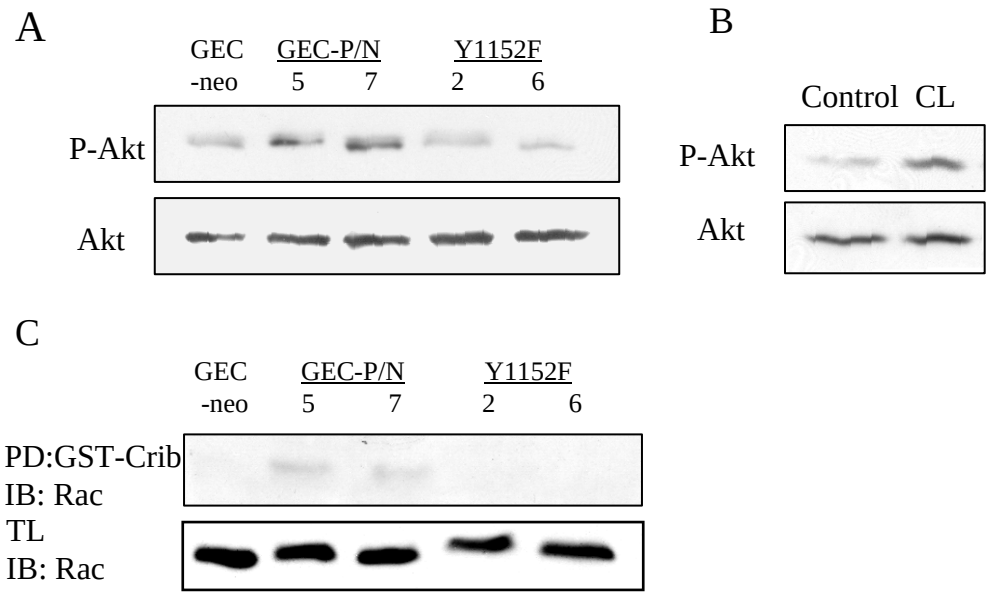
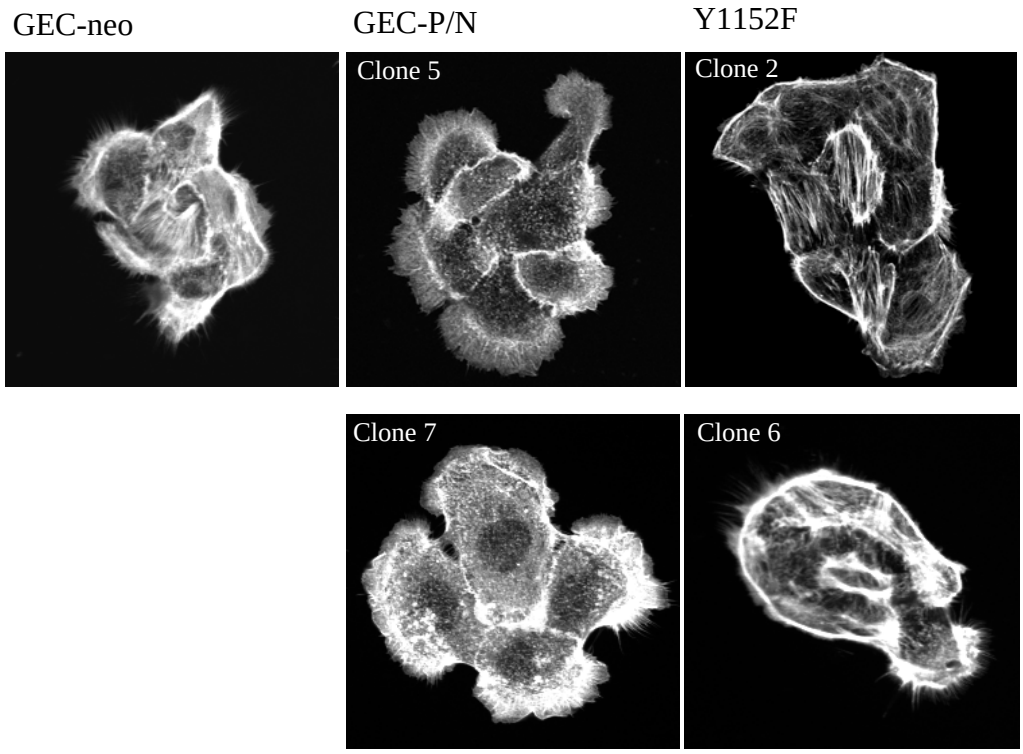
Supplemental Figures



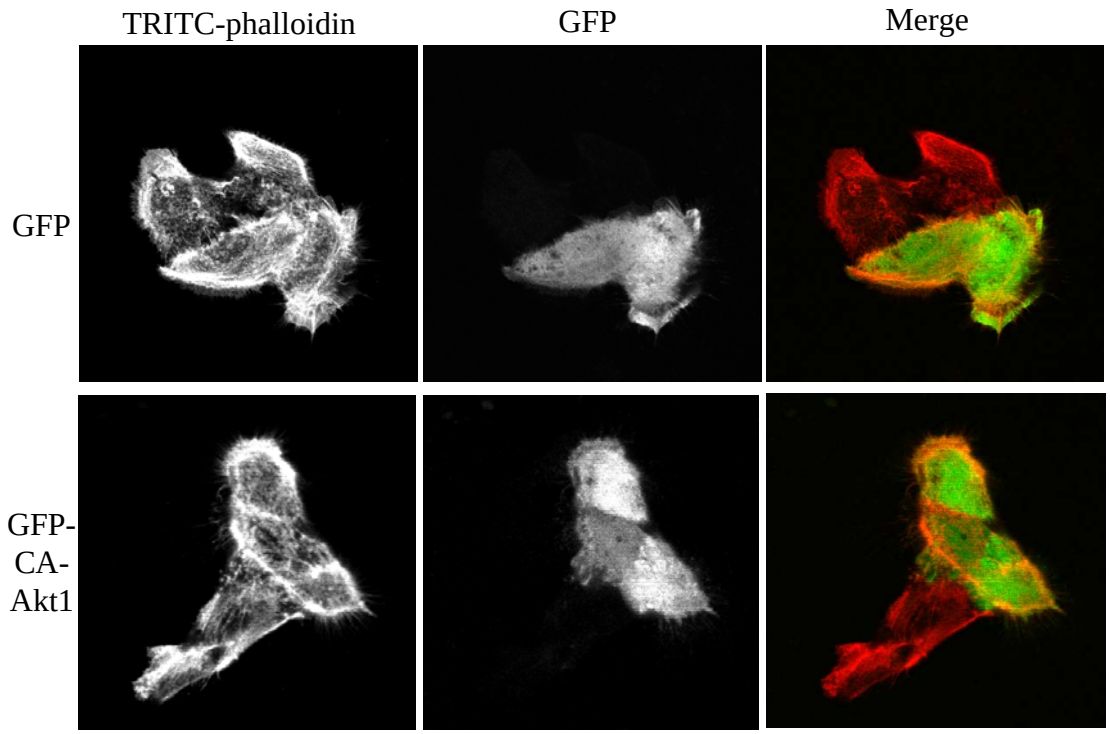
S1. Nephrin activates Akt and Rac1. Serum starved GEC-neo, GEC-P/N clones and GEC-Y1152 clones were lysed and subjected to immunoblotting. Akt phosphorylation was increased in GEC-P/N clones, as compared with GEC-Neo (vector-transfected control cells) or GEC-Y1152F clones. B. Nephrin was cross-linked with antibodies in GEC-P/N as described previously(Li H. et al. BBRC2006,349: 310-316). Cell lysates of GEC-P/N with or without antibody-mediated cross-linking were lysed and subjected to immunoblotting. Akt phosphorylation was increased by cross-linking of nephrin C. GEC-neo, GEC-P/N clones, GEC-Y1152F clones were serum starved for 16 h. Cell lysates were subjected to GST-Crib (Cdc42/Rac1 interaction binding) pull down (PD) followed by immunoblotting with anti-Rac1. Similar to Akt phosphorylation, Rac1 activity was increased in GEC-P/N clones, but not in GEC-Y1152F clones..

S2



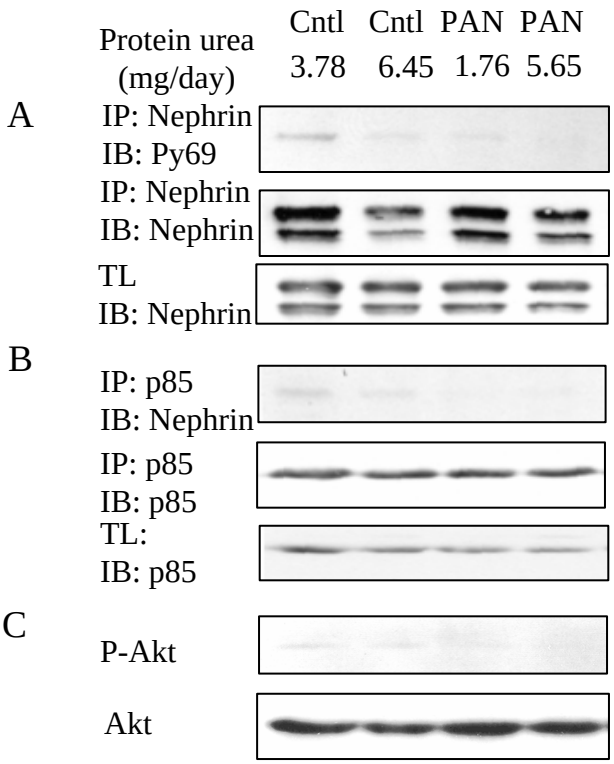
S2. Nephrin expression modulates the actin cytoskeleton in GEC. Serum starved GEC-neo, GEC-P/N, GEC-Y1152F were stained with TRITC-phalloidin. GEC-P/N clones showed more membrane ruffles and less stress fibers, as compared with GEC-Neo and GEC-Y1152F.

S3



S3. Active Akt1 decreases stress fibers in GEC-neo. GEC-neo were transfected with CA-Akt1 (GFP-tagged) or GFP alone. Cells transfected with CA-Akt1 generally did not show prominent stress fibers.

S4



S4. Nephrin-p85 interaction and Akt phosphorylation are decreased in PAN. PAN was induced as in Methods (Chapter 2). Glomerular lysates from rats with PAN (day 3.5) and control rats were immunoprecipitated for nephrin (A) or p85 (B) and precipitates were blotted for phosphotyrosine (A) or nephrin (B). A. Tyrosine phosphorylation of nephrin was decreased in PAN. B. The amount of nephrin co-immunoprecipitated with p85 was decreased in PAN. The amounts of p85 were not different between control and PAN. C. Akt phosphorylation was decreased in PAN.