# Characterization of the CdGAP-β-PIX interaction in glomerular podocytes

Dina Greenberg

Division of Experimental Medicine

McGill University

Montréal, Canada

April 2022

A thesis submitted to McGill University in partial fulfillment
of the requirements of the degree of Master of Science

Abstract

The glomerulus is the filtration unit of the kidney. When its permselectivity (barrier function) is impaired, leakage of proteins in urine (proteinuria) occurs – this is a hallmark of kidney disease and a prognostic of severity. Visceral glomerular epithelial cells (podocytes) are critical for the permselectivity of the glomerulus. Podocytes have actin-based finger-like projections called foot processes that interdigitate at the glomerular capillary to support macromolecular filtration. Dysregulation of the podocyte cytoskeleton can cause foot process effacement or detachment and is characteristic of focal segmental glomerulosclerosis (FSGS). Cytoskeletal regulation is a major function of the Rho family of small GTPases (Rho GTPases); these are in turn activated by guanine nucleotide exchange factors (GEFs) and inactivated by GTPase-activating proteins (GAPs). Dysregulation of the Rho GTPases RhoA, Rac1, and Cdc42 is deleterious to renal function and disruptive to the podocyte actin cytoskeleton.

Recent proximity-dependent biotin identification (Bio-ID) data from the Takano lab has shown that in podocytes, ARHGAP31 (CdGAP) and ARHGEF7 (β-PIX) are the top Rac1-interacting GAP and GEF, respectively. More recently, we have discovered that CdGAP and β-PIX interact physically by coimmunoprecipitation. We therefore speculated that CdGAP and β-PIX may interact physically and functionally to modulate focal adhesion dynamics.

This work identifies the CdGAP-β-PIX interacting domains and investigates the functional nature of the interaction through a combination of gene knockdown and overexpression techniques. On the basis of these findings, I propose a mechanism under which CdGAP and β-PIX form a complex in the cytosol of human podocytes, wherein CdGAP is unable to exert GAP activity on Rac1/Cdc42 at basal conditions. Upon EGF stimulation, CdGAP and β-PIX jointly migrate to the cell membrane, where they exert their respective effects on Rac1/Cdc42 activity and focal adhesion dynamics. Furthermore, CdGAP knockdown in podocytes increases levels of membrane-bound β-PIX, suggesting the GAP retains the GEF cytosolically at basal conditions. These findings collectively support a mechanism for targeted regulation of local Rac1/Cdc42 activity, and thus of focal adhesion dynamics, in human podocytes.

## Résumé

Le glomérule est l'unité de filtration du rein. Lorsque sa “permsélectivité” (fonction de barrière) est altérée, une fuite de protéines dans l'urine (protéinurie) se produit – c'est une caractéristique de la maladie rénale et un indice de gravité de la maladie. Les cellules épithéliales glomérulaires viscérales (podocytes) sont essentielles à la “permsélectivité” du glomérule. Les podocytes ont des projections en forme de doigt à base d'actine appelées « processus du pied » qui s'interdigitent au niveau du capillaire glomérulaire pour soutenir la filtration macromoléculaire. La dérégulation du cytosquelette podocytaire peut causer un effacement ou un décollement des processus du pied et est caractéristique de la glomérulosclérose segmentaire focale. La régulation du cytosquelette est une fonction majeure de la famille des petites Rho GTPases (Rho GTPases); celles-ci sont à leur tour activées par les facteurs d'échange de nucléotides de guanine (GEF) et inactivés par les protéines activatrices de la GTPase (GAPs). La dérégulation des Rho GTPases RhoA, Rac1 et Cdc42 est délétère pour la fonction rénale et perturbe le cytosquelette d'actine podocytaire.

Des données Bio-ID récentes du laboratoire du Dr. Takano ont montré que dans les podocytes, ARHGAP31 (CdGAP) et ARHGEF7 (β-PIX) sont les principales GAP et GEF interagissant avec Rac1, respectivement. Plus récemment, nous avons découvert que CdGAP et β-PIX interagissent physiquement par coimmunoprécipitation. Nous avons donc émis l'hypothèse que CdGAP et β-PIX peuvent interagir physiquement et fonctionnellement pour moduler la dynamique d'adhésion focale.

Ce travail a conduit à identifier les domaines d'interaction CdGAP-β-PIX et à étudier la nature fonctionnelle de cette interaction en utilisant une combinaison de techniques d'élimination et de surexpression des gènes. Sur la base de ces résultats, je propose un mécanisme en vertu duquel CdGAP et β-PIX forment un complexe dans le cytoplasme des podocytes humains, dans lequel CdGAP est incapable d'exercer une activité GAP sur Rac1/Cdc42 dans des conditions basales. Lors de la stimulation par EGF, CdGAP et β-PIX migrent conjointement vers la membrane cellulaire, où ils exercent leurs effets respectifs sur l'activité Rac1/Cdc42 et la dynamique d'adhésion focale. En outre, l’élimination de CdGAP dans les podocytes augmente les niveaux de β-PIX liée à la membrane, ce qui suggère que CdGAP retient cytosoliquement β-PIX dans des conditions basales. Ces résultats soutiennent collectivement un mécanisme de régulation ciblée de l'activité locale Rac1/Cdc42, et donc de la dynamique d'adhésion focale, dans les podocytes humains.

Acknowledgements

This work would not be possible without the support of many people. First and foremost, I am indebted to my supervisors, Dr. Tomoko Takano and Dr. Nathalie Lamarche-Vane. They gave me the opportunity to join their labs, and in my time here, encouraged curiosity, critical thinking, and tenacity in the pursuit of science. Thank you for your patience, encouragement, and tremendous support throughout this journey; it has been pivotal for my growth and has inspired me to continue with research.

To all members of the Takano and Lamarche labs, you have made this time enjoyable. Your advice and support have been invaluable to my success, particularly on occasions of messy immunoblots and failed experiments. I am especially grateful to Lamine Aoudjit and Dr. Jun Matsuda, who taught me many of the skills that allowed me to complete this project. Thank you all for supporting my development as a graduate student, both technically and personally.

I am grateful to my committee members, Dr. Andrey Cybulsky, Dr. Jean-François Côté, and Dr. Joyce Rauch, whose feedback and questions have been incredibly helpful to my progress.

Lastly, I am thankful for all the encouragement I have received along the way from family and friends. Your love and support have made all the difference, and I have been lucky to have it.

Table of Contents

## Contribution of authors

Jun Matsuda performed (a) the coimmunoprecipitation experiment identifying the β-PIX binding domain of CdGAP, (b) the generation, phalloidin staining, and immunofluorescent imaging of CdGAP and β-PIX knockdown podocyte cell lines, and (c) the Rac1/Cdc42 activity assay in EGF-stimulated control and CdGAP knockdown human podocytes. Lamine Aoudjit assisted with initial overexpression of CdGAP and β-PIX in HEK 293 cells. Dina Greenberg performed (a) the coimmunoprecipitation experiment identifying the CdGAP binding domain of β-PIX, (b) overexpression assays of CdGAP and β-PIX in HEK 293 cells, (c) the immunofluorescence staining and localization analysis of endogenous CdGAP and β-PIX in human podocytes, (d) cell migration assays, (e) morphological analyses of CdGAP and β-PIX knockdown podocytes, and basal Rac1/Cdc42 activity assay in HEK 293 cells overexpressing CdGAP and β-PIX.

List of abbreviations

| | |
|---|---|
| ARHGAP24 | Rho GTPase activating protein 24 |
| ARHGAP31 | Rho GTPase activating protein 31, see also: CdGAP |
| ARHGEF6 | Rho guanine nucleotide exchange factor 6, see also: α-PIX |
| ARHGEF7 | Rho guanine nucleotide exchange factor 7, see also: β-PIX |
| Bio-ID | Proximity-dependent biotin identification |
| BR | Basic region |
| CaMKII | $Ca^{2+}$ /calmodulin-dependent protein kinase II |
| CAPZ | F-actin capping protein |
| CD2AP | CD2-associated protein |
| Cdc42 | Cell division control protein 42 homolog |
| CdGAP | Cdc42 GTPase-activating protein, see also: ARHGAP31 |
| COL4A3 | Collagen IV α3 chain |
| COL4A4 | Collagen IV α4 chain |
| COL4A5 | Collagen IV α5 chain |
| CsA | Cyclosporine A |
| DAPI | 4’,6-diamidino-2-phenylindole |
| DH | Dbl homology |
| EGF | Epidermal growth factor |
| EGFR | Epidermal growth factor receptor |
| ERK | Extracellular signal-regulated kinase |
| F-actin | Filamentous actin |
| FAK | Focal adhesion kinase |
| FAT1 | FAT atypical cadherin 1 |
| FSGS | Focal segmental glomerulosclerosis |
| Fyn | Tyrosine-protein kinase Fyn |
| GAP | GTPase-activating protein |
| GBM | Glomerular basement membrane |
| GDI | Guanine nucleotide dissociation inhibitor |
| GEF | Guanine nucleotide exchange factor |
| GSK-3 | Glycogen synthase kinase-3 |
| HEK 293 | Human embryonic kidney 293 |
| HP | Human podocyte |
| HSPG | Heparan sulfate proteoglycan |
| INF2 | Inverted formin 2 |
| IQGAP1 | Iq-motif containing GTPase-activating protein 1 |
| IRSp53 | Insulin receptor substrate 53 kDa |
| ITGB4 | Integrin β4 |
| ITSN1 | Intersectin-1 |

| | |
|---|---|
| ITSN2 | Intersectin-2 |
| LAMB2 | Laminin β2 |
| LPS | Lipopolysaccharide |
| MAPK | Mitogen-activated protein kinase |
| Myo1e | Myosin-1e |
| NEPH1 | Nephrin-like protein 1 |
| PAK | P21-activated kinase |
| PBR | Poly basic region |
| PH | Pleckstrin homology |
| PI(3,4,5)P3 | Phosphatidylinositol 3,4,5-triphosphate |
| PI3K | Phosphoinositide 3-kinase |
| PKA | Protein kinase A |
| PRD | Proline rich domain |
| RAAS | Renin-angiotensin-aldosterone system |
| Rac1 | Ras-related C3 botulinum toxin gene |
| RhoA | Ras homolog family member |
| RhoGDIα | Rho GDP-dissociation inhibitor α |
| ROS | Reactive oxygen species |
| RSK | Ribosomal S6 protein kinase |
| SH3 | Src homology 3 |
| Smurf1 | SMAD specific E3 ubiquitin protein ligase 1 |
| srGAP1 | Slit-robo GTPase activating protein 1 |
| TEM | Transmission electron microscopy |
| TRP6 | Transient receptor potential cation channel 6 |
| WT1 | Wim's tumor 1 |
| YAP | Yes-associated protein |
| α-PIX | PAK-interacting exchange factor alpha: see also, ARHGEF6 |
| β-PIX | PAK-interacting exchange factor beta: see also, ARHGEF7 |

List of figures

# Chapter 1: Introduction and Literature Review

## 1.1 The kidney

The kidney is a paired organ responsible for the filtration of blood – the first step of urine formation. Healthy adult kidneys can filter as many as 180 L of blood per day, and in doing so, they carry out critical homeostatic functions. These include electrolyte and acid-base homeostasis, maintenance of blood pressure, and other endocrine regulatory roles (1).

The nephron is the functional unit of the kidney. It has previously been believed that each kidney contains an approximate 1 million nephrons, yet further investigation has revealed that nephron number can vary drastically between individuals, with studies reporting nephron numbers (per kidney) ranging from 331,000 to 2,702,000 across varied populations (2, 3). Much of the variation in nephron endowment is present from birth, with numerous health factors affecting subsequent nephron loss throughout one's lifetime (2). It follows that an abundance of functional nephrons is critical for kidney health.

The nephron is composed of two major subunits: the glomerulus and the tubule. Blood enters the kidney via renal artery and makes its way to the glomerulus (the proximal-most part of the nephron), where initial filtration takes place (**Fig. 1A**). The resulting ultrafiltrate passes into the proximal tubule, loop of Henle, distal tubule, and finally the collecting duct, by which point it has been transformed into its final urine product (1).

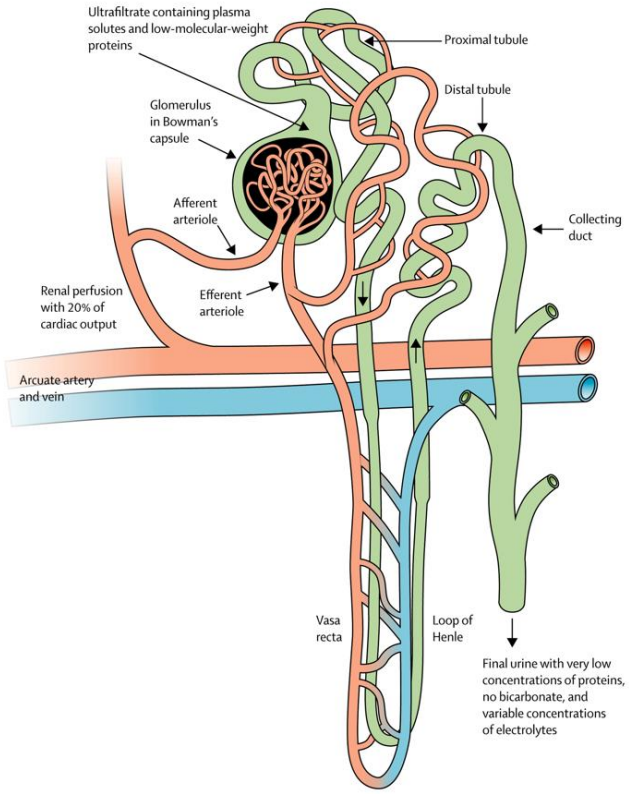
### 1.1.1 The glomerulus

The glomerulus itself consists of a network of tightly wound capillaries seeded within a capsule termed Bowman's capsule. (In fact, the term glomerulus stems from the Latin *glomus*, or ball of yarn, representing the capillary tuft.) Capillary loops are supported by the scaffolding of the mesangium, a structure composed of matrix and mesangial cells. Mesangial cells are smooth muscle-like pericytes that carry out phagocytotic, intercellular signaling, and immunoregulatory

functions in the glomerulus (4). Their contractile properties permit them regulate capillary blood flow, and subsequently, glomerular filtration rate (5).

Glomerular capillaries are characterized by their fenestrated endothelial cells, which allow for the passage of water and small solutes (6). These cells are separated from the urinary space by two additional strata: the glomerular basement membrane (GBM) that sits atop the capillaries, and the overlaying glomerular epithelial cells (or podocytes). Together, these three components make up the glomerular filtration barrier (**Fig. 1B**) (7). This barrier allows for selective permeability, such that water and small solutes (i.e., ions, amino acids, glucose, urea) can pass into the urinary space, while larger plasma components (i.e., albumin) and cells are retained in the capillary and returned to circulation (8). The glomerular filtration barrier also retains anionic macromolecules; it is hypothesized that this property attributes to the exclusion of albumin from ultrafiltrate (9, 10). Conversely, loss of integrity of the glomerular filtration barrier can result in passage of protein into the urine, or proteinuria. This is both a hallmark of kidney disease and a prognostic of severity and highlights the importance of the glomerular filtration barrier to kidney health.

A




B

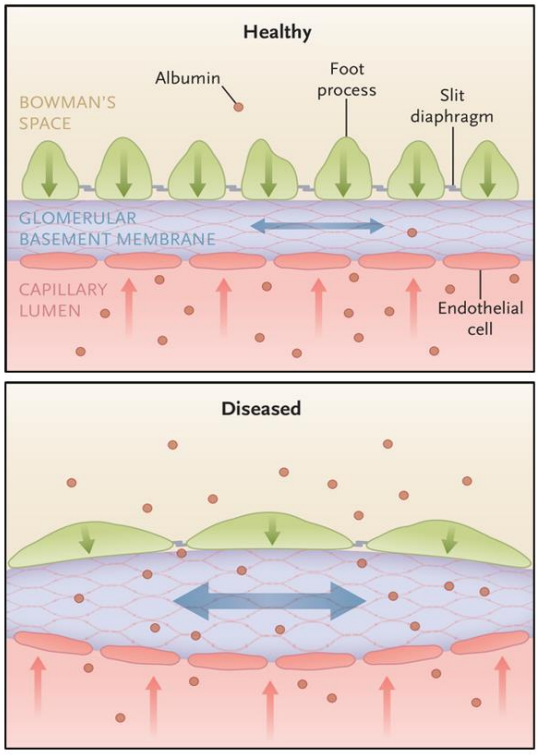



**Figure 1: Anatomy of the nephron and glomerular filtration barrier. A**: Anatomy of the nephron. Blood enters via the afferent arteriole, is filtered into the glomerulus (via the glomerular filtration barrier). Filtrate passes through the proximal tubule, loop of Henle, distal tubule, and makes its way into the collected duct. **B**: The glomerular filtration barrier consists of glomerular endothelial cells (of the capillary), the glomerular basement membrane, and glomerular epithelial cells (or podocytes). The slit diaphragm serves as a fine macromolecular sieve and an intracellular contact point between podocyte foot processes. A healthy filtration barrier (top) retains albumin. In diseased condition (bottom), foot processes widen, the slit diaphragm narrows, and blood albumin is no longer retained in the capillary space, but rather passes into Bowman's space. Arrows illustrate podocyte buttressing forces (green), circumferential wall stress on the glomerular basement membrane (blue), and compressing force of fluid filtration (red).

**A**: This figure was published in The Lancet, Vol 382, Kai-Uwe Eckhardt, Josef Coresh, Olivier Devuyst, Richard J Johndon, Anna Kottgen, Andrew S Levey, Adeera Levin, Evolving importance of kidney disease: from subspecialty to global health burden, 158-169, Copyright Elsevier (2013).

**B**: Reproduced and modified with permission from Benzing, D Salant. N Engl J Med 2021;384:1437-1446, Copyright Massachusetts Medical Society.

#### 1.1.1.1 The glomerular basement membrane (GBM)

The GBM is formed by fusion of the two adjacent endothelial and epithelial basement membranes, followed by further deposition of basement membrane proteins by the epithelium during glomerular maturation (11). Thicker than most basement membranes, it is also unique in its composition. The GBM is made up of type IV collagen, laminin, nidogen, and heparan sulfate proteoglycan (HSPG) (12). However, while perlecan is the most predominant HSPG in most basement membranes, it is present only in low levels in the GBM, where agrin is the dominant HSPG isoform instead (13). Although both nidogens and HPSGs support GBM integrity by scaffolding of other components (13, 14), GBM disorders most often arise from dysregulation of collagens and laminins themselves.

The type IV collagen family comprises six alpha chains which can self-assemble into trimers that form a larger matrix. Of these, the most abundant collagen IV trimer in the GBM is α3α4α5, along with the less abundant α1α2α1 trimer (15). It was found that mutation of the COL4A5 gene (which encodes the collagen IV α5 chain) gives rise the more prevalent X-linked form of Alport syndrome; a condition characterized by hematuria, proteinuria, progressive renal failure and loss of hearing (16, 17). However, mutations in the COL4A3 and COL4A4 genes have also been identified as causing autosomal recessive form of the disease (18). Degradation of the α3α4α5 collagen IV chain in patients with Alport syndrome is accompanied by an increased compensatory abundance of the α1α2α1 collagen chain, which renders the GBM more susceptible to endoproteolysis. This causes gradual and segmental thinning and thickening, splitting, and eventually deterioration of the membrane (19). Some patients, however, do not exhibit the split GBM and instead present with focal segmental glomerulosclerosis (FSGS) accompanied by significant levels of proteinuria (20). Collectively, these pathologies highlight the significance of tightly regulated collagen IV deposition to the structural integrity of the GBM.

Like type IV collagen, laminin exists in a trimer structure consisting of an α, β, and γ chain, which are assembled from a selection of five α, four β, and three γ monomers (21). Of these, the major laminin trimer present in the GBM is laminin-521 (α5β2γ1), however two other laminins (laminin-111 and laminin-511) appear throughout GBM development and maturation (22). A mutation in LAMB2 (the gene encoding laminin β2) has been identified as causing congenital

nephrotic syndrome termed Pierson syndrome (23, 24). Clinical manifestations of the disease are varied, as are the causative LAMB2 mutations. However, most patients present with some degree of nephrotic syndrome that can be accompanied by ocular and neurological abnormality (25–27). *In vivo* studies revealed that the GBM of LAMB2$^{-/-}$ mice contained ectopic deposition of other laminins; however, this compensatory response is insufficient as the GBM remains too porous (i.e., lacking permselectivity), resulting in heavy proteinuria and early death (28). Adequate distribution of laminin is, therefore, critical to GBM function.

#### 1.1.1.2 The podocyte

Atop the GBM lies the final layer of the filtration barrier: the podocytes. Podocytes are terminally differentiated glomerular epithelial cells with a unique architecture that supports filtration. They are made up of three distinct components: a cell body that branches off into primary actin-based processes, which in turn branch off into smaller, secondary processes called foot processes (**Fig. 2A, B**). Major processes extend outward from the cell body and wrap around the glomerular capillaries, while foot processes interdigitate with those of neighboring podocytes (29, 30) – thus, podocytes cover most of the capillary surface area. The small space that remains between adjacent foot processes is the filtration slit. It is bridged by the slit diaphragm; an intercellular contact point between foot processes that can be described as a modified adherens junction (**Fig. 2C**). The slit diaphragm network serves as a filtration mesh, and is the final component of the glomerular filtration barrier (31), leading into the urinary space. Thus, the structural integrity of the slit diaphragm, and in turn of the podocytes that sustain it, is critical to glomerular filtration (**Fig. 2D**). Conversely, dysregulation of podocyte focal adhesions (macromolecular complexes that link the cytoskeleton and extracellular matrix) can result in foot process effacement (thus disrupting the slit diaphragm), or even cell detachment from the GBM, resulting in the degradation of the filtration barrier (**Fig. 2E**).

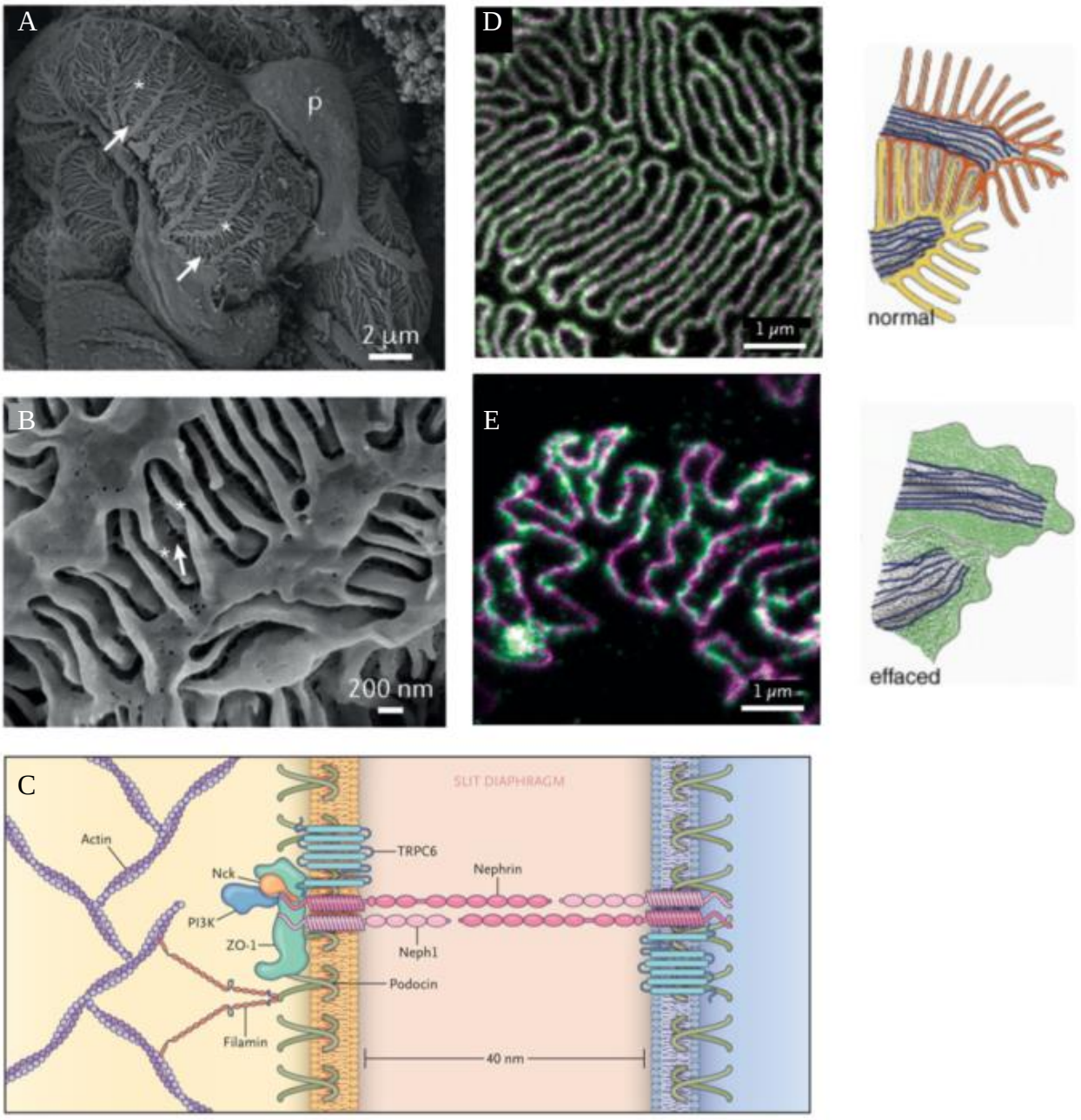



**Figure 2: Podocyte architecture supports slit diaphragm maintenance. A**: Podocytes wrap around glomerular capillaries and their foot processes interdigitate with one another. (P) denotes the podocyte cell body, asterisks (*) demarcate foot processes, and arrows denote slit diaphragm between foot processes. **B**: Enlarged version of (**A**). **C**: The podocyte slit diaphragm is composed of transmembrane proteins and scaffold proteins, which connect the slit diaphragm to the podocyte actin cytoskeleton. **D**: A normal slit diaphragm has extensive surface area that supports filtration (left), which is supported by adjacent interdigitating foot processes (right). **E**: Loss of slit diaphragm area (left) is caused by foot process effacement (right) under disease conditions. Left

panels of (**D**) and (**E**) show immunofluorescent staining for the slit diaphragm markers nephrin (magenta) and podocin (green).

**A**, **B**: Reproduced with permission from Perico, L, Conti, S, Benigi, A, Remuzzi G. Nat Rev Nephrol 2016; 12:692 – 710, Copyright Springer Nature Limited.

**C**, **D** (left), **E** (left): Reproduced with permission from Benzing, D Salant. N Engl J Med 2021;384:1437-1446, Copyright Massachusetts Medical Society.

**D** (right), **E** (right): This figure was published in Trends in Cell Biology, Vol 17, Christian Faul, Katsuhiko Asanuma, Etsuko yanagida-Asanuma, Kwanghee Kim, Peter Mundel, Actin up: regulation of podocyte structure and function by components of the actin cytoskeleton, 428-427, Copyright Elsevier (2007).

We are born with an estimated 800 podocytes per glomerulus, and this number is consistently dropping (32). Podocyte loss is most pronounced in cases of glomerular disease, but these cells are also subject to more routine stressors throughout their lifetime. For example, while they require a physiological level of transcapillary pressure for maintenance, hypertension can be damaging to podocytes. Under excess mechanical stress, podocytes become hyertrophic (thereby obstructing an increasing amount of capillary filtration space), their primary processes thin, and their foot processes efface, leading to eventual cell detachment (33). Overactivation of the renin-angiotensin-aldosterone system (RAAS), which can drive hypertension via production of angiotensin II (a potent vasoconstrictor), causes further damage as it promotes formation of intracellular and mitochondrial reactive oxygen species (ROS) (34, 35). Angiotensin II also promotes secretion of aldosterone, a mineralcorticoid required for electrolyte homeostasis, which can induce further oxidative stress (35). Studies have shown that both angiotensin II and aldosterone contribute to kidney injury, and that in podocytes, injury is driven in part by cytoskeletal rearrangement (36–39). Similarly, immunologic stressors like cytokines and complement can damage podocytes through numerous pathways, some of them affecting cytoskeletal integrity (40–42).

Indeed, the podocyte cytoskeleton is critical to its maintenance of glomerular permselectivity. While the cell body is dense in organelles to support both anabolic and catabolic activity of the podocyte, the primary processes contain far fewer organelles. Their function, rather,

is to support the intricate branching architecture of the podocyte. A network of intermediate filaments (primarily desmin and vinculin) and microtubules extend from the cell body and throughout primary processes, eventually leading to the branching of primary processes into foot processes (30, 43). At the site of foot processes, intermediate filaments meet with an actin network that is composed of both short, branched filamentous actin (F-actin) filaments, as well as long, ordered contractile F-actin filament bundles spanning the length of the foot process (30). F-actin also forms a high arch to connect neighboring foot processes of the same podocyte; it is this arch that serves as a connection point to the intermediate filament and microtubule network that spans the rest of the cell, effectively linking the contractile apparatus of the foot process to the entire podocyte cytoskeleton (29). At the base of the foot process are large macromolecular protein complexes termed focal adhesions; these actin endpoints are critical for anchoring the podocyte to the GBM, but also support actin polymerization and intracellular signal transduction (44).

The actin cytoskeleton is essential to foot process maintenance, and its reorganization is deleterious to podocyte function, often giving rise to foot process effacement (45–48). This is characteristic of many forms of kidney disease, including focal segmental glomerulosclerosis (FSGS). FSGS is a histopathological injury characterized by partial (segmental; affecting only a portion of the glomerular tuft) scarring (sclerosis) that is localized (focal) to some glomeruli (49). Although both cause and clinical presentation can vary, FSGS is often accompanied by proteinuria and foot process effacement (49, 50). FSGS affects the entire glomerulus, but often arises from dysregulation of the podocyte in particular (48).

Indeed, mutations of numerous members of the podocyte cytoskeleton regulatory network have been linked to familial FSGS. α-actinin-4, a protein that mediates connection of the actin cytoskeleton to focal adhesions, is one such example (51). Mutations in the ACTN4 gene have been found to cause a hereditary form of focal segmental glomerulosclerosis (FSGS), and *in vivo* studies have shown the representative mouse model to have GBM alteration and podocyte malformation and effacement that results in proteinuria (52, 53). *In vitro* studies have supported the notion that the ACTN4 mutation impairs the podocyte cytoskeleton, leaving it increasingly brittle, with fewer peripheral projections and impaired spreading and motility (54, 55). α-actinin, in turn, is subject to regulation by synaptopodin – a podocyte specific actin-binding protein that mediates cell contractility (56). In children with idiopathic nephrotic syndrome, synaptopodin

expression was decreased in comparison to health controls, with further decrease that correlated to histopathological changes (57). In other words, cases of nephrotic syndrome that presented with minimal changes in the glomerulus (a histopathology termed "minimal change disease" that seldom precedes renal failure) showed higher synaptopodin expression than cases presenting with FSGS (which can progress to renal failure). The same study found a positive association between synaptopodin expression levels (which were highest in minimal change disease presenting children) and response to steroid therapy (57). Synaptopodin further regulates cytoskeletal dynamics by mediating activation of RhoA and inhibition of Cdc42, underscoring the dynamic nature of podocyte cytoskeleton regulation (58).

FSGS can also arise from mutations in the MYO1E gene, which encodes Myo1e, a nonmuscle class I myosin that is important to actin-dependent endocytosis (59, 60). Biopsies taken from patients with MYO1E mutations revealed disorganization of both podocytes and GBM, and *in vitro* assays showed mislocalization of the mutated form of the protein in cultured podocytes (59). Inverted formin-2 (INF2) is another critical cytoskeleton regulator as it mediates actin polymerization and depolymerization (61). Its mutation has been linked to FSGS, accounting for an estimated 9-17% of autosomal dominant FSGS cases (61–64). *In vitro* studies suggest that the INF2 mutations found in the clinical setting produce a protein with hindered capacity for actin remodeling, and further revealed that INF2 signaling in podocytes is dependent upon Cdc42 activity (62, 63). Collectively, these FSGS causing mutations demonstrate the importance of cytoskeleton regulation to maintenance of podocyte foot processes and their support of the glomerular filtration barrier.

As aforementioned, a common feature of FSGS is foot process effacement that can, in some cases, progress to podocyte detachment from the underlaying GBM (32). While podocyte attachment is reliant upon proper cytoskeletal function, it is also dependent upon the integrins that link foot processes to the GBM. Podocytes anchor to GBM laminins through integrins α3β1 and α6β1, and to collagen through integrins α1β1 and α2β1 (65, 66). Of these, integrin α3β1 is the predominant isotype in the glomerulus, and deletion of the integrin α3 subunit, both systemic and podocyte-specific, causes glomerulosclerosis, tubular dilation, GBM disorganization and foot process effacement (67, 68). (However, systemic α3 integrin knockout mice die neonatally, while podocyte-specific knockout mice develop proteinuria within their first week of life and progress

to nephrotic syndrome by 5-6 weeks (67, 68).) Mice with podocyte-specific deletion of β1 integrin had very similar pathology type to that of podocyte-specific α3 integrin knockout mice, however β1 integrin deletion elicited worsened pathology with earlier onset (at 3 weeks) of end stage renal failure (69). In humans, a homozygous missense mutation in ITGB4 has been found to reduce β4 integrin expression and produce FSGS without full nephrotic syndrome; a finding that is consistent with the notion that α3β1 is the predominant integrin within the glomerulus (70). Integrins serve another important role important role in the podocyte, as their activation triggers numerous downstream signaling cascades. One such example is integrin-mediated autophosphorylation of focal adhesion kinase (FAK), which allows for subsequent activation of Src and Rho GTPase (see below) (71). Furthermore, there is crosstalk between integrins and numerous growth factor receptors (e.g. epidermal growth factor receptor (EGFR)), and this crosstalk supports many processes that rely on spatiotemporal coordination of cytoskeletal activity (71, 72). For this reason, many studies (including our own) use growth factor ligands to promote integrin activation and observe cytoskeletal activity *in vitro*.

As the podocyte is reliant on the structural integrity of the GBM for adequate attachment, the GBM relies on the podocyte for a portion of its matrix deposition (11, 30). For example, some mutations in the WT1 gene (encoding Wilm's tumor 1, a nuclear transcription factor critical to podocyte function) have been linked to Denys-Drash syndrome, a condition characterized by early-onset renal failure and GBM thickening (73). Similarly, WT1 mutant mice show GBM thickening with podocyte foot process effacement and subsequent proteinuria (74). Thus, neither podocyte nor GBM are independent entities, but rather rely on one another for maintenance.

#### 1.1.1.3 The slit diaphragm

Adjacent foot processes of the podocyte are connected to one another by the slit diaphragm; an intracellular contact point that has been described as a modified adherens junction (31, 75). The slit diaphragm participates in numerous intracellular cell signaling pathways (e.g. actin polymerization, integrin β1 activation) (76–78). More importantly, the slit diaphragm serves as the final filtration sieve; seminal transmission electron microscopy (TEM) work by Rodewald and Karnovsky in 1974 suggests the slit diaphragm exists in a zipper-like pattern, with rod-like units

extending from the foot processes towards a central filament that runs parallel to the cell membrane. The rectangular pores that are formed by this cross-linking average 40 by 140 Å in dimension – a space sufficiently small to restrict passage of albumin, suggesting the slit diaphragm may be the principal filtration barrier within the glomerulus (79–81).

The slit diaphragm is composed of both transmembrane proteins (i.e., the proteins that form the extracellular molecular sieve) and cytoplasmic scaffold proteins that anchor the slit diaphragm to its bordering foot processes (78). Of the transmembrane proteins, nephrin was the first to be identified (82). Kestilä and colleagues documented the role of NPHS1 (encoding nephrin) mutations in the development of congenital nephrotic syndrome of the Finnish type, observing fusion of the podocyte foot processes and loss of the slit diaphragm (82). Soon thereafter, nephrin was characterized as a critical component of the molecular sieve complex of the slit diaphragm (83–85). Extensive investigation has revealed that nephrin participates in numerous signaling cascades as it interacts with Nck, Iq-motif containing GTPase-activating protein 1 (IQGAP1), phosphoinositide 3-kinase (PI3K), tyrosine-protein kinase Fyn (Fyn), and others to modulate the actin cytoskeleton (48, 86–90). Nephrin also interacts with another key component of the slit diaphragm, nephrin-like protein 1 (NEPH1). Aptly named to indicate its close homology to nephrin, NEPH1 cooperates with nephrin to maintain glomerular permeability (91, 92). Indeed, like nephrin, NEPH1 is a transmembrane protein that is essential to the slit diaphragm; NEPH1 null mice experience early postnatal lethality and extensive foot process effacement (93). Critically, nephrin and NEPH1 form a complex that supports outside-in cell signaling ultimately leading to actin polymerization; disruption of either protein, therefore, is deleterious to slit diaphragm integrity (76).

The extracellular portion of the slit diaphragm is supported by an intracellular network of slit diaphragm adaptor proteins such as podocin, CD2-associated protein (CD2AP), and others (48). FAT atypical cadherin 1 (FAT1) and transient receptor potential cation channel 6 (TRP6) are likewise important components of the slit diaphragm (48). Podocin was originally identified as the product of the NPHS2 gene that is mutated in autosomal recessive steroid-resistant nephrotic syndrome (94). Podocin is a membrane protein expressed exclusively in the podocyte, localized at the insertion site of the slit diaphragm (94, 95). It associates with nephrin to facilitate signaling and may support structural organization of the slit diaphragm (95, 96). Podocin associates with

another slit adaptor protein, CD2AP (97). CD2AP supports podocyte cytoskeletal remodeling, cell survival, and endocytosis (98–100). CD2AP knockout mice die at 6 to 7 weeks of age from renal failure, and notably, show foot process misconfiguration, highlighting the importance of CD2AP to glomerular filtration (101). In fact, CD2AP associates not only with podocin, but also directly with nephrin, F-actin capping protein (CAPZ), cortactin, and Rac1 to regulate cytoskeletal dynamics (30, 102, 103). FAT1 is an adhesion protein that supports slit diaphragm structural integrity and contributes to actin regulation via its extracellular domain. (104). Global FAT1 deletion in mice causes perinatal lethality, with visible podocyte fusion and subsequent loss of the slit diaphragm in embryos (105). Meanwhile, restriction of the deletion to podocytes induces glomerular filtration barrier abnormalities and foot process effacement (106). Of interest, FAT1 knockout in cultured renal tubule cells decreased Rac1 and Cdc42 activity, and injection of Rac1/Cdc42 activators into FAT1 mutant zebrafish allowed for partial rescue of the pronephric cysts caused by the mutation (106). Another slit diaphragm protein, TRPC6, is required for podocyte chemosensation and mechanosensation, and its mutations have been linked to FSGS (107–109). Individuals with TRPC6 mutations have varied phenotypes, but most experience gain of function mutations resulting in calcium-induced podocyte apoptosis (110, 111). Collectively, these findings illustrate the importance of slit diaphragm maintenance to glomerular health, as well as the dual nature of the relationship between the slit diaphragm and podocyte cytoskeleton.

### 1.2 Rho GTPases

The Rho family of small guanosine triphosphatases (Rho GTPases) are a collection of ubiquitously expressed regulatory proteins that act as molecular switches, toggling between an active GTP-loaded and inactive GDP-loaded form (**Fig. 3**) (112, 113). They are one of five groups of the larger Ras-like superfamily, containing Arf/Sar, Rab, Ran, Ras, and Rho GTPases (114). Rho GTPases modulate a wide array of cell functions: cell migration and adhesion, polarity, chemotaxis and durotaxis, morphogenesis, axonal guidance, cell cycle progression, endocytosis, and transcriptional regulation – in other words, they are master cytoskeletal regulators (112, 113, 115–117). Rho GTPases themselves are regulated by three classes of proteins: guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation

inhibitors (GDIs). GEFs catalyze the exchange of GDP to GTP, thus activating their Rho GTPase target (118, 119). Conversely, GAPs stimulate intrinsic GTP hydrolysis activity, thereby deactivating Rho GTPase activity (120, 121). GDIs sequester inactive Rho GTPases, preventing the dissociation of GDP (122). 82 GEFs, 69 GAPs, and 3 GDIs have been identified to date, many of which are expressed in a localized fashion (119, 121, 123–125). Given that Rho GTPases are ubiquitously expressed, GAPs, GEFs, and GDIs allow for their tight spatiotemporal regulation (112). Intricate control of Rho GTPase signaling is made only more dynamic by the discovery of extensive crosstalk between different Rho GTPase members (126, 127).

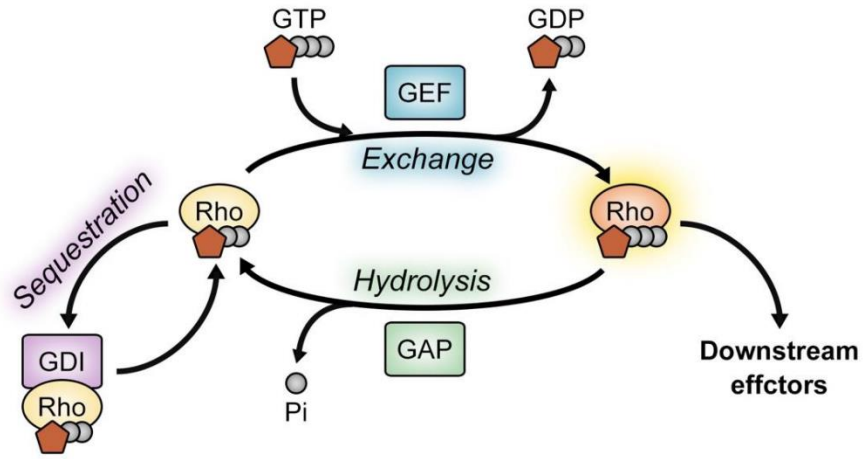



**Figure 3: A summary of Rho GTPase regulation.** Rho GTPases cycle between an active, GTP-bound, and inactive, GDP-bound state. They are activated by guanine nucleotide exchange factors (GEFs), inactivated via hydrolysis by GTPase-activating proteins (GAPs), and sequestered and thus rendered inactive by guanine nucleotide dissociation inhibitors (GDIs).



Of the 20 members of the Rho GTPase family, RhoA, Rac1, and Cdc42 are some of the most conserved and best studied (117). All three are crucial to cell motility; RhoA promotes actin stress fibers and supports focal adhesion maturation to induce a contractile phenotype, while Rac1 and Cdc42 promote a migratory phenotype by the induction of lamellipodia and filopodia, respectively (116, 128, 129). Together, these three Rho GTPases support coordinated cell migration. One study has used a computational multiplexing approach to show activities of RhoA,

Rac1, and Cdc42 in migrating mouse fibroblasts. They found that RhoA is active at the cell edge at time initiation of cellular protrusion, while Rac1 and Cdc42 are activated 40 seconds later, 2 μm behind the cell edge (130). Thus, the three Rho GTPases coordinate cell motility. RhoA, Rac1, and Cdc42 participate in numerous pathways and have therefore been the subject of extensive research in many facets of human development and disease, including nephrology (131–138). Indeed, the complex cytoskeletal architecture of the podocyte lends itself to extensive regulation by Rho GTPases, particularly in podocyte morphology and attachment to the GBM.

### 1.2.1 RhoA in podocytes

RhoA is expressed in immortalized podocytes and this expression is upregulated upon their differentiation. In these cells, RhoA promotes formation of contractile actin-myosin containing stress fibers in a synaptopodin dependent manner; synaptopodin binds RhoA, thereby rescuing it from SMAD specific E3 ubiquitin protein ligase 1 (Smurf1)-mediated ubiquitination (a process leading to consecutive degradation) (139). Synaptopodin, in turn, is protected from degradation by PKA or CaMKII-mediated phosphorylation and subsequent binding with 14-3-3. Calcineurin can competitively bind synaptopodin and dephosphorylate it to promote its degradation, together with loss of RhoA and stress fibers *in vitro* and proteinuria *in vivo*. This effect can be rescued *in vitro* and *in vivo* by treatment with calcineurin inhibitor cyclosporine A (CsA), a pharmacological agent used in treatment of nephrotic syndrome (140). In a similar vein, mice expressing podocyte-specific dominant negative RhoA developed proteinuria and foot process effacement, while podocyte specific RhoA knockout mice saw no phenotype until 3 months of age (141, 142). The difference in genetic background of the two mouse models may account for some of the difference in phenotypes, as could potential compensation by other Rho GTPase proteins (143). It remains that, on at least some level, RhoA activity is required for the maintenance of podocytes.

Overactivation of RhoA in podocytes can be just as harmful as insufficiency. Complement C5b-9-mediated cell injury contributes to pathogenicity of many kidney diseases; treatment of cultured podocytes with C5b-9 causes increased activity of RhoA but decreased Rac1 and Cdc42 activities (144, 145). C5b-9 treated podocytes also had decreased p190RhoGAP, a RhoA GAP, potentially explaining the increase in RhoA activity in these cells. Podocytes expressing

constitutively active RhoA were smaller in size and showed strong cortical F-actin staining (144). Another study found that expression of constitutively active RhoA in human embryonic kidney (HEK) 293 cells caused increased expression of fibronectin (146). The same study designed mice expressing doxycycline-inducible podocyte-specific constitutively active RhoA and found the mice developed proteinuria and foot process effacement in a manner directly correlating with RhoA activity. Elevated RhoA activity in these mice was also associated with transcriptional upregulation of fibronectin and collagen IA1, reflecting the development of glomerulosclerosis. Of importance, withdrawal of doxycycline treatment reversed proteinuria in some but not all mice, suggesting that sustained RhoA overactivation is harmful to podocytes *in vivo* (146). Rho-associated protein kinase (ROCK) is a major RhoA target and cytoskeletal regulator that has been linked to glomerular malfunction; accordingly, ROCK inhibition is a major line of experimental treatment in kidney disease (114, 147–150). Another major RhoA effector, mDia, is a formin subject to negative regulation by INF2 (151, 152). However, in INF2-linked FSGS, INF2 is unable to bind mDia, resulting in its hyperactivation and contributing to actin cytoskeleton disorganization in podocytes (62). Lastly, gain of function TRPC6 mutations, which have been linked to FSGS, increase RhoA activity to promote cell migration *in vitro* (110, 111, 153). Collectively, these studies suggest that tight regulation of RhoA signaling is critical for glomerular health.

### 1.2.2 Rac1 in podocytes

Like RhoA, Rac1 expression is increased upon podocyte differentiation. Unlike RhoA, Rac1 supports a migratory cell phenotype by promoting actin polymerization and subsequent lamellipodia and ruffle formation at the leading edge of a migrating cell, allowing for epithelial fusion (129, 151, 154). Rac1 activity in mouse podocytes has been shown to promote formation of lamellipodia and cellular processes (characteristics of podocyte differentiation) in a manner that is further potentiated by nephrin (via IQGAP1) (155, 156). Indeed, nephrin mediates actin reorganization through binding of PI3K and subsequent activation of both Rac1 and Akt (88). PI3K then contributes to a positive feedback cycle of Rac1 activity maintenance (via intermediary Dbl family Rac1 GEFs), as active Rac1 in turn promotes accumulation of PI3K, thus sustaining a cellular polarization response that permits directional motility (151, 157). This asymmetrical signal

is further maintained by Rac1 promoting phosphorylation of the RhoA GAP p190RhoGAP to inhibit RhoA-mediated stress fiber formation and focal adhesion maturation (158).

*In vitro* studies have revealed that inhibition of Rac1 in immortalized podocytes leads to disruption of their F-actin cytoskeleton and thinning of their processes (155). Interestingly, this is not immediately reflected in *in vivo* studies; podocyte specific Rac1 knockout mice have normal podocyte morphology and renal function into adulthood, suggesting Rac1 does not have a significant role in glomerular development. Furthermore, microarray analyses showed low levels of Rac2 and Rac3 (both Rac1 homologues), implying that the normal glomerular development in these mice is unlikely to be a product of compensatory response. Furthermore, when podocyte specific Rac1 knockout mice were challenged under a protamine sulfate model, they fared better than their control counterparts who developed foot process effacement and slit diaphragm loss (159). Collectively, these data implicate a potential pathogenic role for Rac1 in podocyte injury.

Rac1 overactivation, however, yields a stronger phenotype than that of Rac1 insufficiency. Overexpression of constitutively active Rac1 in cultured podocytes increased cell size, number of processes, and prominence of microtubules and F-actin (155). Rac1 overactivation also reduces podocytes' adhesion to laminin and causes redistribution of β1 integrin (160). This is reflected *in vivo*; mice expressing doxycycline-inducible constitutively active Rac1 develop proteinuria within five days of doxycycline treatment with histology resembling minimal change disease, and this is reversible with doxycycline withdrawal. Doxycycline treatment lasting two months, however, caused proteinuria, FSGS, and urinary shedding of podocytes, suggesting that changes in glomerular pathology generally correlate with duration of Rac1 hyperactivation (160). Other studies using constitutively active Rac1 models have supported the conclusion that the degree of podocyte and glomerular dysregulation is reversible in shorter periods of Rac1 activation, but more permanent (e.g. podocyte detachment) when Rac1 activity is sustained (161, 162).

Rac1 hyperactivation has been observed in the clinical setting. Biopsies collected from patients with FSGS showed elevated levels (in comparison to healthy controls) of GTP-bound Rac1 by immunofluorescent staining, and their sera stimulated Rac1 activity in cultured podocytes (160). Loss of function mutations in the ARHGDIA gene encoding Rho GDP-dissociation inhibitor α (RhoGDIα) give rise to congenital nephrotic syndrome; the resulting mutated protein is unable to bind and sequester its Rho GTPase targets, causing podocyte cytoskeletal

dysregulation (163, 164). Although it interacts with RhoA, Rac1, and Cdc42, mutation of RhoGDIα in cultured caused greater elevation to Rac1 and Cdc42 activity than it did to RhoA, and treatment with Rac1 inhibitor attenuated the hypermigratory phenotype caused by the mutation (163). In an opposing line of evidence, expression of disease-associated mutations in RhoGDIα in podocytes caused decreased cell migration, although this study also reported elevated Rac1 and Cdc42 in the mutant cells, suggesting that the loss of function disease-causing ARHGDIA mutations are not accurately modeled by simplistic RhoGDIα protein knockout (164). Loss of function Rho GTPase Activating Protein 24 (Arhgap24) mutation has also been associated with FSGS. *In vitro*, Arhgap24 is upregulated in differentiating podocytes and its ablation causes elevated Rac1 and Cdc42 (but not RhoA) activity, together with increased membrane ruffling and cell motility (165). Thus, the study of Rho GTPase regulators is pertinent to the understanding of how Rac1 maintains the podocyte cytoskeleton.

### 1.2.3 Cdc42 in podocytes

Cdc42 generally operates in concert with Rac1 as it supports actin polymerization in the cell (151). Cdc42 is also a critical modulator of cell polarity. This was originally observed in yeast; *Saccharomyces cerevisiae* expressing a loss of function Cdc42 mutant were unable to establish cell polarity (e.g., actin asymmetry) (166). Further studies have revealed Cdc42 plays a critical role in regulating cell polarization and directionality in response to extracellular cues (e.g., integrin activation), and that Cdc42 (together with Rac1) activity is required for epithelial fusion (113, 154). As previously mentioned, in podocytes, nephrin interacts with IQGAP1 to activate not only Rac1, but also Cdc42, thus supporting concurrent activity of Rac1 and Cdc42 (156). Conversely, synaptopodin, which promotes activation of RhoA, inhibits Cdc42 activity by sequestering the adaptor protein IRSp53 that is required for Cdc42-IRSp53-Mena complex mediated filopodia formation (58, 139). Inhibition of Mena suppressed Cdc42-induced filopodia and attenuated LPS-induced proteinuria in synaptopodin deficient mice, suggesting that aberrant Cdc42 activity contributes to the pathogenesis of proteinuria (58).

Notwithstanding, there is a strong body of evidence showing Cdc42 is critical to glomerular function. Podocyte specific Cdc42 knockout mice generated by Scott *et al.* developed tubular and

glomerular dilatation as early as postnatal day 0, which progressed to glomerulosclerosis at postnatal day 5. By day 12, the mice had proteinaceous casts and collapsed glomeruli , and died within two weeks due to renal failure (141). Podocyte specific Cdc24 knockout mice studied by Blattner *et al.* similarly showed glomerulosclerosis and foot process effacement starting postnatal day 10, but curiously the authors observed progression of glomerular disease that was less severe than that found by Scott and colleagues, citing potential differences in environment and genetic background heterogeneity (141, 159). Both studies however observed reduced expression of the slit diaphragm components nephrin and podocin, and Blattner *et al.* further observed their redistribution by immunofluorescent staining of kidney sections. Podocyte-specific Cdc42 knockout also led to increased synaptopodin expression, and dephosphorylation of cofilin, resulting in alteration of podocyte cytoskeletal architecture (141, 159). Collectively, these studies demonstrate that Cdc42 is necessary for functional glomerular development and maintenance. This notion is upheld in the clinical setting; missense mutations in the scaffolding proteins and Cdc42 GEFs Intersectin-1 (ITSN1) and Intersectin-2 (ITSN2) have been found in patients with congenital nephrotic syndrome (167–169). Cultured podocytes with the disease-causing mutations showed reduced Cdc42 activity and blunted filopodia formation. ITSN2 null mice developed normal kidneys, but developed stronger proteinuria and foot process effacement than their wildtype control counterparts when challenged with LPS (169). This underscores the requirement for tight regulation of Cdc42 in the maintenance of glomerular function.

### 1.2.4 Regulators of Rho GTPases

There is an abundance of literature pertaining to the three prototypical Rho GTPases (RhoA, Rac1, and Cdc42) in podocytes. However, the mechanisms by which these Rho GTPases are regulated are only partially understood. The relationship between many regulators and their target Rho GTPases has been characterized, but our understanding of the Rho GTPase interactome remains insufficient. In particular, there is a gap in our knowledge of (1) interactors of Rho GTPases and their regulators, and (2) the spatiotemporal organization of the Rho GTPase interactome.

To address these queries, the lab of Dr. Takano implemented BioID, a protein-protein interaction screening method that uses proximity ligation and subsequent proteomics, to

investigate which Rho regulatory proteins interact with Rac1 in podocytes (**Fig. 4**) (170). A nucleotide-free mutant of Rac1 with preferential binding for GEFs (Rac1G15A) was used as bait (171). In brief, Rac1G15A was fused with a promiscuous *E. coli* biotin ligase (BirA) and expressed in immortalized human podocytes. Treatment with biotin caused biotinylation of proteins in close proximity to Rac1G15A, which were subsequently isolated by streptavidin beads and subjected to analysis by mass spectrometry analyses. 125 Rac1G15A interactors were identified, many of which were known Rho GTPase regulators. Further sorting revealed that in podocytes, Rho guanine nucleotide exchange factor 7 (ARHGEF7, henceforth referred to as β-PIX) was the top Rac1-interacting GEF, while Rho GTPase activating protein 31 (ARHGAP 31, henceforth referred to as CdGAP) was the top Rac1-interacting GAP.

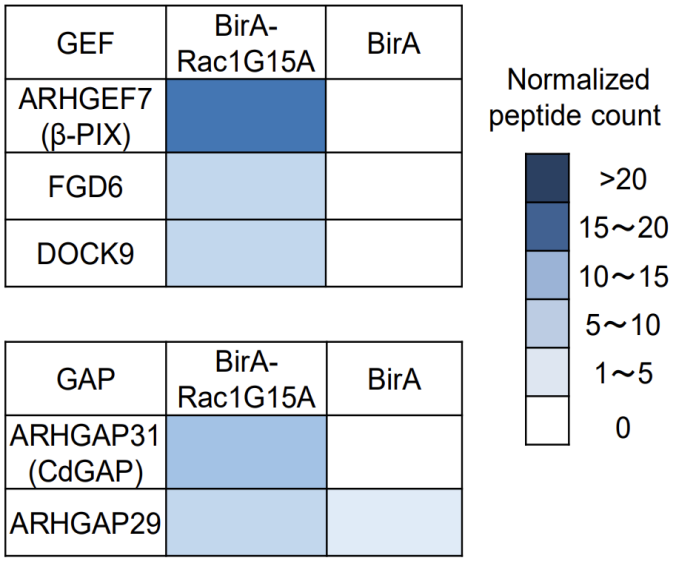



**Figure 4: Bio-ID identifies ARHGEF7 (β-PIX) and ARHGAP31 (CdGAP) as the top Rac1-interacting GEF and GAP in podocytes, respectively.** Heatmap of normalized peptide count towards three GEFs and two GAPs showing significant enrichment in BirA-Rac1G15A expressing podocytes vs. in control podocytes. BioID experiment was done by the Takano lab.

1.2.4.1 CdGAP

ARHGAP31 was initially discovered as a ubiquitously expressed (with greater prominence in heart and lung tissue) GTPase-activating protein towards Rac1 and Cdc42, but not RhoA. Thus, it was appropriately named Cdc42 GTPase-activating protein, or CdGAP. Initial microinjection of CdGAP in Swiss 3T3 cells abolished membrane ruffling and filopodia formation, demonstrating its effects on focal adhesion dynamics (172). Since then, CdGAP has been extensively studied for its role in cancer, as it regulates functions like cell spreading, lamellipodia formation, migration, and maintenance of cell polarity, but also modulates E-cadherin transcription (173–177). CdGAP has also been implicated in angiogenesis and organogenesis (178, 179).

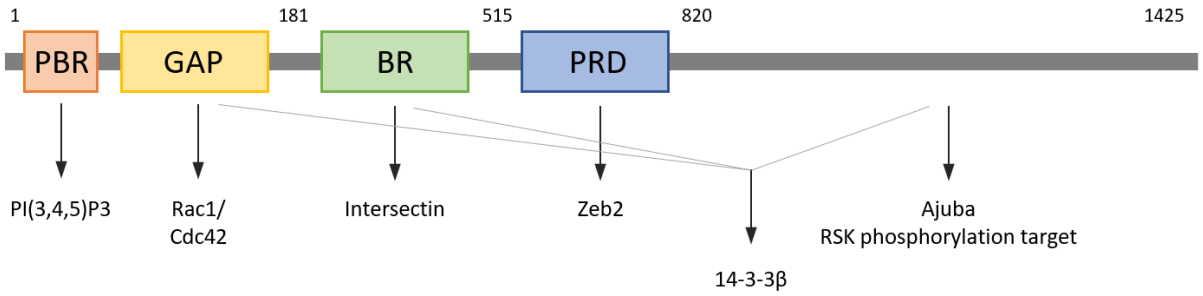



**Figure 5: Domain structure of CdGAP and associated binding partners.** CdGAP contains a N-terminal stretch of polybasic residues (PBR), a GAP domain, a basic-rich (BR) domain, a proline-rich domain (PRD), and an extended C-terminus. Interactions of individual CdGAP domains to their corresponding binding partners are denoted by arrows. Amino acid mapping is indicated above each domain.

CdGAP is a large multidomain protein subject to many interactions (**Fig. 5**). At its N-terminal is a GAP domain preceded by a stretch of polybasic residues (PBR) that mediates binding to negatively charged phospholipids. Phosphatidylinositol 3,4,5-triphosphate (PI(3,4,5)P3) can bind this PBR to stimulate GAP activity of CdGAP, thus targeting CdGAP activity to the plasma membrane (180). The basic-rich (BR) domain that follows likewise mediates CdGAP activity; Intersectin (a scaffolding protein and Cdc42 GEF) can bind the BR to inhibit CdGAP GAP activity, likely though conformational change involving the PBR (167, 177, 181–183). 14-3-3β likewise interacts with the BR (as well as the GAP domain and C-terminal RSK-phosphorylated serine

residues), sequestering it into the cytoplasm and inhibiting its GAP activity and transcriptional repression of E-cadherin (174, 176). This transcriptional repression is mediated by the proline-rich domain (PRD) that is adjacent to the BR; the PRD of nuclear CdGAP interacts with Zeb2 and, in a GAP-independent manner, downregulates E-cadherin expression, consequently disrupting adherens junctions and increasing cell migration, invasion, and cancer metastasis (176).

The PRD is followed by the C-terminus, which does not have any defined domains, but is an important site that allows CdGAP to act as an intermediary of the Ras/MAPK and Rac1 pathways; Ras activation promotes downstream phosphorylation on Thr-776 of the CdGAP PRD by ERK and RSK, thus downregulating its GAP activity and allowing for increased Rac1 activation (184). Thr-776 is also subject to phosphorylation by GSK-3, resulting in the serum-dependent stability of CdGAP protein levels and allowing for tight regulation of CdGAP activity under varying cellular conditions (185). The PRD is also a binding site for 14-3-3β (alongside the BR domain), as well as the scaffolding protein Ajuba, which binds CdGAP and Rac1 at two distinct domains to facilitate spatiotemporal regulation of Rac1 activity and cell-cell junction stability (176, 186). Collectively, these interactions demonstrate the complex regulation of CdGAP activity in cytoskeletal dynamics.

*In vivo,* a CdGAP truncation mutant has been linked to a disease called Adams-Oliver syndrome (179). Although varied in phenotype, this condition is characterized by terminal transverse limb defects and aplasia cutis congenita (a congenital absence of skin) (187). Molecularly, the responsible CdGAP mutant lacks the C-terminus and part of the PRD, and causes a gain of function of GAP activity, disrupting the actin cytoskeleton (179). In mice, CdGAP knockout causes incompletely penetrant (44%) embryonic/perinatal lethality that can be attributed to vascular defects and edema (178). Study of renal phenotype in surviving CdGAP knockout mice generated by the Takano lab revealed that males, but not females, developed mild proteinuria at 6 months. Podocyte-specific CdGAP knockout mice followed a similar trend, with males developing proteinuria at 7 months of age, together with foot process effacement at 12 months of age, while females showed neither. Together, these findings highlight the importance of CdGAP regulation in cytoskeletal dynamics, and in the context of renal pathology, to podocyte health.

1.2.4.2 β-PIX

β-PIX was originally discovered, together with its homologous protein ARHGEF6 (α-PIX), as a p21-acivated kinase (PAK)-binding protein that bound a conserved proline-rich domain within PAK and localized to focal adhesions. Overexpression of β-PIX in NIH 3T3 cells promoted the formation of membrane ruffles. Although it is a Rac1/Cdc42 GEF, its main function was originally believed to be a scaffolding focal adhesion protein (188–190). In fact, β-PIX is a multidomain protein whose functions extend beyond PAK binding (**Fig. 6**). Structurally, α-PIX and β-PIX share most of their domains, apart from the calponin homology (CH) domain found at the N-terminal of α-PIX, which is absent from β-PIX. This CH domain can bind β-parvin (an integrin signaling adaptor protein), and a deletion mutation within this domain has been linked to X-linked mental retardation, highlighting the importance of the α-PIX-β-parvin interaction in the brain (191, 192).

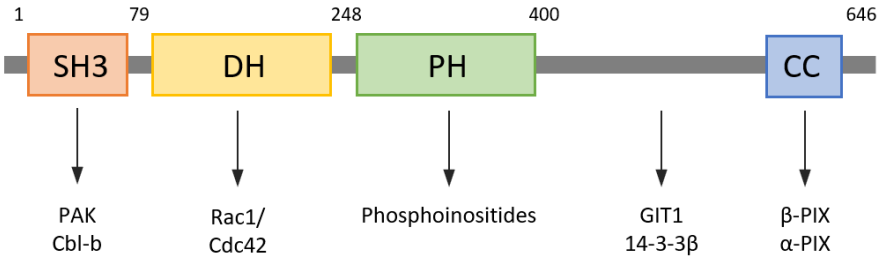



**Figure 6: Domain structure of β-PIX and associated binding partners.** β-PIX contains a Src homology 3 (SH3) domain, a Dbl homology (DH) domain, a pleckstrin homology (PH) domain, and a C-terminus containing a GIT1-binding domain (GBD; indicated only by arrow) and a coiled-coil (CC) domain. Interactions of individual β-PIX domains to their corresponding binding partners are denoted by arrows. Amino acid mapping is indicated above each domain.

Next to the CH domain, and at the N-terminal of the β-PIX protein is the Src homology 3 (SH3) domain, which binds to the focal adhesion protein p21-activated kinase (PAK) (190). The β-PIX-PAK binding interaction allows for the targeting of PAKs to focal adhesion complexes, promoting their reorganization (193). Although PAKs are regulatory targets of both Rac1 and Cdc42, a mechanism has emerged whereby membrane localized β-PIX activates Cdc42, which in turn causes the activation, autophosphorylation, and dissociation of PAK from β-PIX. This in turn

allows β-PIX to bind Rac1 and target it to the membrane, promoting actin polymerization, cell spreading, and directional cell migration (194). The SH3 domain of β-PIX however can bind not only PAK, but also the ubiquitin ligase Cbl, which plays a role in EGFR downregulation. In this feedback loop, β-PIX (in complex with Cdc42) sequesters Cbl and prevents it from downregulating EGFR, while free Cbl in turn promotes degradation of β-PIX. This mechanism highlights the role of β-PIX in the regulation of EGFR-mediated signaling (195).

Next to the SH3 domain of β-PIX is the Dbl homology (DH) domain, which is required for nucleotide exchange on Rac1 and Cdc42 (196). The DH domain is followed by the pleckstrin homology (PH) domain, which allows the targeting of β-PIX to membrane phosphoinositides, thus mediating local Rac1/Cdc42 activity (197). Next to the PH domain of β-PIX is the C-terminus, which mediates several protein interactions**.** Here, the G protein-coupled receptor kinase-interacting target (GIT) binds the β-PIX and assists with recruitment of the PIX-PAK complex to focal adhesions. The GAP activity of p95-APP1 (the chicken homologue of human GIT1) towards the endosomal compartmental localized Arf6 has shed further light on the GIT-PIX-PAK complex as it shuttles between endosomal compartments, focal adhesions, and the leading edge of cells. This shuttling allows the complex to mediate actin reorganization at the leading edge of lamellipodia and, upon the breakdown of focal adhesions (i.e., in cellular migration), return to the cell body (198, 199). Thus, β-PIX plays an important role in the targeting of actin reorganization via the GIT-PIX-PAK complex.

In addition to GIT binding, the C-terminus of β-PIX allows to dimerize (via a leucine zipper coil-coil (CC) domain) (200). This dimerization is believed to enhance the PH domain-mediated membrane targeting, and although typically results in the form of a β-PIX homodimer, has also been demonstrated as an α/β-PIX heterodimer *in vivo* (191, 197). Lastly, the C-terminus of dimeric, but not monomeric $\beta_1$-PIX (the predominant β-PIX isoform in the kidney) has also exhibited PKA-dependent binding to 14-3-3β, which in turn downregulates the GEF activity of $\beta_1$-PIX (201, 202). The same study revealed that dimerization is required for mediation of subcellular localization as well as high GEF activity towards Rac1 but not Cdc42 (202). Thus, the C-terminus of β-PIX is an important regulatory site that can affect localization as well as Rac1/Cdc42 activity.

*In vivo*, systemic β-PIX knockout is embryonic lethal owing to defective anterior visceral endoderm migration (203, 204). Podocyte specific β-PIX knockout mice are instead born with low

body weight and between 8-13 weeks of age, develop onset of progressive proteinuria, glomerulosclerosis, foot process effacement, and podocyte loss. They likewise have greater susceptibility than wildtype mice to LPS-mediated kidney injury. In cultured podocytes, β-PIX deficiency promotes decreased Cdc42 activity, which in turn blunts Yes-associated protein (YAP) transcriptional co-activation of cell proliferation, thereby contributing to increased cell apoptosis (205). Other studies have supported the notion that β-PIX promotes a migratory cell phenotype; it prevents maturation of focal adhesions and instead supports lamellipodial and ruffle formation as well as focal adhesion turnover (201, 206). Thus, β-PIX is required for podocyte maintenance, and may support glomerular function through both its roles as a focal adhesion protein and Cdc42/YAP regulator.

## 1.3 Study rationale and aims

**Rationale:** The identification of CdGAP and β-PIX as the top Rac1-modulating GAP and GEF in glomerular podocytes prompted further investigation by the Takano lab. Further investigation revealed that CdGAP and β-PIX interact by co-immunoprecipitation, although it is unknown whether this interaction is direct. Functional GAP-GEF interactions have, however, been identified between (1) CdGAP and Intersectin, and (2) β-PIX and Slit-Robo Rho GTPase activating protein 1 (srGAP1) (182, 183, 206), raising the possibility that there may be a functional link between CdGAP and β-PIX. This formed the basis for the hypothesis that CdGAP and β-PIX interact physically and functionally in the regulation of the downstream Rho GTPase targets Rac1 and Cdc42.

**Aim 1**: Investigate the biochemical nature of the CdGAP-β-PIX interaction.

**Aim 2**: Investigate the functional nature of the CdGAP-β-PIX interaction.

# Chapter 2: Materials and Methods

*Cell culture, transient transfection, and cell lysis*

HEK 293 cells were cultured in DMEM containing 10% fetal bovine serum (FBS) with 1% penicillin/streptomycin (Gibco) at 37˚C in 5% $CO^2$. Immortalized human podocytes from Dr. Moin Saleem (207) were cultured in RPMI containing 10% fetal bovine serum (FBS) with 1% penicillin/streptomycin (Gibco) at permissive conditions (33˚C in 5% $CO_2$). Where applicable, podocytes were differentiated for 7 days at non-permissive conditions (37˚C in 5% $CO_2$).

For all transfection experiments, HEK 293 cells were transfected using Lipofectamine 2000 (Invitrogen), following manufacturer's protocol. Cells were transfected 24 hours prior to lysis, and transfection efficiency was verified by immunofluorescence where possible.

HEK 293 cells and podocytes were lysed with ice-cold lysis buffer (10 mM Tris [pH 7.5], 1 mM EDTA [pH 8.0], 1 mM EGTA [pH 8.0], 125 mM sodium chloride, 10 mM sodium pyrophosphate, 25 mM sodium fluoride, 2 mM sodium orthovanadate, 1% Triton X-100 [Sigma-Aldrich], and protease inhibitor cocktail [Roche]). Following cell lysis, lysates were centrifuged at 10,000 x *g* for 15 minutes at 4˚C to remove insoluble materials, protein concentrations were determined using Pierce BCA Protein Assay Kit (Thermo Fisher).

*Generation of CdGAP and β-PIX knockdown cell lines*

β-PIX knockdown mouse podocytes and CdGAP knockdown human podocytes were established using MISSION short hairpin RNA (shRNA) Lentiviral Transduction Particles (Sigma Aldrich) with puromycin (Wisent Inc.) selection.

Podocytes with CdGAP and β-PIX KD and their controls were established using MISSION Lentiviral shRNA (Sigma-Aldrich, TRCN0000047640, TRCN0000110029, and SHC001, respectively). HEK293T cells were transiently transfected using the lentiviral packaging system (abm, LV003) according to the manufacturer's instructions. Virus-containing supernatants were added to podocytes under permissive conditions for 16 hours. Puromycin (Wisent Inc.) was added 48 hours later and puromycin-resistant cells were pooled for further experiments.

Knockdown of β-PIX in mouse podocytes was verified by immunoblotting for β-PIX alongside tubulin control. Knockdown of CdGAP in human podocytes was verified by immunoblotting for CdGAP alongside tubulin control.

*Immunofluorescence*

HEK 293 cells and undifferentiated podocytes were passaged and immediately plated on glass cover slips coated with 5% laminin 521 (Corning). Podocytes were allowed to differentiate for 7 days on the cover slips at non-permissive conditions prior to fixing. HEK 293 cells were transfected using Lipofectamine 2000 (Invitrogen), following manufacturer's protocols, 24 hours prior to fixing. Where applicable, cells were stimulated with epidermal growth factor (EGF) (100 ng/ml) prior to fixing. (EGF stimulation was 5 minutes long for Rho GTPase activity assays and 30 minutes long for immunofluorescent staining of CdGAP and β-PIX.) Cells were fixed with 4% PFA and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich), blocked with 3% bovine serum albumin in PBS, immunostained with relevant antibodies (see table below), and stained with 4',6-diamidino-2-phenylindole (DAPI) and phalloidin. Immunofluorescence images were obtained using a Zeiss LSM780 laser scanning confocal microscope with the Zeiss Plan Apochromat 63x 1.40 Oil DIC objective. All imaging parameters were maintained constant throughout image acquisition of all samples.

*IncuCyte wound healing assay*

HEK 293 cells were transfected using Lipofectamine 2000 (Invitrogen), following manufacturer's protocols. 24 hours later, transfection was validated by microscopy. Cells were transferred to a 96-well plate coated with 5% fibronectin (Sigma-Aldrich) into full serum media, and once at a confluent state, a scratch wound was made using the IncuCyte 96-well Wound Maker (Sartorius). Cell scanning was initiated immediately for a period of 24 hours, and migration rate was analyzed using the IncuCyte Analysis Software (Sartorius).

*Co-immunoprecipitation assay*

HEK 293 cells were transfected using Lipofectamine 2000 (Invitrogen) following manufacturer's protocol. Lysates were incubated with 0.5 μg anti-GFP antibody overnight at 4˚C, and then with protein A agarose beads (Santa Cruz, sc-2001) for 2 hours at 4˚C. Beads were washed three times with lysis buffer. Proteins were eluted into SDS loading buffer and boiled for 5 minutes at 95˚C. Immunoprecipitation was assessed by SDS-PAGE and subsequent immunoblotting.

*Rho-GTPase activity assay*

HEK 293 cells were transfected using Lipofectamine 2000 (Invitrogen) following manufacturer's protocol, 24 hours prior to lysis. Podocytes were differentiated for 7 days prior to lysis. Lysates (~1 mg) were incubated with GST beads (GE Healthcare Bio-Sciences) fused to Cdc42-Rac1 interactive binding domain for 1 hour at 4˚C. Beads were washed three times with binding buffer (25 mM Hepes, 30 mM magnesium chloride, 40 mM sodium chloride, 0.5% Triton X-100 [Sigma-Aldrich], 1mM DDT). Proteins were eluted into SDS loading buffer and boiled for 5 minutes at 95˚C. Rac1/Cdc42 activity was assessed by SDS-PAGE and subsequent immunoblotting.

*Immunoblotting*

Equal amounts of samples were resolved by electrophoresis on a sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gel with a concentration of 7.5% for coimmunoprecipitation experiments and 12% for Rho-GTPase activity assay experiments. Proteins were transferred to a nitrocellulose membrane which was then blocked in 5% non-fat dry milk/TBS solution for one hour at room temperature and incubated in the primary antibody (in a solution of 5% BSA/TBS) overnight at 4˚C. The following day, the membrane was subjected to three five-minute washes in wash buffer and incubated in the secondary antibody for two hours (in a solution of 5% BSA/TBS with 0.2% Tween) at room temperature. After three more five-minute washes in wash buffer, the membrane was imaged using a LiCOR fluorescence imager.

*Localization analyses*

Localization was assessed via Zen Black Image Analysis Software (Zeiss). Regions of interest were manually traced with the tracing tool and mean channel intensity of each channel was calculated. Colocalization was assessed by Pearson's Colocalization Coefficient (PCC). Costes for PCC were set manually and held constant between experiments.

*Cell morphology analyses*

Cell area (morphology) was assessed via ImageJ (NIH). The tracing tool was used to manually trace the perimeter of every cell, thereby generating measurement, and calculating area. Area was assessed as a unit of pixels.

*Statistics*

Statistical analyses were performed using GraphPad Prism 9.2.0 (La Jolla California, USA). Results are expressed as the mean ± standard error of the mean (SEM). Multiple-group comparisons were performed using analysis of variance (ANOVA) with post-testing using the Tukey-Kramer test. Differences between two experimental values were assessed using the Student's T-test. $p < 0.05$ was considered statistically significant.

*Antibodies and reagents*

| **Antibodies and reagents** | **Supplier and catalogue number** |
|---|---|
| Cdc42 | NEB, 2466T |
| CdGAP | Sigma-Aldrich, HPA036380 |
| DAPI | Invitrogen, D21490 |
| Epidermal growth factor (EGF) | BioShop, E012 |
| GFP | Invitrogen, G10362 for IP |
| GFP | Invitrogen, A6455 for IB |
| mCherry | Invitrogen, 16D7 |
| Promo-Fluor-647-phalloidin | PromoKine, PK-PF647P-7-01 |
| Rac1 | Millipore, 05-389 |
| α-tubulin | Sigma-Aldrich, T5168 |

**Secondary antibodies**

Alexa 488-conjugated secondary anti-mouse antibody (CST, 4408)
Alexa 555-conjugated secondary anti-rabbit antibody (CST, 4413)
LiCOR IRDye 680RD
    926-68072, anti-mouse
    926, 68073, anti-rabbit
LiCOR IRDye, 800CW
    926-32210, anti-mouse
    926, 32211, anti-rabbit

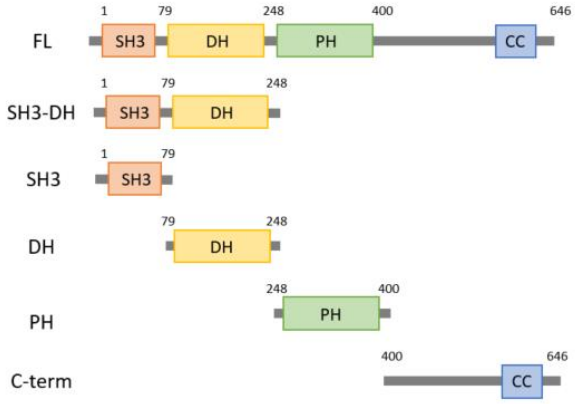
# Chapter 3: Results

## 3.1 Investigating the biochemical nature of the CdGAP-β-PIX interaction

### *β-PIX binds CdGAP via its SH3-DH domain, and in an inconsistent manner, its C-terminus*

To better understand the biochemical nature of the CdGAP-β-PIX interaction, we aimed to resolve the interacting domain within each protein. Jun Matsuda had previously identified (via coimmunoprecipitation) the BR domain of CdGAP to be responsible for binding β-PIX. However, the CdGAP-binding domain of β-PIX was unresolved. To determine which domain(s) of β-PIX were responsible for the interaction with CdGAP, mCherry-tagged constructs of the SH3, DH, PH, and C-terminus domains of β-PIX were co-transfected with GFP-CdGAP into HEK 293 cells (**Fig. 7A**). Owing to the low transfection efficiency (likely due to cytotoxicity) of the DH domain, a joint mCherry-SH3-DH domain construct was also included to help assess DH domain binding to CdGAP. Following transfection, lysates were subjected to immunoprecipitation with an anti-GFP antibody, and mCherry immunoblotting revealed CdGAP-binding β-PIX constructs (**Fig. 7B**). It was found that full length mCherry- β-PIX, together with mCherry-SH3-DH, bound to GFP-CdGAP. The SH3 domain alone did not bind to β-PIX, but the DH domain binding could not be verified owing to either a weak expression or signal overlap with heavy IgG banding at the expected molecular weight. Lastly, the mCherry-C-terminus construct exhibited binding in some, but not all experiments, suggesting an inconsistent or weak interaction with CdGAP.

A




B

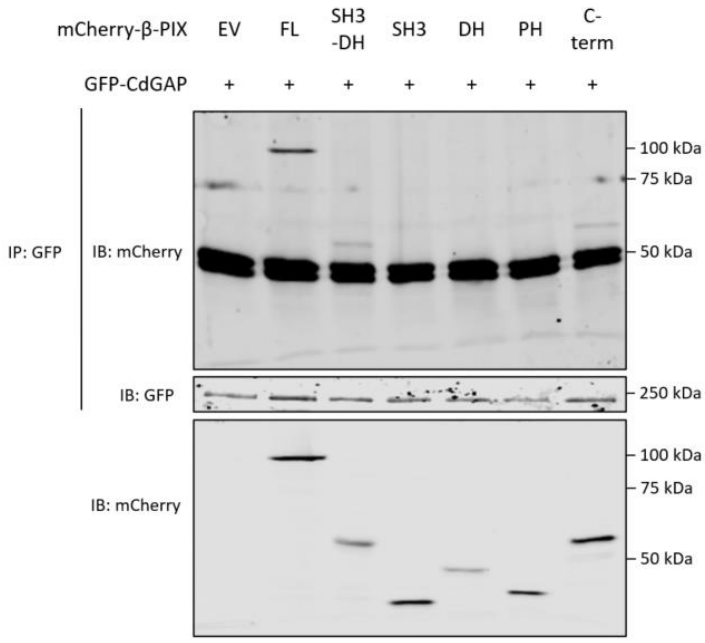



**Figure 7: The SH3-DH domain and C-terminus of β-PIX can coimmunoprecipitate with CdGAP.** (**A**) Schematic representation of the β-PIX deletion mutants used in this coimmunoprecipitation experiment. (**B**) Cell lysates of HEK 293 cells overexpressing mCherry-β-PIX deletion constructs and full length GFP-CdGAP were immunoprecipitated with anti-GFP antibody (for CdGAP) followed by immunoblotting for mCherry (for β-PIX). EV: empty vector; SH3-DH: src homology 3 and Dbl homology; SH3: Src homology 3; DH: Dbl homology; PH: pleckstrin homology; C-term: C-terminus.

3.2 Investigating the functional nature of the CdGAP- β-PIX interaction

*CdGAP and β-PIX colocalize in the cytosol of HEK 293 cells*

In investigating the functional interaction of CdGAP and β-PIX, we began by studying their localization. Studies were initiated in HEK 293 cells for technical simplicity. In brief, GFP-CdGAP and FLAG-β-PIX were overexpressed in HEK 293 cells, fixed, immunostained, and visualized by confocal microscopy. We found that β-PIX was distributed throughout the cytosol while CdGAP was either in the nucleus, cytosol (where it colocalized with β-PIX), or both (**Fig. 8**). CdGAP localization data was in line with observations from the lab of Dr. Lamarche.

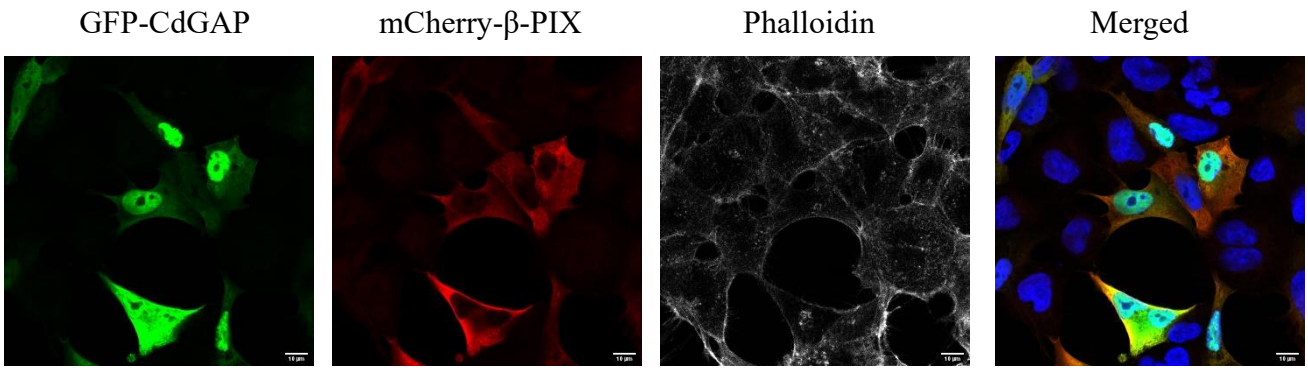



**Figure 8: CdGAP and β-PIX colocalize in the cytosol of HEK 293 cells.** GFP-CdGAP and FLAG-β-PIX were overexpressed in HEK 293 cells. Cells were subjected to staining by antibody and localization of proteins was assessed via confocal microscopy. Overexpression of GFP-CdGAP (green) shows nuclear and cytosolic localization, while FLAG-β-PIX (red) is restricted to the cytosol. Bar: 10 μm.

*CdGAP and β-PIX colocalize in the cytosol of human podocytes and jointly migrate to the cell periphery upon EGF stimulation*

CdGAP and β-PIX are both known focal adhesion proteins (177, 190, 201, 208). Given their capacity to regulate focal adhesion dynamics, as well as their binding interaction *in vitro*, we hypothesized that they may interact at the site of focal adhesions. To test this, differentiated human podocytes were stimulated with EGF for 30 minutes (or left untreated) and subsequent localization of endogenous CdGAP and β-PIX was studied (**Fig. 9A**). A stimulation period of 30 minutes was chosen as it was found to be sufficient to induce a change in localization, while shorter stimulation periods (e.g., 15 minutes) resulted in fewer changes (data not shown). In control podocytes, CdGAP signal was nuclear and cytosolic, the latter colocalizing with β-PIX. Very low levels of additional β-PIX could be found at the plasma membrane. However, upon EGF stimulation, both CdGAP and β-PIX signal at the plasma membrane increased (**Fig. 9B-C**) and appeared to colocalize. Quantification of CdGAP and β-PIX colocalization at the cellular periphery (as defined by the outer 10 μm of the cell) revealed that EGF stimulation promoted a modest but statistically significant increase of colocalization of peripheral CdGAP and β-PIX (**Fig. 9D**), while whole cell colocalization (i.e., cytosolic) remained unchanged (**Fig. 9E**). These data suggest that CdGAP and β-PIX jointly translocate to the plasma membrane upon EGF stimulation.

Of note, CdGAP knockdown in human podocytes significantly increased the levels of peripheral β-PIX localization when compared to control podocytes (**Fig. 9F**). This finding suggests that CdGAP preserves cytosolic β-PIX in the absence of EGF, but upon EGF stimulation, the two proteins migrate to the plasma membrane where they likely participate in the regulation of focal adhesion dynamics.

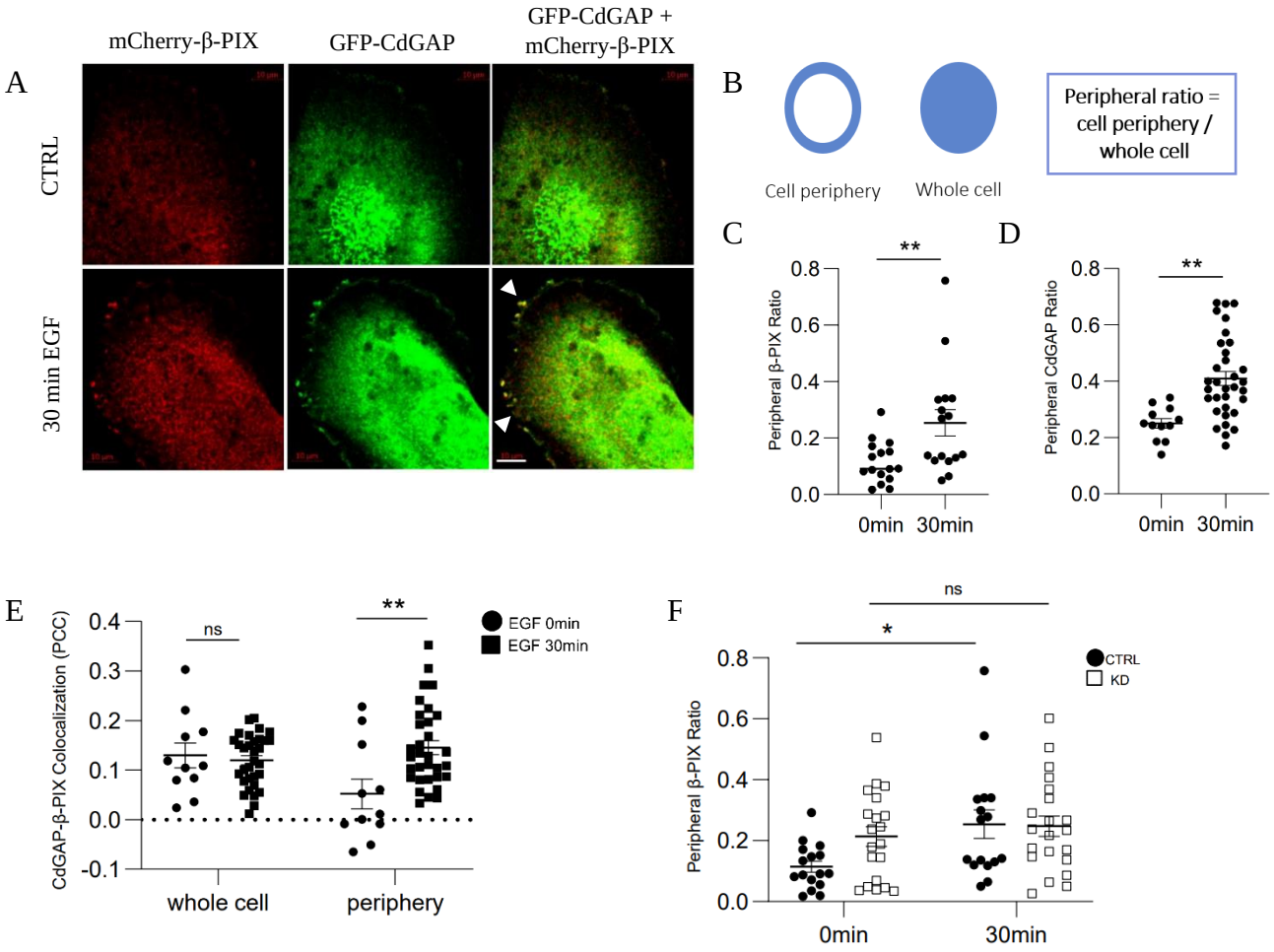



**Figure 9: EGF stimulation promotes translocation and subsequent colocalization of CdGAP and β-PIX at the cell membrane.** Cultured human podocytes were stimulated with EGF (30 min) and compared to an unstimulated (0 min) control condition. (**A**) Staining of endogenous CdGAP (green) and β-PIX (red) in control and EGF-stimulated human podocytes. Arrowheads in lower right panel point to areas of CdGAP and β-PIX colocalization at cell periphery. (**B**) Schematic illustrating the selection of regions of interest and subsequent calculation, whereby the cell periphery encompasses the outer 10 µm at the cell membrane. (**C**) Quantification of peripheral β-PIX signal in EGF-stimulated and control podocytes. (**D**) Quantification of peripheral CdGAP signal in EGF-stimulated and control podocytes. (**E**) Quantification via Pearson's Colocalization Coefficient (PCC) of CdGAP-β-PIX colocalization in whole cell and cell periphery in control and EGF-stimulated podocytes. (**F**) Quantification of peripheral β-PIX signal in control vs CdGAP knockdown podocytes, with and without EGF stimulation. n = 12, 33 (C); 16 (D); 11-32 (E); 16-21 (F). Statistically significant differences (*$P$ < 0.05, **$P$ < 0.01, ns = not significant) are indicated. Bar: 10 µm (A).

*Overexpression of CdGAP and β-PIX jointly, but not of β-PIX alone, promotes cell migration*

To assess the effects of CdGAP and β-PIX on focal adhesion dynamics, we began by testing their effect on cell migration. In brief, CdGAP alone, β-PIX alone, or CdGAP and β-PIX together (alongside an empty vector (EV) control condition) were overexpressed in HEK 293 cells. After validation of transfection efficiency (**Fig. 10A**), cells were seeded to full confluency into a 96-well plate and a scratch wound was created in each well (**Fig. 10B**). Wound closure, or cell migration, was assessed over the following 24 hours. Quantification of relative wound density at 12 hours (**Fig. 10C**) revealed that doubly overexpressing CdGAP and β-PIX cells, as well as CdGAP overexpressing cells, migrated significantly faster than the empty vector control condition. β-PIX overexpressing cells appeared to migrate slower than empty vector control cells, though this was not statistically significant. Together, these results suggest that CdGAP, both alone and together with β-PIX, promotes cellular migration.

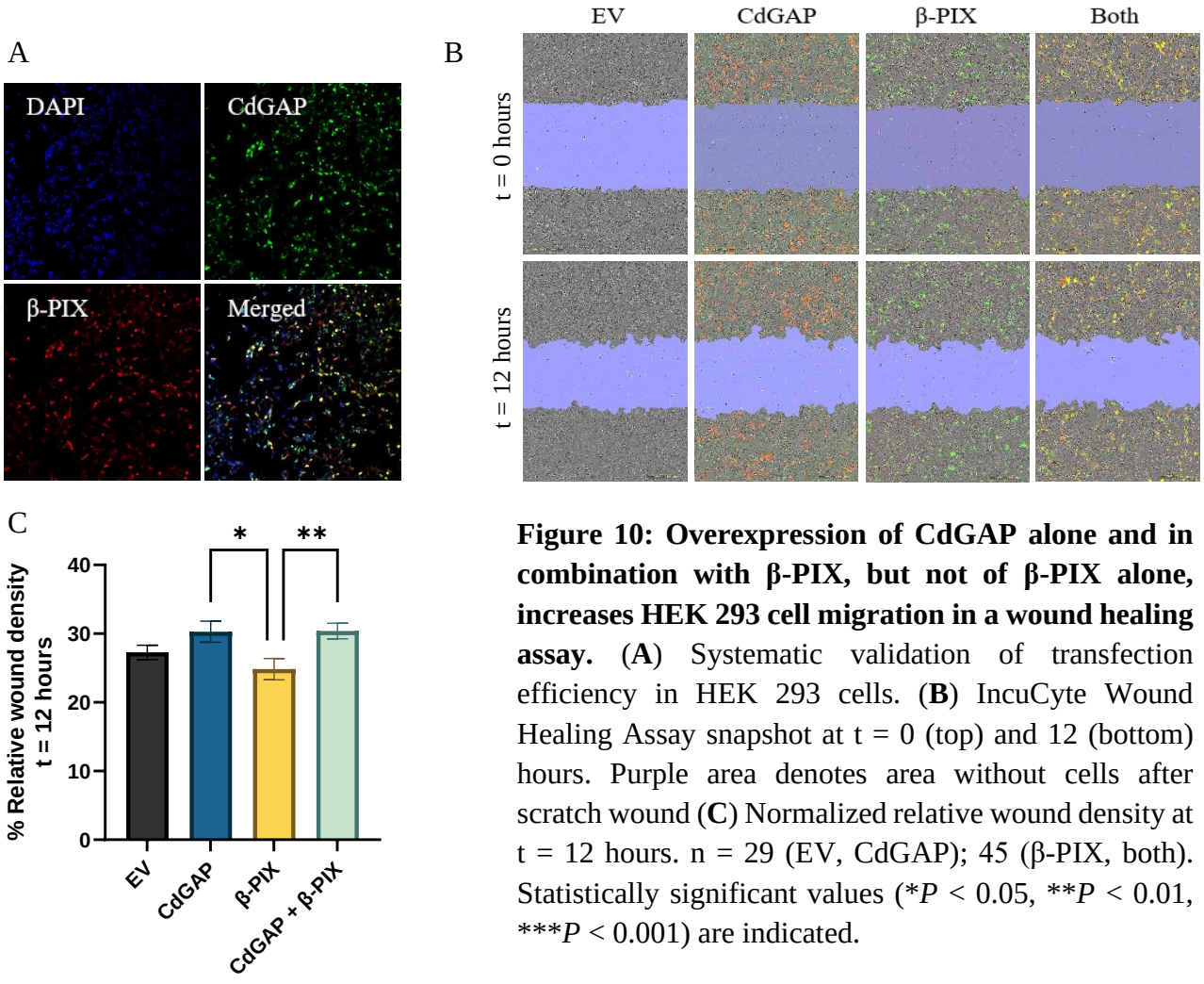



**Figure 10: Overexpression of CdGAP alone and in combination with β-PIX, but not of β-PIX alone, increases HEK 293 cell migration in a wound healing assay.** (**A**) Systematic validation of transfection efficiency in HEK 293 cells. (**B**) IncuCyte Wound Healing Assay snapshot at t = 0 (top) and 12 (bottom) hours. Purple area denotes area without cells after scratch wound (**C**) Normalized relative wound density at t = 12 hours. n = 29 (EV, CdGAP); 45 (β-PIX, both). Statistically significant values (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) are indicated.

*CdGAP knockdown promotes cell spreading while β-PIX knockdown promotes mild cell rounding*

To assess the effects of CdGAP and β-PIX on cell morphology, two podocyte knockdown lines were generated by the Takano lab. Knockdown of both CdGAP and β-PIX was confirmed by immunoblotting (data not shown). Morphology, or cell rounding versus spreading, was assessed by measurement of cell area. This was accomplished by manual tracing of the cell perimeter in ImageJ. In human podocytes, CdGAP knockdown caused a mild spreading effect with a prominent increase in stress fibers (**Fig. 11A**). Quantification demonstrated that CdGAP knockdown cells had a larger cell area than control cells (**Fig. 11B**). Conversely, β-PIX knockdown in mouse podocytes induced a decrease in cell ruffling, and with it, a mild rounding effect (**Fig. 11C**). This translated to a mild but statistically significant decrease in cell area (**Fig. 11D**). Thus, knockdown of CdGAP and β-PIX in podocytes appears to have opposite effects on cell area, whereby CdGAP knockdown promotes cell spreading and β-PIX knockdown promotes mild cell rounding.

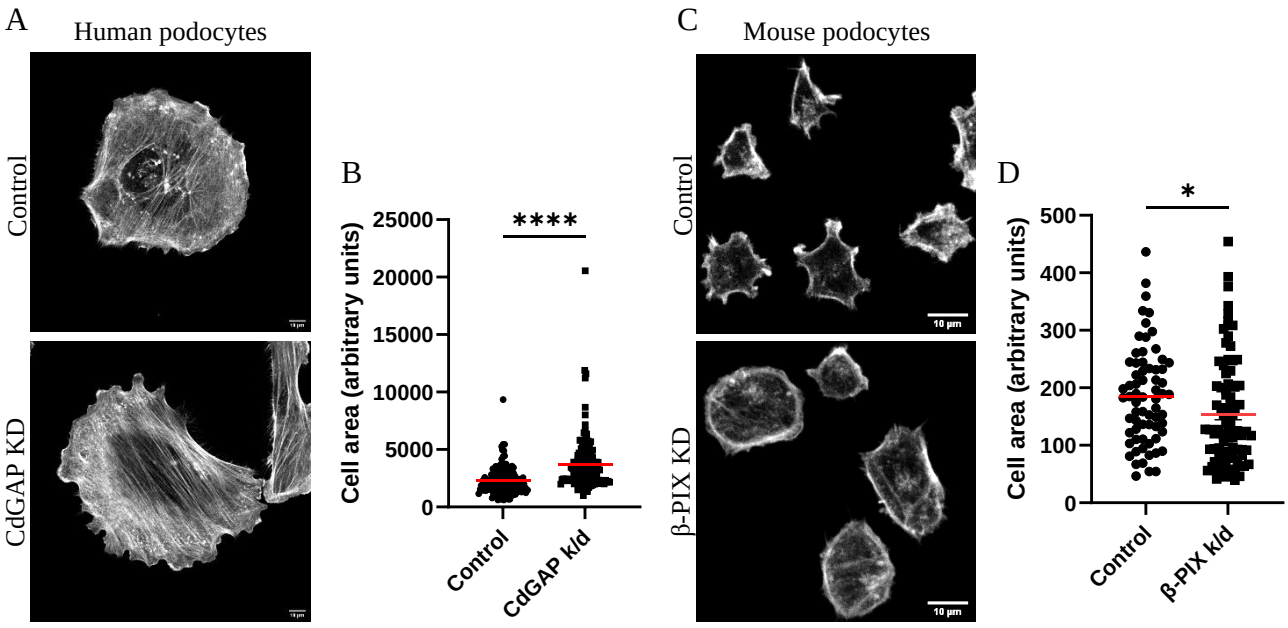



**Figure 11: CdGAP and β-PIX affect morphology in podocytes.** (**A**) Control (top) and CdGAP knockdown (bottom) human pocoytes stained for phalloidin (far red). (**B**) Quantification of cell area in arbitrary units (pixels) of control and CdGAP knockdown human podocytes. (**C**) Control (top) and β-PIX knockdown (bottom) mouse podocytes stained for phalloidin (far red). (**D**) Quantification of cell area in arbitrary units (pixels) of control and β-PIX knockdown mouse podocytes. n = 155, 164 (A); 68, 85 (B). Statistically significant values (*$P$ < 0.05, **$P$ < 0.01, ***$P$ < 0.001, ****$P$ < 0.0001) are indicated. Bar: 10 µm (A, C). Mean bars coloured for increased visibility (B, D).

*CdGAP is required for EGF-mediated Rac1 and Cdc42 activation*

Thus far, CdGAP knockdown has resulted in translocation of β-PIX to the cell membrane (**Fig. 9A**, **F**), as well as increased cell spreading (**Fig. 11A**, **B**). Given its known GAP activity towards Rac1 and Cdc42, the Takano lab examined whether CdGAP knockdown results in an increase of Rac1/Cdc42 activities in podocytes. Control or CdGAP knockdown podocytes were either left at basal conditions or stimulated with EGF for 5 minutes (a sufficiently short stimulation period to observe transient Rho GTPase activity). Rac1 and Cdc42 activity levels were detected via GST-CRIB pulldown. As expected, CdGAP knockdown podocytes exhibited greater basal Rac1 and Cdc42 activities than control podocytes (**Fig. 12**). When stimulated with EGF, control podocytes demonstrated an increase in Rac1 and Cdc42 activity (**Fig. 12**). CdGAP knockdown podocytes, however, failed to respond to EGF stimulation, as their Rac1 and Cdc42 activity levels did not change (**Fig. 12**). This suggests that CdGAP maintains low basal Rac1 and Cdc42 activity levels but is required for EGF-mediated stimulation of Rac1 and Cdc42.

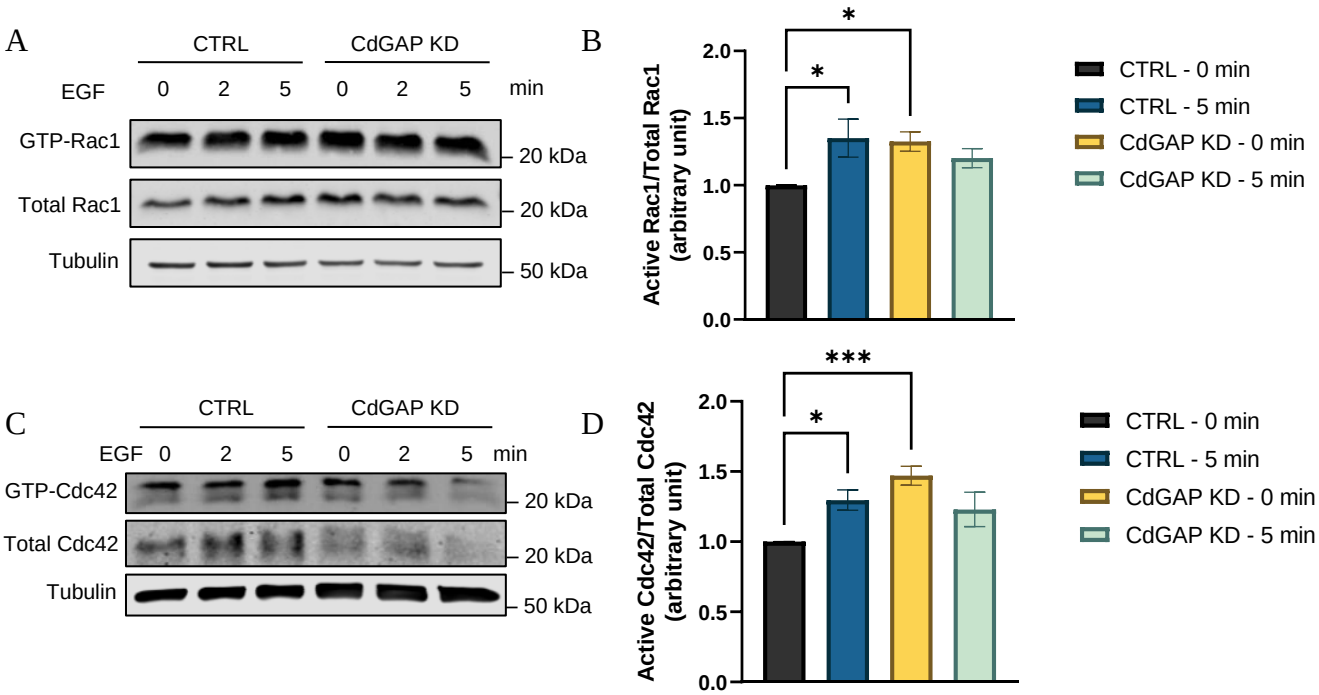



**Figure 12: CdGAP knockdown enhances basal Rac1 and Cdc42 activities but blunts further EGF-induced Rac1 and Cdc42 activation.** (**A** and **C**) Cell lysates from CdGAP knockdown and control (CTRL) podocytes, treated or untreated with 100 ng/ml EGF, were subjected to pull-down with GST-CRIB for active (GTP-bound) Rac1 (**A**) and Cdc42 (**C**). Representative immunoblots are shown. (**B** and **D**) Densitometric quantification of active GTP-bound forms of Rac1 (**B**) and Cdc42 (**D**) normalized to total Rac1 and Cdc42, respectively. n = 7 (B); 9 (D). Statistically significant values (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) are indicated.

*CdGAP does not inhibit β-PIX mediated Rac1 activation*

Given the requirement for CdGAP in EGF-mediated Rac1 and Cdc42 stimulation in podocytes (**Fig. 12**), we assessed the effects of CdGAP and β-PIX on Rac1 and Cdc42 activity under basal conditions. We hypothesized that if CdGAP is required for EGF-mediated activation of Rac1 and Cdc42, it may act synergistically with β-PIX, to support β-PIX Rac1/Cdc42 GEF activity. HEK 293 cells were transfected with either an empty vector (EV) control, β-PIX alone, CdGAP alone, or β-PIX and CdGAP together. Rac1 and Cdc42 activity levels were detected via GST-CRIB pulldown (**Fig. 13**). As expected, β-PIX showed GEF activity towards both Rac1 and Cdc42, with greater activation of Cdc42 than Rac1. CdGAP, conversely, blunted activity levels of Cdc42, but unexpectedly did not show any detectable GAP activity towards Rac1 (**Fig. 13**). Double overexpression of CdGAP and β-PIX resulted in Rac1 and Cdc42 activity that was comparable to that of β-PIX alone (**Fig. 13**). Thus, CdGAP and β-PIX did not appear to have an additive effect on Rac1 nor Cdc42 activity. Instead, this finding suggests that despite its known GAP activity, CdGAP does not inhibit the β-PIX-mediated activation of Rac1.

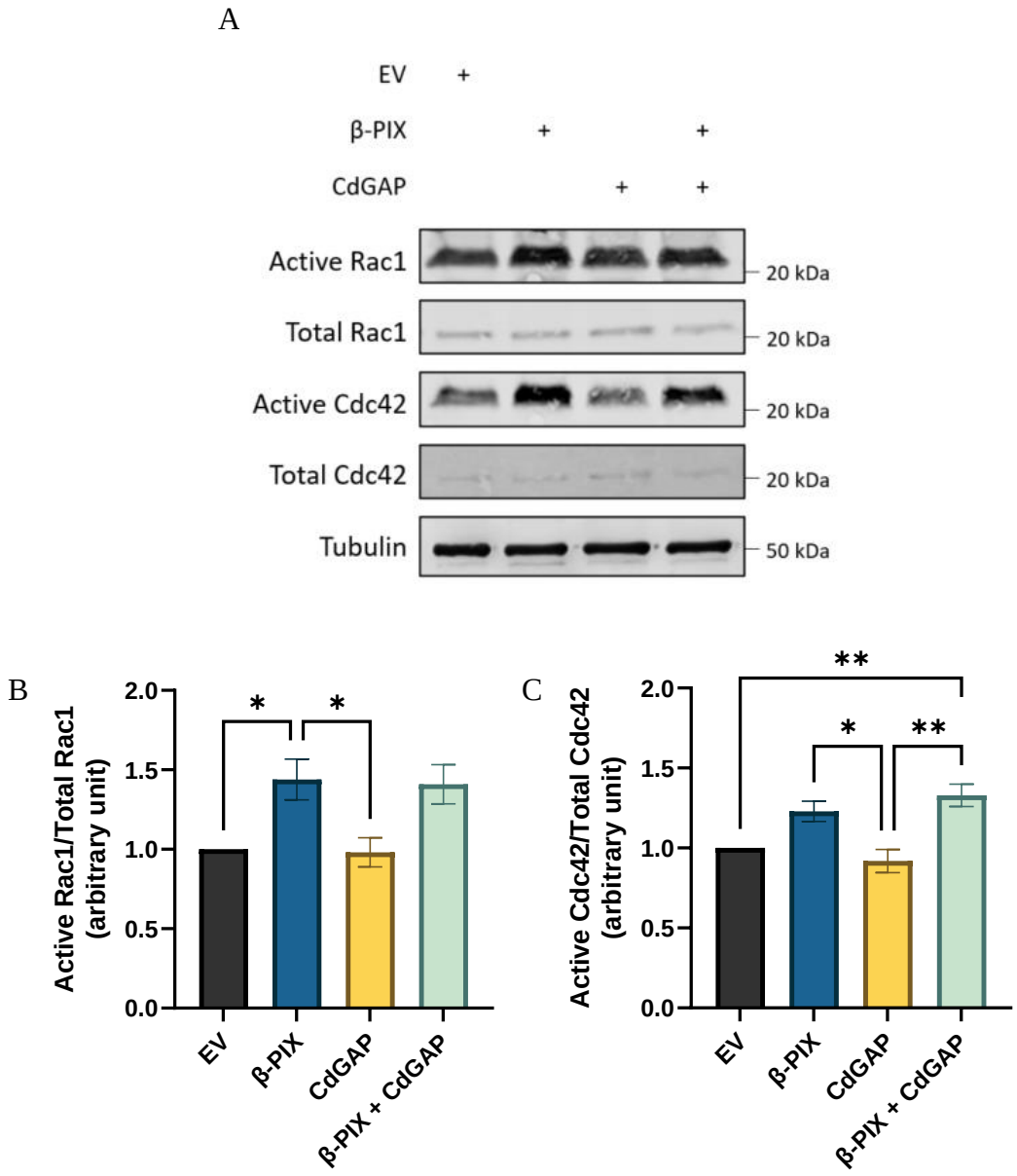



**Figure 13: CdGAP does not inhibit β-PIX mediated Rac1/Cdc42 activation.** HEK 293 cells overexpressing an empty vector (EV) control, β-PIX, CdGAP, or β-PIX with CdGAP were lysed and subjected to pull-down with GST-CRIB for active (GTP-bound) Rac1 and Cdc42. (**A**) Active and total Rac1 and Cdc42, together with tubulin, were detected by immunoblotting. A representative immunoblot is shown. (**B**, **C**) Densitometric quantification of Rac1 (**B**) and Cdc42 (**C**) activity, normalized to tubulin. n = 3-5 (B, C). Statistically significant values (*$P < 0.05$) are indicated.

# Chapter 4: Discussion

The Takano lab has discovered that (1) CdGAP and β-PIX are the top Rac1-interacting GAP and GEF in podocytes respectively, and that (2) this GAP and GEF physically interact *in vitro* by coimmunoprecipitation. Given the existence of other functional GAP-GEF interactions involving these two proteins (e.g., CdGAP-Intersectin and srGAP1-β-PIX), we were inclined to hypothesize the CdGAP-β-PIX interaction was likewise functional. Indeed, we found that CdGAP and β-PIX colocalize in human podocytes, and that their interaction affects intracellular localization, Rho GTPase activity, as well as cytoskeleton-dependent functions like cell morphology and motility.

Previous work from the lab has shown that CdGAP binds β-PIX via its BR domain. Notably, this is the same domain through which CdGAP interacts with Intersectin. Binding of Intersectin to CdGAP results in a loss of GAP activity, likely through conformational change involving the PRD domain. It is possible that β-PIX behaves in a similar fashion, and in its binding to CdGAP, inhibits GAP activity. Such a possibility would explain the failure of CdGAP to blunt basal Rac1/Cdc42 activity in HEK 293 cells overexpressing CdGAP and β-PIX, when compared to cells overexpressing β-PIX alone (**Fig. 13**). Given that the PRD of CdGAP is required for the Intersectin-mediated downregulation of GAP activity, it would be interesting to explore whether a PRD deficient mutant of CdGAP might overcome β-PIX-mediated inhibition. Nonetheless, the preliminary finding that β-PIX mediates CdGAP GAP activity suggests that this GEF and GAP may operate under a sophisticated regulatory mechanism.

β-PIX was found to bind CdGAP through its SH3-DH domain with inconsistent binding (present in some experiments but not others) of the C-terminus (**Fig. 7**). In trying to resolve the roles of the individual SH3 and DH domains in this interaction, we found that SH3 alone is not sufficient to bind CdGAP. The capacity of the DH domain alone to bind CdGAP could not be resolved due to its low expression level, and under the immunoblotting system in place (owing to IgG band signal overlap at the predicted molecular weight). The SH3 domain of β-PIX is important to its scaffolding activity, as it is required for shuttling of the PAK-PIX-GIT complex from the cell body to the membrane. Notably, Intersectin binds CdGAP via its SH3 domain. It is therefore tempting to propose that the SH3 domain β-PIX may be required, but not sufficient, for the binding of CdGAP.

The DH domain of β-PIX, which likely also participates in the CdGAP binding interaction, is required for mediating nucleotide exchange on Rac1 and Cdc42 (**Fig. 7**). ten Klooster *et al.* found that β-PIX is able to bind both active and inactive Rac1 on its SH3 domain, and that this interaction is essential for targeted localization of Rac1 and its subsequent activation (194). It is possible that CdGAP binding to the SH3 domain of β-PIX accommodates for a similar targeting mechanism, whereby integrin-induced EGF stimulation promotes translocation of β-PIX to the cell membrane for subsequent Cdc42 and Rac1 regulation, thus pulling CdGAP along to allow for the targeted regulation of focal adhesion turnover. This might explain the joint translocation of CdGAP and β-PIX to the cell membrane of podocytes that took place upon EGF stimulation (**Fig. 9**).

The other site of CdGAP interaction on β-PIX was its C-terminus (**Fig. 7**). This interaction is likely context specific, as it appeared in some coimmunoprecipitation experiments but not others. The C-terminus of β-PIX regulates its dimerization, and as such, its translocation to the cell membrane. Chadi and Sorokin reported that dimerization of β-PIX is essential for its localization to the cell membrane, as well as strong GEF activity towards Rac1 (202). It is possible that situational binding of CdGAP to the β-PIX C-terminus impairs its ability to dimerize. In this situation, we might expect a reduction in β-PIX membrane localization. This aligns with our finding that at basal conditions, membrane-localized β-PIX levels are low, but increase upon CdGAP knockdown (**Fig. 9F**). It would therefore be interesting to determine whether the interaction of CdGAP with the C-terminus of β-PIX affects β-PIX dimerization.

There is, however, an alternate interpretation of the significance of CdGAP binding the β-PIX C-terminus. The C-terminus is an important β-PIX regulatory domain containing two phosphorylation sites, Ser-516 and Thr-526. The study by Kutys and Yamada that identified the β-PIX-srGAP1 interaction also discovered that this interaction was promoted by dephosphorylation of Thr-526. They found that when fibroblasts are seeded on collagen, activation of α2 integrin promotes protein phosphatase 2A (PP2A) activity, causing dephosphorylation of the Thr-526 residue. This, in turn, allowed for binding of β-PIX and srGAP1 and subsequent activation of Cdc42, but not Rac1 (206). Rac1 activation by β-PIX, instead, required that the Thr-526 residue of β-PIX is phosphorylated; one study has demonstrated that this can happen in response to fibroblast growth factor that triggers a Ras/ERK/PAK2 signaling cascade (209). It appears that

Rac1 activation by β-PIX takes place downstream of Cdc42 activation (194). It could therefore be that dephosphorylation of the Thr-526 residue on β-PIX supports its association with CdGAP, as it does in the case of srGAP1. However, if this is the case, it is not immediately clear what happens upon EGF stimulation. Phosphorylation of Thr-526 allows PAK1 to bind β-PIX. Subsequent β-PIX-mediated activation of Cdc42 causes PAK1 autophosphorylation and dissociation from the β-PIX DH domain, which in turn binds and activates Rac1 (194). Yet, collagen plated fibroblasts exhibiting β-PIX-srGAP1 interaction had reduced PAK1 binding to β-PIX (206). It could therefore be that under basal conditions, CdGAP-β-PIX interaction is modulated by Thr-526 dephosphorylation with little PAK1 binding, but that upon EGF stimulation, Thr-526 is phosphorylated and CdGAP is somehow recruited to the cell periphery in complex with PAK1. Further examination of the effects of Thr-526 phosphorylation on β-PIX interaction with CdGAP and PAK1 could, therefore, provide useful mechanistic insight.

Whatever the mechanism of joint CdGAP-β-PIX translocation to the cellular periphery may be, it is likely that it culminates in the release of β-PIX-mediated inhibition of CdGAP GAP activity. This may happen through dissociation of the CdGAP-β-PIX complex, or perhaps through conformational change that releases the previously folded CdGAP PRD-GAP domains into an open confirmation, such that the GAP domain is accessible for Rac1/Cdc42 inhibition. This is supported by the observation that, despite their physical interaction, CdGAP and β-PIX still exert opposite effects on cytoskeletal dynamics, as they promote two different types of cell morphology. CdGAP knockdown in human podocytes resulted in an increase of cell area (i.e., cell spreading) (**Fig. 11**). This finding is in line with a previous report from the lab of Dr. Lamarche that shows that expression of a GAP-null CdGAP mutant in fibroblasts supports cell spreading, as well as other reports demonstrating that CdGAP overexpression (in numerous cell types) causes reduction in cell area (i.e., cell rounding) (177, 179, 180, 183). To assess the effects of β-PIX activity on podocyte morphology, we had to generate a mouse shRNA knockdown cell line, as β-PIX ablation causes human podocytes to detach from their substrate, making subsequent experiments difficult. Knockdown of β-PIX in mouse podocytes resulted in a mild decrease in cell area (i.e., cell rounding). A similar finding has been observed in collagen-plated fibroblasts (206). β-PIX knockdown podocytes also exhibited fewer membrane ruffles, a finding that supports this GEF's known role in ruffle formation (190). Collectively, these findings support the previously

established effects of CdGAP and β-PIX on cell morphology, especially given their respective GAP and GEF activities.

While CdGAP and β-PIX knockdown gave rise to opposing effects on cell morphology in accordance to their respective GAP and GEF activities, their overexpression did not create such a strong opposing effect. To assess cell migration, HEK 293 cells were overexpressed with either an empty vector control, CdGAP, β-PIX, or CdGAP and β-PIX together. Cells were seeded on a laminin-521 substrate, a wound was made, and migration was observed over the following 12 hours, as it was expected that proliferation was unlikely to impact cell migration over such a short time period. In our assay, overexpression of CdGAP promoted cell migration, while β-PIX overexpression showed a mild inhibitory effect (**Fig. 10**). A previous study has shown, very similarly, that CdGAP overexpression in HEK 293 cells gives rise to increased cell migration (176). Another study of Neu-NT-expressing mammary tumor cells found that while CdGAP knockdown itself didn't alter cell motility, it rendered the cells unresponsive to TGF-β induced cell migration (173). This is in line with the finding that CdGAP is required for durotaxis, as CdGAP deficient human osteosarcoma U2OS cells are unresponsive to changes in matrix rigidity (210). These data suggest that CdGAP is a driver of cell motility, as was the case in our migration assay. Yet, another report has found that CdGAP overexpression in U2OS cells deceased cell migration, while CdGAP knockdown promoted it (208). The cause for this discrepancy may simply be that CdGAP operates in a context-dependent manner. Indeed, tumor cells often operate on an altered genetic program, and it is well established that dysregulation of Rho GTPase activity is a common factor to many cancer types (211). That said, in the context of our experiment, CdGAP was a clear driver of cell migration.

Conversely, β-PIX overexpression appeared to slow cellular migration in our wound healing assay (**Fig. 10**). This is not in line with other studies that have found β-PIX knockdown to blunt both total and directional cell migration (by impaired activation of both Rac1 and Cdc42) (202, 212). Given the poor effects on cell health seen in β-PIX knockdown human podocytes, as well as HEK 293 cells overexpressing the DH domain of β-PIX, it is possible that the HEK 293 overexpression system was not optimal for assessment of β-PIX effects on cell motility. Indeed, the overexpression protocol was designed with maximum transfection efficiency in mind and did not account for the potential of resulting cellular toxicity. Although our findings do not lend

themselves to a concrete conclusion on the role of β-PIX in cell migration, dual overexpression of CdGAP and β-PIX in HEK 293 cells yielded migration that was similar to that of cells overexpressing CdGAP alone. This suggests that between this GEF and GAP, CdGAP is the primary driver of cell migration. Such a possibility would support the view that the functional purpose of the CdGAP-β-PIX interaction is β-PIX-mediated shuttling of the complex to the site of focal adhesions upon extracellular stimulation (such as that created by a wound in a wound healing assay), where CdGAP can exert its functional effects upon cytoskeletal dynamics.

In assaying CdGAP GAP activity, we also discovered that CdGAP knockdown blunts EGF-induced Rac1/Cdc42 activation in podocytes (**Fig. 12**). While it may be that the already-elevated basal Rac1/Cdc42 activities of CdGAP knockdown podocytes have reached their maximum threshold, there is another interesting possibility: that CdGAP is somehow required for EGF-induced β-PIX GEF activity. As aforementioned, CdGAP has been identified as an important mediator of TGF-β induced cell migration and focal adhesion-based mechanosensing (173, 210). On the basis of this, we might speculate that CdGAP contributes to EGF-induced integrin signal transduction and may be necessary for the functional translocation of β-PIX. It would be interesting to compare other β-PIX activation markers (e.g. phosphorylation of β-PIX itself and its downstream effectors) between control and CdGAP knockdown podocytes to determine the role of CdGAP in this GAP-GEF partnership.

As in any study that aims to ascertain the nature of disease *in vitro*, the question of physiological relevance is a critical one. In this study, we used HEK 293 cells where the use of podocytes was technically challenging, i.e., overexpression studies. As aforementioned, β-PIX knockdown in human podocytes caused challenges owing to immediate detachment from substrate. However, in considering the relevance of the CdGAP-β-PIX relationship to glomerular disease, we must remember that this GAP and GEF do not operate in a vacuum, but are rather part of an intricate Rho GTPase regulatory network (213). Both CdGAP and β-PIX interact with numerous signaling partners in podocytes, and dysregulation of both is deleterious to glomerular function *in vivo*. This study sheds light on two Rho GTPase regulatory members of the podocyte network, and the need for their balanced activities.

In conclusion, the findings of this work support a mechanism that allows for targeted regulation of Rac1 and Cdc42 by CdGAP and β-PIX in podocytes. Under a basal state, CdGAP

retains β-PIX in the cytosol, where β-PIX binding may inhibit GAP activity of CdGAP via the BR domain. Upon integrin-mediated EGF signaling, the two proteins jointly translocate to the cell membrane. Given the known role of β-PIX in Cdc42/Rac1 targeting to focal adhesions, it is likely that the joint translocation of CdGAP and β-PIX in this context facilitates the targeting of CdGAP to focal adhesions. At this point, CdGAP may undergo a conformational change or dissociate from β-PIX to render its GAP activity available for inactivation of Rac1/Cdc42. Thus, the CdGAP-β-PIX interaction may facilitate the precise regulation of focal adhesion assembly and disassembly, and sheds light on the complex regulatory network of Rho GTPases in podocytes by their upstream GEFs and GAPs. Indeed, untangling the Rho GTPase network is critical for our understanding of proteinuric kidney diseases and may have implications in the development of small molecule treatments.

References


1. Eckardt, K.-U., Coresh, J., Devuyst, O., Johnson, R. J., Köttgen, A., Levey, A. S., and Levin, A. (2013) Evolving importance of kidney disease: from subspecialty to global health burden. *The Lancet*. **382**, 158–169
2. Bertram, J. F., Douglas-Denton, R. N., Diouf, B., Hughson, M. D., and Hoy, W. E. (2011) Human nephron number: implications for health and disease. *Pediatr Nephrol*. **26**, 1529–1533
3. Puelles, V. G., Hoy, W. E., Hughson, M. D., Diouf, B., Douglas-Denton, R. N., and Bertram, J. F. (2011) Glomerular number and size variability and risk for kidney disease. *Current Opinion in Nephrology and Hypertension*. **20**, 7–15
4. Ebefors, K., Bergwall, L., and Nyström, J. (2022) The Glomerulus According to the Mesangium. *Frontiers in Medicine*. [online] https://www.frontiersin.org/article/10.3389/fmed.2021.740527 (Accessed March 3, 2022)
5. Schlöndorff, D., and Banas, B. (2009) The Mesangial Cell Revisited: No Cell Is an Island. *JASN*. **20**, 1179–1187
6. Satchell, S. C., and Braet, F. (2009) Glomerular endothelial cell fenestrations: an integral component of the glomerular filtration barrier. *Am J Physiol Renal Physiol*. **296**, F947–F956
7. Haraldsson, B., Nyström, J., and Deen, W. M. (2008) Properties of the Glomerular Barrier and Mechanisms of Proteinuria. *Physiological Reviews*. **88**, 451–487
8. Scott, R. P., and Quaggin, S. E. (2015) The cell biology of renal filtration. *Journal of Cell Biology*. **209**, 199–210
9. Thomson, S. C., and Blantz, R. C. (2010) A New Role for Charge of the Glomerular Capillary Membrane. *JASN*. **21**, 2011–2013
10. Deen, W. M., Lazzara, M. J., and Myers, B. D. (2001) Structural determinants of glomerular permeability. *American Journal of Physiology-Renal Physiology*. **281**, F579–F596
11. Abrahamson, D. R. (1985) Origin of the glomerular basement membrane visualized after in vivo labeling of laminin in newborn rat kidneys. *Journal of Cell Biology*. **100**, 1988–2000
12. Miner, J. H. (2012) The glomerular basement membrane. *Experimental Cell Research*. **318**, 973–978
13. Groffen, A. J., Ruegg, M. A., Dijkman, H., van de Velden, T. J., Buskens, C. A., van den Born, J., Assmann, K. J., Monnens, L. A., Veerkamp, J. H., and van den Heuvel, L. P. (1998) Agrin Is a Major Heparan Sulfate Proteoglycan in the Human Glomerular Basement Membrane. *J Histochem Cytochem.* **46**, 19–27
14. Bader, B. L., Smyth, N., Nedbal, S., Miosge, N., Baranowsky, A., Mokkapati, S., Murshed, M., and Nischt, R. (2005) Compound Genetic Ablation of Nidogen 1 and 2 Causes Basement Membrane Defects and Perinatal Lethality in Mice. *Molecular and Cellular Biology*. **25**, 6846–6856
15. Kashtan, C. E., and Kim, Y. (1992) Distribution of the α1 and α2 chains of collagen IV and of collagens V and VI in Alport syndrome. *Kidney International*. **42**, 115–126
16. Barker, D. F., Hostikka, S. L., Zhou, J., Chow, L. T., Oliphant, A. R., Gerken, S. C., Gregory, M. C., Skolnick, M. H., Atkin, C. L., and Tryggvason, K. (1990) Identification of mutations in the COL4A5 collagen gene in Alport syndrome. *Science*. **248**, 1224–1227

17. Williamson, D. A. J. (1961) ALPORT'S SYNDROME OF HEREDITARY NEPHRITIS WITH DEAFNESS. *The Lancet*. **278**, 1321–1323
18. Gubler, M.-C., Knebelmann, B., Beziau, A., Broyer, M., Pirson, Y., Haddoum, F., Kleppel, M. M., and Antignac, C. (1995) Autosomal recessive Alport syndrome: Immunohistochemical study of type IV collagen chain distribution. *Kidney International*. **47**, 1142–1147
19. Kalluri, R., Shield, C. F., Todd, P., Hudson, B. G., and Neilson, E. G. (1997) Isoform switching of type IV collagen is developmentally arrested in X-linked Alport syndrome leading to increased susceptibility of renal basement membranes to endoproteolysis. 10.1172/JCI119431
20. Voskarides, K., Damianou, L., Neocleous, V., Zouvani, I., Christodoulidou, S., Hadjiconstantinou, V., Ioannou, K., Athanasiou, Y., Patsias, C., Alexopoulos, E., Pierides, A., Kyriacou, K., and Deltas, C. (2007) COL4A3/COL4A4 Mutations Producing Focal Segmental Glomerulosclerosis and Renal Failure in Thin Basement Membrane Nephropathy. *JASN*. **18**, 3004–3016
21. Aumailley, M., Bruckner-Tuderman, L., Carter, W. G., Deutzmann, R., Edgar, D., Ekblom, P., Engel, J., Engvall, E., Hohenester, E., Jones, J. C. R., Kleinman, H. K., Marinkovich, M. P., Martin, G. R., Mayer, U., Meneguzzi, G., Miner, J. H., Miyazaki, K., Patarroyo, M., Paulsson, M., Quaranta, V., Sanes, J. R., Sasaki, T., Sekiguchi, K., Sorokin, L. M., Talts, J. F., Tryggvason, K., Uitto, J., Virtanen, I., von der Mark, K., Wewer, U. M., Yamada, Y., and Yurchenco, P. D. (2005) A simplified laminin nomenclature. *Matrix Biology*. **24**, 326–332
22. Miner, J. H. (1998) Developmental biology of glomerular basement membrane components. *Curr Opin Nephrol Hypertens*. **7**, 13–19
23. Matejas, V., Hinkes, B., Alkandari, F., Al-Gazali, L., Annexstad, E., Aytac, M. B., Barrow, M., Bláhová, K., Bockenhauer, D., Cheong, H. I., Maruniak-Chudek, I., Cochat, P., Dötsch, J., Gajjar, P., Hennekam, R. C., Janssen, F., Kagan, M., Kariminejad, A., Kemper, M. J., Koenig, J., Kogan, J., Kroes, H. Y., Kuwertz-Bröking, E., Lewanda, A. F., Medeira, A., Muscheites, J., Niaudet, P., Pierson, M., Saggar, A., Seaver, L., Suri, M., Tsygin, A., Wühl, E., Zurowska, A., Uebe, S., Hildebrandt, F., Antignac, C., and Zenker, M. (2010) Mutations in the human laminin β2 (LAMB2) gene and the associated phenotypic spectruma. *Human Mutation*. **31**, 992–1002
24. VanDeVoorde, R., Witte, D., Kogan, J., and Goebel, J. (2006) Pierson Syndrome: A Novel Cause of Congenital Nephrotic Syndrome. *Pediatrics*. **118**, e501–e505
25. Choi, H. J., Lee, B. H., Kang, J. H., Jeong, H. J., Moon, K. C., Ha, I. S., Yu, Y. S., Matejas, V., Zenker, M., Choi, Y., and Cheong, H. I. (2008) Variable phenotype of Pierson syndrome. *Pediatr Nephrol*. **23**, 995–1000
26. Kagan, M., Cohen, A. H., Matejas, V., Vlangos, C., and Zenker, M. (2008) A milder variant of Pierson syndrome. *Pediatr Nephrol*. **23**, 323–327
27. Zenker, M., Aigner, T., Wendler, O., Tralau, T., Müntefering, H., Fenski, R., Pitz, S., Schumacher, V., Royer-Pokora, B., Wühl, E., Cochat, P., Bouvier, R., Kraus, C., Mark, K., Madlon, H., Dötsch, J., Rascher, W., Maruniak-Chudek, I., Lennert, T., Neumann, L. M., and Reis, A. (2004) Human laminin β2 deficiency causes congenital nephrosis with mesangial sclerosis and distinct eye abnormalities. *Human Molecular Genetics*. **13**, 2625–2632

28. Jarad, G., Cunningham, J., Shaw, A. S., and Miner, J. H. (2006) Proteinuria precedes podocyte abnormalities in*Lamb2*$^{-/-}$ mice, implicating the glomerular basement membrane as an albumin barrier. *J Clin Invest*. **116**, 2272–2279
29. Pavenstädt, H., Kriz, W., and Kretzler, M. (2003) Cell Biology of the Glomerular Podocyte. *Physiological Reviews*. **83**, 253–307
30. Faul, C., Asanuma, K., Yanagida-Asanuma, E., Kim, K., and Mundel, P. (2007) Actin up: regulation of podocyte structure and function by components of the actin cytoskeleton. *Trends Cell Biol*. **17**, 428–437
31. Reiser, J., Kriz, W., Kretzler, M., and Mundel, P. (2000) The Glomerular Slit Diaphragm Is a Modified Adherens Junction. *JASN*. **11**, 1–8
32. Kriz, W., Shirato, I., Nagata, M., LeHir, M., and Lemley, K. V. (2013) The podocyte's response to stress: the enigma of foot process effacement. *American Journal of Physiology-Renal Physiology*. **304**, F333–F347
33. Nagata, M., and Kriz, W. (1992) Glomerular damage after uninephrectomy in young rats. II. Mechanical stress on podocytes as a pathway to sclerosis. *Kidney International*. **42**, 148–160
34. Ruggenenti, P., Cravedi, P., and Remuzzi, G. (2010) The RAAS in the pathogenesis and treatment of diabetic nephropathy. *Nat Rev Nephrol*. **6**, 319–330
35. Sachse, A., and Wolf, G. (2007) Angiotensin II–Induced Reactive Oxygen Species and the Kidney. *JASN*. **18**, 2439–2446
36. Hsu, H.-H., Hoffmann, S., Endlich, N., Velic, A., Schwab, A., Weide, T., Schlatter, E., and Pavenstädt, H. (2008) Mechanisms of angiotensin II signaling on cytoskeleton of podocytes. *J Mol Med*. **86**, 1379–1394
37. Kawarazaki, W., Nagase, M., Yoshida, S., Takeuchi, M., Ishizawa, K., Ayuzawa, N., Ueda, K., and Fujita, T. (2012) Angiotensin II- and Salt-Induced Kidney Injury through Rac1-Mediated Mineralocorticoid Receptor Activation. *J Am Soc Nephrol*. **23**, 997–1007
38. Shibata, S., Nagase, M., Yoshida, S., Kawachi, H., and Fujita, T. (2007) Podocyte as the Target for Aldosterone. *Hypertension*. **49**, 355–364
39. Brewster, U. C., and Perazella, M. A. (2004) The renin-angiotensin-aldosterone system and the kidney: effects on kidney disease. *The American Journal of Medicine*. **116**, 263–272
40. Locatelli, M., Buelli, S., Pezzotta, A., Corna, D., Perico, L., Tomasoni, S., Rottoli, D., Rizzo, P., Conti, D., Thurman, J. M., Remuzzi, G., Zoja, C., and Morigi, M. (2014) Shiga Toxin Promotes Podocyte Injury in Experimental Hemolytic Uremic Syndrome via Activation of the Alternative Pathway of Complement. *JASN*. **25**, 1786–1798
41. Milas, O., Gadalean, F., Vlad, A., Dumitrascu, V., Velciov, S., Gluhovschi, C., Bob, F., Popescu, R., Ursoniu, S., Jianu, D. C., Matusz, P., Pusztai, A.-M., Secara, A., Simulescu, A., Stefan, M., Patruica, M., Petrica, F., Vlad, D., and Petrica, L. (2020) Pro-inflammatory cytokines are associated with podocyte damage and proximal tubular dysfunction in the early stage of diabetic kidney disease in type 2 diabetes mellitus patients. *Journal of Diabetes and its Complications*. **34**, 107479
42. Wright, R. D., and Beresford, M. W. (2018) Podocytes contribute, and respond, to the inflammatory environment in lupus nephritis. *American Journal of Physiology-Renal Physiology*. **315**, F1683–F1694
43. Cortes, P., Méndez, M., Riser, B. L., Guérin, C. J., Rodríguez-Barbero, A., Hassett, C., and Yee, J. (2000) F-actin fiber distribution in glomerular cells: Structural and functional implications. *Kidney International*. **58**, 2452–2461

44. Sever, S., and Schiffer, M. (2018) Actin dynamics at focal adhesions: a common endpoint and putative therapeutic target for proteinuric kidney diseases. *Kidney Int*. **93**, 1298–1307
45. Schmieder, S., Nagai, M., Orlando, R. A., Takeda, T., and Farquhar, M. G. (2004) Podocalyxin Activates RhoA and Induces Actin Reorganization through NHERF1 and Ezrin in MDCK Cells. *JASN*. **15**, 2289–2298
46. Takeda, T., McQuistan, T., Orlando, R. A., and Farquhar, M. G. (2001) Loss of glomerular foot processes is associated with uncoupling of podocalyxin from the actin cytoskeleton. *J Clin Invest*. **108**, 289–301
47. Bijian, K., Takano, T., Papillon, J., Le Berre, L., Michaud, J.-L., Kennedy, C. R. J., and Cybulsky, A. V. (2005) Actin cytoskeleton regulates extracellular matrix-dependent survival signals in glomerular epithelial cells. *Am J Physiol Renal Physiol*. **289**, F1313-1323
48. Perico, L., Conti, S., Benigni, A., and Remuzzi, G. (2016) Podocyte–actin dynamics in health and disease. *Nat Rev Nephrol*. **12**, 692–710
49. D'Agati, V. D., Fogo, A. B., Bruijn, J. A., and Jennette, J. C. (2004) Pathologic classification of focal segmental glomerulosclerosis: a working proposal. *American Journal of Kidney Diseases*. **43**, 368–382
50. Vriese, A. S. D., Sethi, S., Nath, K. A., Glassock, R. J., and Fervenza, F. C. (2018) Differentiating Primary, Genetic, and Secondary FSGS in Adults: A Clinicopathologic Approach. *JASN*. **29**, 759–774
51. Yao, N. Y., Becker, D. J., Broedersz, C. P., Depken, M., MacKintosh, F. C., Pollak, M. R., and Weitz, D. A. (2011) Nonlinear Viscoelasticity of Actin Transiently Cross-linked with Mutant α-Actinin-4. *Journal of Molecular Biology*. **411**, 1062–1071
52. Kaplan, J. M., H Kim, S., North, K. N., Rennke, H., A Correia, L., Tong, H.-Q., Mathis, B. J., Rodríguez-Pérez, J.-C., Allen, P. G., Beggs, A. H., and Pollak, M. R. (2000) Mutations in ACTN4, encoding α-actinin-4, cause familial focal segmental glomerulosclerosis. *Nat Genet*. **24**, 251–256
53. Feng, D., Kumar, M., Muntel, J., Gurley, S. B., Birrane, G., Stillman, I. E., Ding, L., Wang, M., Ahmed, S., Schlondorff, J., Alper, S. L., Ferrante, T., Marquez, S. L., Ng, C. F., Novak, R., Ingber, D. E., Steen, H., and Pollak, M. R. (2020) Phosphorylation of ACTN4 Leads to Podocyte Vulnerability and Proteinuric Glomerulosclerosis. *J Am Soc Nephrol*. **31**, 1479–1495
54. Michaud, J.-L., Lemieux, L. I., Dubé, M., Vanderhyden, B. C., Robertson, S. J., and Kennedy, C. R. J. (2003) Focal and Segmental Glomerulosclerosis in Mice with Podocyte-Specific Expression of Mutant α-Actinin-4. *JASN*. **14**, 1200–1211
55. Feng, D., Notbohm, J., Benjamin, A., He, S., Wang, M., Ang, L.-H., Bantawa, M., Bouzid, M., Del Gado, E., Krishnan, R., and Pollak, M. R. (2018) Disease-causing mutation in α-actinin-4 promotes podocyte detachment through maladaptation to periodic stretch. *Proc Natl Acad Sci U S A*. **115**, 1517–1522
56. Asanuma, K., Kim, K., Oh, J., Giardino, L., Chabanis, S., Faul, C., Reiser, J., and Mundel, P. (2005) Synaptopodin regulates the actin-bundling activity of alpha-actinin in an isoform-specific manner. *J Clin Invest*. **115**, 1188–1198
57. Srivastava, T., Garola, R. E., Whiting, J. M., and Alon, U. S. (2001) Synaptopodin expression in idiopathic nephrotic syndrome of childhood. *Kidney International*. **59**, 118–125
58. Yanagida-Asanuma, E., Asanuma, K., Kim, K., Donnelly, M., Young Choi, H., Hyung Chang, J., Suetsugu, S., Tomino, Y., Takenawa, T., Faul, C., and Mundel, P. (2007)

Synaptopodin Protects Against Proteinuria by Disrupting Cdc42:IRSp53:Mena Signaling Complexes in Kidney Podocytes. *Am J Pathol*. **171**, 415–427
59. Mele, C., Iatropoulos, P., Donadelli, R., Calabria, A., Maranta, R., Cassis, P., Buelli, S., Tomasoni, S., Piras, R., Krendel, M., Bettoni, S., Morigi, M., Delledonne, M., Pecoraro, C., Abbate, I., Capobianchi, M. R., Hildebrandt, F., Otto, E., Schaefer, F., Macciardi, F., Ozaltin, F., Emre, S., Ibsirlioglu, T., Benigni, A., Remuzzi, G., and Noris, M. (2011) MYO1E Mutations and Childhood Familial Focal Segmental Glomerulosclerosis. *N Engl J Med*. **365**, 295–306
60. Krendel, M., Osterweil, E. K., and Mooseker, M. S. (2007) Myosin 1E interacts with synaptojanin-1 and dynamin via its SH3 domain. *FEBS Lett*. **581**, 644–650
61. Zhao, Y., Zhang, H., Wang, H., Ye, M., and Jin, X. (2022) Role of formin INF2 in human diseases. *Mol Biol Rep*. **49**, 735–746
62. Sun, H., Schlondorff, J. S., Brown, E. J., Higgs, H. N., and Pollak, M. R. (2011) Rho activation of mDia formins is modulated by an interaction with inverted formin 2 (INF2). *Proc Natl Acad Sci U S A*. **108**, 2933–2938
63. Madrid, R., Aranda, J. F., Rodríguez-Fraticelli, A. E., Ventimiglia, L., Andrés-Delgado, L., Shehata, M., Fanayan, S., Shahheydari, H., Gómez, S., Jiménez, A., Martín-Belmonte, F., Byrne, J. A., and Alonso, M. A. (2010) The Formin INF2 Regulates Basolateral-to-Apical Transcytosis and Lumen Formation in Association with Cdc42 and MAL2. *Developmental Cell*. **18**, 814–827
64. Brown, E. J., Schlöndorff, J. S., Becker, D. J., Tsukaguchi, H., Tonna, S. J., Uscinski, A. L., Higgs, H. N., Henderson, J. M., and Pollak, M. R. (2010) Mutations in the formin gene INF2 cause focal segmental glomerulosclerosis. *Nat Genet*. **42**, 72–76
65. Hynes, R. O. (2002) Integrins: Bidirectional, Allosteric Signaling Machines. *Cell*. **110**, 673–687
66. Kreidberg, J. A., and Symons, J. M. (2000) Integrins in kidney development, function, and disease. *American Journal of Physiology-Renal Physiology*. **279**, F233–F242
67. Kreidberg, J. A., Donovan, M. J., Goldstein, S. L., Rennke, H., Shepherd, K., Jones, R. C., and Jaenisch, R. (1996) Alpha 3 beta 1 integrin has a crucial role in kidney and lung organogenesis. *Development*. **122**, 3537–3547
68. Sachs, N., Kreft, M., van den Bergh Weerman, M. A., Beynon, A. J., Peters, T. A., Weening, J. J., and Sonnenberg, A. (2006) Kidney failure in mice lacking the tetraspanin CD151. *Journal of Cell Biology*. **175**, 33–39
69. Pozzi, A., Jarad, G., Moeckel, G. W., Coffa, S., Zhang, X., Gewin, L., Eremina, V., Hudson, B. G., Borza, D.-B., Harris, R. C., Holzman, L. B., Phillips, C. L., Fassler, R., Quaggin, S. E., Miner, J. H., and Zent, R. (2008) β1 integrin expression by podocytes is required to maintain glomerular structural integrity. *Developmental Biology*. **316**, 288–301
70. Kambham, N., Tanji, N., Seigle, R. L., Markowitz, G. S., Pulkkinen, L., Uitto, J., and D'Agati, V. D. (2000) Congenital focal segmental glomerulosclerosis associated with β4 integrin mutation and epidermolysis bullosa. *American Journal of Kidney Diseases*. **36**, 190–196
71. Huveneers, S., and Danen, E. H. J. (2009) Adhesion signaling – crosstalk between integrins, Src and Rho. *Journal of Cell Science*. **122**, 1059–1069
72. Yamada, K. M., and Even-Ram, S. (2002) Integrin regulation of growth factor receptors. *Nat Cell Biol*. **4**, E75–E76

73. Roca, N., Muñoz, M., Cruz, A., Vilalta, R., Lara, E., and Ariceta, G. (2019) Long-term outcome in a case series of Denys–Drash syndrome. *Clinical Kidney Journal*. **12**, 836–839
74. Gao, F., Maiti, S., Sun, G., Ordonez, N. G., Udtha, M., Deng, J. M., Behringer, R. R., and Huff, V. (2004) The Wt1+/R394W Mouse Displays Glomerulosclerosis and Early-Onset Renal Failure Characteristic of Human Denys-Drash Syndrome. *Molecular and Cellular Biology*. **24**, 9899–9910
75. Kerjaschki, D. (2001) Caught flat-footed: podocyte damage and the molecular bases of focal glomerulosclerosis. *J Clin Invest*. **108**, 1583–1587
76. Garg, P., Verma, R., Nihalani, D., Johnstone, D. B., and Holzman, L. B. (2007) Neph1 Cooperates with Nephrin To Transduce a Signal That Induces Actin Polymerization. *Mol Cell Biol*. **27**, 8698–8712
77. Dlugos, C. P., Picciotto, C., Lepa, C., Krakow, M., Stöber, A., Eddy, M.-L., Weide, T., Jeibmann, A., P Krahn, M., Van Marck, V., Klingauf, J., Ricker, A., Wedlich-Söldner, R., Pavenstädt, H., Klämbt, C., and George, B. (2019) Nephrin Signaling Results in Integrin β1 Activation. *J Am Soc Nephrol*. **30**, 1006–1019
78. Kawachi, H., and Fukusumi, Y. (2020) New insight into podocyte slit diaphragm, a therapeutic target of proteinuria. *Clin Exp Nephrol*. **24**, 193–204
79. Rodewald, R., and Karnovsky, M. J. (1974) PORous SUBSTRUCTURE OF THE GLOMERULAR SLIT DIAPHRAGM IN THE RAT AND MOUSE. *Journal of Cell Biology*. **60**, 423–433
80. Sugio, S., Kashima, A., Mochizuki, S., Noda, M., and Kobayashi, K. (1999) Crystal structure of human serum albumin at 2.5 Å resolution. *Protein Engineering, Design and Selection*. **12**, 439–446
81. Gagliardini, E., Conti, S., Benigni, A., Remuzzi, G., and Remuzzi, A. (2010) Imaging of the Porous Ultrastructure of the Glomerular Epithelial Filtration Slit. *J Am Soc Nephrol*. **21**, 2081–2089
82. Kestilä, M., Lenkkeri, U., Männikkö, M., Lamerdin, J., McCready, P., Putaala, H., Ruotsalainen, V., Morita, T., Nissinen, M., Herva, R., Kashtan, C. E., Peltonen, L., Holmberg, C., Olsen, A., and Tryggvason, K. (1998) Positionally cloned gene for a novel glomerular protein--nephrin--is mutated in congenital nephrotic syndrome. *Mol Cell*. **1**, 575–582
83. Wartiovaara, J., Öfverstedt, L.-G., Khoshnoodi, J., Zhang, J., Mäkelä, E., Sandin, S., Ruotsalainen, V., Cheng, R. H., Jalanko, H., Skoglund, U., and Tryggvason, K. (2004) Nephrin strands contribute to a porous slit diaphragm scaffold as revealed by electron tomography. *J Clin Invest*. **114**, 1475–1483
84. Ruotsalainen, V., Ljungberg, P., Wartiovaara, J., Lenkkeri, U., Kestilä, M., Jalanko, H., Holmberg, C., and Tryggvason, K. (1999) Nephrin is specifically located at the slit diaphragm of glomerular podocytes. *Proc Natl Acad Sci U S A*. **96**, 7962–7967
85. Grahammer, F., Wigge, C., Schell, C., Kretz, O., Patrakka, J., Schneider, S., Klose, M., Kind, J., Arnold, S. J., Habermann, A., Bräuniger, R., Rinschen, M. M., Völker, L., Bregenzer, A., Rubbenstroth, D., Boerries, M., Kerjaschki, D., Miner, J. H., Walz, G., Benzing, T., Fornoni, A., Frangakis, A. S., and Huber, T. B. (2016) A flexible, multilayered protein scaffold maintains the slit in between glomerular podocytes. *JCI Insight*. **1**, 86177
86. Jones, N., Blasutig, I. M., Eremina, V., Ruston, J. M., Bladt, F., Li, H., Huang, H., Larose, L., Li, S. S.-C., Takano, T., Quaggin, S. E., and Pawson, T. (2006) Nck adaptor proteins link nephrin to the actin cytoskeleton of kidney podocytes. *Nature*. **440**, 818–823

87. Liu, Y., Liang, W., Yang, Y., Pan, Y., Yang, Q., Chen, X., Singhal, P. C., and Ding, G. (2015) IQGAP1 regulates actin cytoskeleton organization in podocytes through interaction with nephrin. *Cell Signal*. **27**, 867–877
88. Zhu, J., Sun, N., Aoudjit, L., Li, H., Kawachi, H., Lemay, S., and Takano, T. (2008) Nephrin mediates actin reorganization via phosphoinositide 3-kinase in podocytes. *Kidney Int*. **73**, 556–566
89. Verma, R., Wharram, B., Kovari, I., Kunkel, R., Nihalani, D., Wary, K. K., Wiggins, R. C., Killen, P., and Holzman, L. B. (2005) Fyn Binds to and Phosphorylates the Kidney Slit Diaphragm Component Nephrin *. *Journal of Biological Chemistry*. 10.1074/jbc.M301689200
90. Verma, R., Kovari, I., Soofi, A., Nihalani, D., Patrie, K., and Holzman, L. B. (2006) Nephrin ectodomain engagement results in Src kinase activation, nephrin phosphorylation, Nck recruitment, and actin polymerization. *J Clin Invest*. **116**, 1346–1359
91. Sellin, L., Huber, T. B., Gerke, P., Quack, I., Pavenstädt, H., and Walz, G. (2003) NEPH1 defines a novel family of podocin interacting proteins. *FASEB J*. **17**, 115–117
92. Liu, G., Kaw, B., Kurfis, J., Rahmanuddin, S., Kanwar, Y. S., and Chugh, S. S. (2003) Neph1 and nephrin interaction in the slit diaphragm is an important determinant of glomerular permeability. *J Clin Invest*. **112**, 209–221
93. Donoviel, D. B., Freed, D. D., Vogel, H., Potter, D. G., Hawkins, E., Barrish, J. P., Mathur, B. N., Turner, C. A., Geske, R., Montgomery, C. A., Starbuck, M., Brandt, M., Gupta, A., Ramirez-Solis, R., Zambrowicz, B. P., and Powell, D. R. (2001) Proteinuria and perinatal lethality in mice lacking NEPH1, a novel protein with homology to NEPHRIN. *Mol Cell Biol*. **21**, 4829–4836
94. Boute, N., Gribouval, O., Roselli, S., Benessy, F., Lee, H., Fuchshuber, A., Dahan, K., Gubler, M. C., Niaudet, P., and Antignac, C. (2000) NPHS2, encoding the glomerular protein podocin, is mutated in autosomal recessive steroid-resistant nephrotic syndrome. *Nat Genet*. **24**, 349–354
95. Huber, T. B., Simons, M., Hartleben, B., Sernetz, L., Schmidts, M., Gundlach, E., Saleem, M. A., Walz, G., and Benzing, T. (2003) Molecular basis of the functional podocin-nephrin complex: mutations in the NPHS2 gene disrupt nephrin targeting to lipid raft microdomains. *Hum Mol Genet*. **12**, 3397–3405
96. Asanuma, K., and Mundel, P. (2003) The role of podocytes in glomerular pathobiology. *Clin Exp Nephrol*. **7**, 255–259
97. Schwarz, K., Simons, M., Reiser, J., Saleem, M. A., Faul, C., Kriz, W., Shaw, A. S., Holzman, L. B., and Mundel, P. (2001) Podocin, a raft-associated component of the glomerular slit diaphragm, interacts with CD2AP and nephrin. *J Clin Invest*. **108**, 1621–1629
98. Lynch, D. K., Winata, S. C., Lyons, R. J., Hughes, W. E., Lehrbach, G. M., Wasinger, V., Corthals, G., Cordwell, S., and Daly, R. J. (2003) A Cortactin-CD2-associated protein (CD2AP) complex provides a novel link between epidermal growth factor receptor endocytosis and the actin cytoskeleton. *J Biol Chem*. **278**, 21805–21813
99. Schiffer, M., Mundel, P., Shaw, A. S., and Böttinger, E. P. (2004) A novel role for the adaptor molecule CD2-associated protein in transforming growth factor-beta-induced apoptosis. *J Biol Chem*. **279**, 37004–37012
100. Kirsch, K. H., Georgescu, M. M., Ishimaru, S., and Hanafusa, H. (1999) CMS: an adapter molecule involved in cytoskeletal rearrangements. *Proc Natl Acad Sci U S A*. **96**, 6211–6216

101. Shih, N. Y., Li, J., Karpitskii, V., Nguyen, A., Dustin, M. L., Kanagawa, O., Miner, J. H., and Shaw, A. S. (1999) Congenital nephrotic syndrome in mice lacking CD2-associated protein. *Science*. **286**, 312–315
102. Shih, N. Y., Li, J., Cotran, R., Mundel, P., Miner, J. H., and Shaw, A. S. (2001) CD2AP localizes to the slit diaphragm and binds to nephrin via a novel C-terminal domain. *Am J Pathol*. **159**, 2303–2308
103. van Duijn, T. J., Anthony, E. C., Hensbergen, P. J., Deelder, A. M., and Hordijk, P. L. (2010) Rac1 recruits the adapter protein CMS/CD2AP to cell-cell contacts. *J Biol Chem*. **285**, 20137–20146
104. Inoue, T., Yaoita, E., Kurihara, H., Shimizu, F., Sakai, T., Kobayashi, T., Ohshiro, K., Kawachi, H., Okada, H., Suzuki, H., Kihara, I., and Yamamoto, T. (2001) FAT is a component of glomerular slit diaphragms. *Kidney International*. **59**, 1003–1012
105. Ciani, L., Patel, A., Allen, N. D., and ffrench-Constant, C. (2003) Mice Lacking the Giant Protocadherin mFAT1 Exhibit Renal Slit Junction Abnormalities and a Partially Penetrant Cyclopia and Anophthalmia Phenotype. *Mol Cell Biol*. **23**, 3575–3582
106. Gee, H. Y., Sadowski, C. E., Aggarwal, P. K., Porath, J. D., Yakulov, T. A., Schueler, M., Lovric, S., Ashraf, S., Braun, D. A., Halbritter, J., Fang, H., Airik, R., Vega-Warner, V., Cho, K. J., Chan, T. A., Morris, L. G. T., ffrench-Constant, C., Allen, N., McNeill, H., Büscher, R., Kyrieleis, H., Wallot, M., Gaspert, A., Kistler, T., Milford, D. V., Saleem, M. A., Keng, W. T., Alexander, S. I., Valentini, R. P., Licht, C., Teh, J. C., Bogdanovic, R., Koziell, A., Bierzynska, A., Soliman, N. A., Otto, E. A., Lifton, R. P., Holzman, L. B., Sibinga, N. E. S., Walz, G., Tufro, A., and Hildebrandt, F. (2016) FAT1 mutations cause a glomerulotubular nephropathy. *Nat Commun*. **7**, 10822
107. Möller, C. C., Flesche, J., and Reiser, J. (2009) Sensitizing the Slit Diaphragm with TRPC6 ion channels. *J Am Soc Nephrol*. **20**, 950–953
108. Reiser, J., Polu, K. R., Möller, C. C., Kenlan, P., Altintas, M. M., Wei, C., Faul, C., Herbert, S., Villegas, I., Avila-Casado, C., McGee, M., Sugimoto, H., Brown, D., Kalluri, R., Mundel, P., Smith, P. L., Clapham, D. E., and Pollak, M. R. (2005) TRPC6 is a glomerular slit diaphragm-associated channel required for normal renal function. *Nat Genet*. **37**, 739–744
109. Winn, M. P., Conlon, P. J., Lynn, K. L., Farrington, M. K., Creazzo, T., Hawkins, A. F., Daskalakis, N., Kwan, S. Y., Ebersviller, S., Burchette, J. L., Pericak-Vance, M. A., Howell, D. N., Vance, J. M., and Rosenberg, P. B. (2005) A mutation in the TRPC6 cation channel causes familial focal segmental glomerulosclerosis. *Science*. **308**, 1801–1804
110. Sun, Z. J., Ng, K. H., Liao, P., Zhang, Y., Ng, J. L., Liu, I. D., Tan, P. H., Chong, S. S. C., Chan, Y. H., Liu, J., Davila, S., Heng, C. K., Jordan, S. C., Soong, T. W., and Yap, H. K. (2015) Genetic Interactions Between TRPC6 and NPHS1 Variants Affect Posttransplant Risk of Recurrent Focal Segmental Glomerulosclerosis. *Am J Transplant*. **15**, 3229–3238
111. Riehle, M., Büscher, A. K., Gohlke, B.-O., Kaßmann, M., Kolatsi-Joannou, M., Bräsen, J. H., Nagel, M., Becker, J. U., Winyard, P., Hoyer, P. F., Preissner, R., Krautwurst, D., Gollasch, M., Weber, S., and Harteneck, C. (2016) TRPC6 G757D Loss-of-Function Mutation Associates with FSGS. *JASN*. **27**, 2771–2783
112. Aelst, L. V., and D'Souza-Schorey, C. (1997) Rho GTPases and signaling networks. *Genes Dev.* **11**, 2295–2322
113. Etienne-Manneville, S., and Hall, A. (2002) Rho GTPases in cell biology. *Nature*. **420**, 629–635

114. Burridge, K., and Wennerberg, K. (2004) Rho and Rac Take Center Stage. *Cell*. **116**, 167–179
115. Hall, A. (1998) Rho GTPases and the actin cytoskeleton. *Science*. **279**, 509–514
116. Nobes, C. D., and Hall, A. (1995) Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell*. **81**, 53–62
117. Heasman, S. J., and Ridley, A. J. (2008) Mammalian Rho GTPases: new insights into their functions from in vivo studies. *Nat Rev Mol Cell Biol*. **9**, 690–701
118. Cerione, R. A., and Zheng, Y. (1996) The Dbl family of oncogenes. *Curr Opin Cell Biol*. **8**, 216–222
119. Laurin, M., and Côté, J.-F. (2014) Insights into the biological functions of Dock family guanine nucleotide exchange factors. *Genes Dev*. **28**, 533–547
120. Garrett, M. D., Self, A. J., van Oers, C., and Hall, A. (1989) Identification of distinct cytoplasmic targets for ras/R-ras and rho regulatory proteins. *J Biol Chem*. **264**, 10–13
121. Tcherkezian, J., and Lamarche-Vane, N. (2007) Current knowledge of the large RhoGAP family of proteins. *Biol Cell*. **99**, 67–86
122. Olofsson, B. (1999) Rho guanine dissociation inhibitors: pivotal molecules in cellular signalling. *Cell Signal*. **11**, 545–554
123. Fort, P., and Blangy, A. (2017) The Evolutionary Landscape of Dbl-Like RhoGEF Families: Adapting Eukaryotic Cells to Environmental Signals. *Genome Biol Evol*. **9**, 1471–1486
124. Amin, E., Jaiswal, M., Derewenda, U., Reis, K., Nouri, K., Koessmeier, K. T., Aspenström, P., Somlyo, A. V., Dvorsky, R., and Ahmadian, M. R. (2016) Deciphering the Molecular and Functional Basis of RHOGAP Family Proteins: A SYSTEMATIC APPROACH TOWARD SELECTIVE INACTIVATION OF RHO FAMILY PROTEINS. *J Biol Chem*. **291**, 20353–20371
125. Garcia-Mata, R., Boulter, E., and Burridge, K. (2011) The "invisible hand": regulation of RHO GTPases by RHOGDIs. *Nat Rev Mol Cell Biol*. **12**, 493–504
126. Salloum, G., Jaafar, L., and El-Sibai, M. (2020) Rho A and Rac1: Antagonists moving forward. *Tissue Cell*. **65**, 101364
127. Antón, I. M., Gómez-Oro, C., Rivas, S., and Wandosell, F. (2020) Crosstalk between WIP and Rho family GTPases. *Small GTPases*. **11**, 160–166
128. Ridley, A. J., and Hall, A. (1992) The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell*. **70**, 389–399
129. Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D., and Hall, A. (1992) The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell*. **70**, 401–410
130. Machacek, M., Hodgson, L., Welch, C., Elliott, H., Pertz, O., Nalbant, P., Abell, A., Johnson, G. L., Hahn, K. M., and Danuser, G. (2009) Coordination of Rho GTPase activities during cell protrusion. *Nature*. **461**, 99–103
131. Shi, J., and Wei, L. (2022) Rho Kinases in Embryonic Development and Stem Cell Research. *Arch Immunol Ther Exp (Warsz)*. **70**, 4
132. Crosas-Molist, E., Samain, R., Kohlhammer, L., Orgaz, J. L., George, S. L., Maiques, O., Barcelo, J., and Sanz-Moreno, V. (2022) Rho GTPase signaling in cancer progression and dissemination. *Physiol Rev*. **102**, 455–510

133. Rodríguez-Fdez, S., and Bustelo, X. R. (2021) Rho GTPases in Skeletal Muscle Development and Homeostasis. *Cells*. **10**, 2984
134. Lee, C. F., Carley, R. E., Butler, C. A., and Morrison, A. R. (2021) Rac GTPase Signaling in Immune-Mediated Mechanisms of Atherosclerosis. *Cells*. **10**, 2808
135. Cai, R., Wang, Y., Huang, Z., Zou, Q., Pu, Y., Yu, C., and Cai, Z. (2021) Role of RhoA/ROCK signaling in Alzheimer's disease. *Behav Brain Res*. **414**, 113481
136. Ngo, A. T. P., Parra-Izquierdo, I., Aslan, J. E., and McCarty, O. J. T. (2021) Rho GTPase regulation of reactive oxygen species generation and signalling in platelet function and disease. *Small GTPases*. **12**, 440–457
137. Niftullayev, S., and Lamarche-Vane, N. (2019) Regulators of Rho GTPases in the Nervous System: Molecular Implication in Axon Guidance and Neurological Disorders. *Int J Mol Sci*. **20**, E1497
138. Saleem, M. A., and Welsh, G. I. (2019) Podocyte RhoGTPases: new therapeutic targets for nephrotic syndrome? *F1000Res*. **8**, F1000 Faculty Rev-1847
139. Asanuma, K., Yanagida-Asanuma, E., Faul, C., Tomino, Y., Kim, K., and Mundel, P. (2006) Synaptopodin orchestrates actin organization and cell motility via regulation of RhoA signalling. *Nat Cell Biol*. **8**, 485–491
140. Faul, C., Donnelly, M., Merscher-Gomez, S., Chang, Y. H., Franz, S., Delfgaauw, J., Chang, J.-M., Choi, H. Y., Campbell, K. N., Kim, K., Reiser, J., and Mundel, P. (2008) The actin cytoskeleton of kidney podocytes is a direct target of the antiproteinuric effect of cyclosporine A. *Nat Med*. **14**, 931–938
141. Scott, R. P., Hawley, S. P., Ruston, J., Du, J., Brakebusch, C., Jones, N., and Pawson, T. (2012) Podocyte-specific loss of Cdc42 leads to congenital nephropathy. *J Am Soc Nephrol*. **23**, 1149–1154
142. Wang, L., Ellis, M. J., Gomez, J. A., Eisner, W., Fennell, W., Howell, D. N., Ruiz, P., Fields, T. A., and Spurney, R. F. (2012) Mechanisms of the proteinuria induced by Rho GTPases. *Kidney International*. **81**, 1075–1085
143. Lal, M. A., and Tryggvason, K. (2012) Knocking Out Podocyte Rho GTPases: And the Winner Is…. *JASN*. **23**, 1128–1129
144. Zhang, H., Cybulsky, A. V., Aoudjit, L., Zhu, J., Li, H., Lamarche-Vane, N., and Takano, T. (2007) Role of Rho-GTPases in complement-mediated glomerular epithelial cell injury. *Am J Physiol Renal Physiol*. **293**, F148-156
145. Koopman, J. J. E., van Essen, M. F., Rennke, H. G., de Vries, A. P. J., and van Kooten, C. (2020) Deposition of the Membrane Attack Complex in Healthy and Diseased Human Kidneys. *Front Immunol*. **11**, 599974
146. Zhu, L., Jiang, R., Aoudjit, L., Jones, N., and Takano, T. (2011) Activation of RhoA in Podocytes Induces Focal Segmental Glomerulosclerosis. *JASN*. **22**, 1621–1630
147. Komers, R. (2013) Rho kinase inhibition in diabetic kidney disease. *British Journal of Clinical Pharmacology*. **76**, 551–559
148. Wang, W., Wang, Y., Long, J., Wang, J., Haudek, S. B., Overbeek, P., Chang, B. H. J., Schumacker, P. T., and Danesh, F. R. (2012) Mitochondrial fission triggered by hyperglycemia is mediated by ROCK1 activation in podocytes and endothelial cells. *Cell Metab*. **15**, 186–200
149. Wang, S., Chen, C., Su, K., Zha, D., Liang, W., Hillebrands, J. L., Goor, H. van, and Ding, G. (2016) Angiotensin II induces reorganization of the actin cytoskeleton and myosin

light-chain phosphorylation in podocytes through rho/ROCK-signaling pathway. *Ren Fail*. **38**, 268–275
150. Lv, Z., Hu, M., Ren, X., Fan, M., Zhen, J., Chen, L., Lin, J., Ding, N., Wang, Q., and Wang, R. (2016) Fyn Mediates High Glucose-Induced Actin Cytoskeleton Reorganization of Podocytes via Promoting ROCK Activation In Vitro. *J Diabetes Res*. **2016**, 5671803
151. Raftopoulou, M., and Hall, A. (2004) Cell migration: Rho GTPases lead the way. *Developmental Biology*. **265**, 23–32
152. Chesarone, M. A., DuPage, A. G., and Goode, B. L. (2010) Unleashing formins to remodel the actin and microtubule cytoskeletons. *Nat Rev Mol Cell Biol*. **11**, 62–74
153. Tian, D., Jacobo, S. M. P., Billing, D., Rozkalne, A., Gage, S. D., Anagnostou, T., Pavenstädt, H., Hsu, H.-H., Schlondorff, J., Ramos, A., and Greka, A. (2010) Antagonistic regulation of actin dynamics and cell motility by TRPC5 and TRPC6 channels. *Sci Signal*. **3**, ra77
154. Jacinto, A., Martinez-Arias, A., and Martin, P. (2001) Mechanisms of epithelial fusion and repair. *Nat Cell Biol*. **3**, E117–E123
155. Attias, O., Jiang, R., Aoudjit, L., Kawachi, H., and Takano, T. (2010) Rac1 contributes to actin organization in glomerular podocytes. *Nephron Exp Nephrol*. **114**, e93–e106
156. Liu, X. L., Kilpeläinen, P., Hellman, U., Sun, Y., Wartiovaara, J., Morgunova, E., Pikkarainen, T., Yan, K., Jonsson, A. P., and Tryggvason, K. (2005) Characterization of the interactions of the nephrin intracellular domain. *The FEBS Journal*. **272**, 228–243
157. Wang, F., Herzmark, P., Weiner, O. D., Srinivasan, S., Servant, G., and Bourne, H. R. (2002) Lipid products of PI(3)Ks maintain persistent cell polarity and directed motility in neutrophils. *Nat Cell Biol*. **4**, 513–518
158. Nimnual, A. S., Taylor, L. J., and Bar-Sagi, D. (2003) Redox-dependent downregulation of Rho by Rac. *Nat Cell Biol*. **5**, 236–241
159. Blattner, S. M., Hodgin, J. B., Nishio, M., Wylie, S. A., Saha, J., Soofi, A. A., Vining, C., Randolph, A., Herbach, N., Wanke, R., Atkins, K. B., Gyung Kang, H., Henger, A., Brakebusch, C., Holzman, L. B., and Kretzler, M. (2013) Divergent functions of the Rho GTPases Rac1 and Cdc42 in podocyte injury. *Kidney Int*. **84**, 920–930
160. Robins, R., Baldwin, C., Aoudjit, L., Côté, J.-F., Gupta, I. R., and Takano, T. (2017) Rac1 activation in podocytes induces the spectrum of nephrotic syndrome. *Kidney Int*. **92**, 349–364
161. Yu, H., Suleiman, H., Kim, A. H. J., Miner, J. H., Dani, A., Shaw, A. S., and Akilesh, S. (2013) Rac1 activation in podocytes induces rapid foot process effacement and proteinuria. *Mol Cell Biol*. **33**, 4755–4764
162. Brähler, S., Yu, H., Suleiman, H., Krishnan, G. M., Saunders, B. T., Kopp, J. B., Miner, J. H., Zinselmeyer, B. H., and Shaw, A. S. (2016) Intravital and Kidney Slice Imaging of Podocyte Membrane Dynamics. *JASN*. **27**, 3285–3290
163. Gee, H. Y., Saisawat, P., Ashraf, S., Hurd, T. W., Vega-Warner, V., Fang, H., Beck, B. B., Gribouval, O., Zhou, W., Diaz, K. A., Natarajan, S., Wiggins, R. C., Lovric, S., Chernin, G., Schoeb, D. S., Ovunc, B., Frishberg, Y., Soliman, N. A., Fathy, H. M., Goebel, H., Hoefele, J., Weber, L. T., Innis, J. W., Faul, C., Han, Z., Washburn, J., Antignac, C., Levy, S., Otto, E. A., and Hildebrandt, F. (2013) ARHGDIA mutations cause nephrotic syndrome via defective RHO GTPase signaling. *J Clin Invest*. **123**, 3243–3253
164. Gupta, I. R., Baldwin, C., Auguste, D., Ha, K. C. H., El Andalousi, J., Fahiminiya, S., Bitzan, M., Bernard, C., Akbari, M. R., Narod, S. A., Rosenblatt, D. S., Majewski, J., and

Takano, T. (2013) ARHGDIA: a novel gene implicated in nephrotic syndrome. *J Med Genet*. **50**, 330–338
165. Akilesh, S., Suleiman, H., Yu, H., Stander, M. C., Lavin, P., Gbadegesin, R., Antignac, C., Pollak, M., Kopp, J. B., Winn, M. P., and Shaw, A. S. (2011) Arhgap24 inactivates Rac1 in mouse podocytes, and a mutant form is associated with familial focal segmental glomerulosclerosis. *J Clin Invest*. **121**, 4127–4137
166. Adams, A. E., Johnson, D. I., Longnecker, R. M., Sloat, B. F., and Pringle, J. R. (1990) CDC42 and CDC43, two additional genes involved in budding and the establishment of cell polarity in the yeast Saccharomyces cerevisiae. *J Cell Biol*. **111**, 131–142
167. Hussain, N. K., Jenna, S., Glogauer, M., Quinn, C. C., Wasiak, S., Guipponi, M., Antonarakis, S. E., Kay, B. K., Stossel, T. P., Lamarche-Vane, N., and McPherson, P. S. (2001) Endocytic protein intersectin-l regulates actin assembly via Cdc42 and N-WASP. *Nat Cell Biol*. **3**, 927–932
168. McGavin, M. K. H., Badour, K., Hardy, L. A., Kubiseski, T. J., Zhang, J., and Siminovitch, K. A. (2001) The Intersectin 2 Adaptor Links Wiskott Aldrich Syndrome Protein (WASp)-mediated Actin Polymerization to T Cell Antigen Receptor Endocytosis. *Journal of Experimental Medicine*. **194**, 1777–1787
169. Ashraf, S., Kudo, H., Rao, J., Kikuchi, A., Widmeier, E., Lawson, J. A., Tan, W., Hermle, T., Warejko, J. K., Shril, S., Airik, M., Jobst-Schwan, T., Lovric, S., Braun, D. A., Gee, H. Y., Schapiro, D., Majmundar, A. J., Sadowski, C. E., Pabst, W. L., Daga, A., van der Ven, A. T., Schmidt, J. M., Low, B. C., Gupta, A. B., Tripathi, B. K., Wong, J., Campbell, K., Metcalfe, K., Schanze, D., Niihori, T., Kaito, H., Nozu, K., Tsukaguchi, H., Tanaka, R., Hamahira, K., Kobayashi, Y., Takizawa, T., Funayama, R., Nakayama, K., Aoki, Y., Kumagai, N., Iijima, K., Fehrenbach, H., Kari, J. A., El Desoky, S., Jalalah, S., Bogdanovic, R., Stajić, N., Zappel, H., Rakhmetova, A., Wassmer, S.-R., Jungraithmayr, T., Strehlau, J., Kumar, A. S., Bagga, A., Soliman, N. A., Mane, S. M., Kaufman, L., Lowy, D. R., Jairajpuri, M. A., Lifton, R. P., Pei, Y., Zenker, M., Kure, S., and Hildebrandt, F. (2018) Mutations in six nephrosis genes delineate a pathogenic pathway amenable to treatment. *Nat Commun*. **9**, 1960
170. Roux, K. J., Kim, D. I., Raida, M., and Burke, B. (2012) A promiscuous biotin ligase fusion protein identifies proximal and interacting proteins in mammalian cells. *J Cell Biol*. **196**, 801–810
171. Bagci, H., Sriskandarajah, N., Robert, A., Boulais, J., Elkholi, I. E., Tran, V., Lin, Z.-Y., Thibault, M.-P., Dubé, N., Faubert, D., Hipfner, D. R., Gingras, A.-C., and Côté, J.-F. (2020) Mapping the proximity interaction network of the Rho-family GTPases reveals signalling pathways and regulatory mechanisms. *Nat Cell Biol*. **22**, 120–134
172. Lamarche-Vane, N., and Hall, A. (1998) CdGAP, a novel proline-rich GTPase-activating protein for Cdc42 and Rac. *J Biol Chem*. **273**, 29172–29177
173. He, Y., Northey, J. J., Primeau, M., Machado, R. D., Trembath, R., Siegel, P. M., and Lamarche-Vane, N. (2011) CdGAP is required for transforming growth factor β- and Neu/ErbB-2-induced breast cancer cell motility and invasion. *Oncogene*. **30**, 1032–1045
174. He, Y., Northey, J. J., Pelletier, A., Kos, Z., Meunier, L., Haibe-Kains, B., Mes-Masson, A.-M., Côté, J.-F., Siegel, P. M., and Lamarche-Vane, N. (2017) The Cdc42/Rac1 regulator CdGAP is a novel E-cadherin transcriptional co-repressor with Zeb2 in breast cancer. *Oncogene*. **36**, 3490–3503

175. Mehra, C., Chung, J.-H., He, Y., Lara-Márquez, M., Goyette, M.-A., Boufaied, N., Barrès, V., Ouellet, V., Guérard, K.-P., Delliaux, C., Saad, F., Lapointe, J., Côté, J.-F., Labbé, D. P., and Lamarche-Vane, N. (2021) CdGAP promotes prostate cancer metastasis by regulating epithelial-to-mesenchymal transition, cell cycle progression, and apoptosis. *Commun Biol*. **4**, 1042
176. Ouadda, A. B. D., He, Y., Calabrese, V., Ishii, H., Chidiac, R., Gratton, J.-P., Roux, P. P., and Lamarche-Vane, N. (2018) CdGAP/ARHGAP31 is regulated by RSK phosphorylation and binding to 14-3-3β adaptor protein. *Oncotarget*. **9**, 11646–11664
177. LaLonde, D. P., Grubinger, M., Lamarche-Vane, N., and Turner, C. E. (2006) CdGAP associates with actopaxin to regulate integrin-dependent changes in cell morphology and motility. *Curr Biol*. **16**, 1375–1385
178. Caron, C., DeGeer, J., Fournier, P., Duquette, P. M., Luangrath, V., Ishii, H., Karimzadeh, F., Lamarche-Vane, N., and Royal, I. (2016) CdGAP/ARHGAP31, a Cdc42/Rac1 GTPase regulator, is critical for vascular development and VEGF-mediated angiogenesis. *Sci Rep*. **6**, 27485
179. Southgate, L., Machado, R. D., Snape, K. M., Primeau, M., Dafou, D., Ruddy, D. M., Branney, P. A., Fisher, M., Lee, G. J., Simpson, M. A., He, Y., Bradshaw, T. Y., Blaumeiser, B., Winship, W. S., Reardon, W., Maher, E. R., FitzPatrick, D. R., Wuyts, W., Zenker, M., Lamarche-Vane, N., and Trembath, R. C. (2011) Gain-of-function mutations of ARHGAP31, a Cdc42/Rac1 GTPase regulator, cause syndromic cutis aplasia and limb anomalies. *Am J Hum Genet*. **88**, 574–585
180. Karimzadeh, F., Primeau, M., Mountassif, D., Rouiller, I., and Lamarche-Vane, N. (2012) A stretch of polybasic residues mediates Cdc42 GTPase-activating protein (CdGAP) binding to phosphatidylinositol 3,4,5-trisphosphate and regulates its GAP activity. *J Biol Chem*. **287**, 19610–19621
181. Guipponi, M., Scott, H. S., Chen, H., Schebesta, A., Rossier, C., and Antonarakis, S. E. (1998) Two isoforms of a human intersectin (ITSN) protein are produced by brain-specific alternative splicing in a stop codon. *Genomics*. **53**, 369–376
182. Primeau, M., Ben Djoudi Ouadda, A., and Lamarche-Vane, N. (2011) Cdc42 GTPase-activating protein (CdGAP) interacts with the SH3D domain of Intersectin through a novel basic-rich motif. *FEBS Lett*. **585**, 847–853
183. Jenna, S., Hussain, N. K., Danek, E. I., Triki, I., Wasiak, S., McPherson, P. S., and Lamarche-Vane, N. (2002) The activity of the GTPase-activating protein CdGAP is regulated by the endocytic protein intersectin. *J Biol Chem*. **277**, 6366–6373
184. Tcherkezian, J., Danek, E. I., Jenna, S., Triki, I., and Lamarche-Vane, N. (2005) Extracellular signal-regulated kinase 1 interacts with and phosphorylates CdGAP at an important regulatory site. *Mol Cell Biol*. **25**, 6314–6329
185. Danek, E. I., Tcherkezian, J., Triki, I., Meriane, M., and Lamarche-Vane, N. (2007) Glycogen synthase kinase-3 phosphorylates CdGAP at a consensus ERK 1 regulatory site. *J Biol Chem*. **282**, 3624–3631
186. McCormack, J. J., Bruche, S., Ouadda, A. B. D., Ishii, H., Lu, H., Garcia-Cattaneo, A., Chávez-Olórtegui, C., Lamarche-Vane, N., and Braga, V. M. M. (2017) The scaffold protein Ajuba suppresses CdGAP activity in epithelia to maintain stable cell-cell contacts. *Sci Rep*. **7**, 9249

187. Isrie, M., Wuyts, W., Van Esch, H., and Devriendt, K. (2014) Isolated terminal limb reduction defects: extending the clinical spectrum of Adams-Oliver syndrome and ARHGAP31 mutations. *Am J Med Genet A*. **164A**, 1576–1579
188. Oh, W. K., Yoo, J. C., Jo, D., Song, Y. H., Kim, M. G., and Park, D. (1997) Cloning of a SH3 domain-containing proline-rich protein, p85SPR, and its localization in focal adhesion. *Biochem Biophys Res Commun*. **235**, 794–798
189. Bagrodia, S., Taylor, S. J., Jordon, K. A., Van Aelst, L., and Cerione, R. A. (1998) A novel regulator of p21-activated kinases. *J Biol Chem*. **273**, 23633–23636
190. Manser, E., Loo, T.-H., Koh, C.-G., Zhao, Z.-S., Chen, X.-Q., Tan, L., Tan, I., Leung, T., and Lim, L. (1998) PAK Kinases Are Directly Coupled to the PIX Family of Nucleotide Exchange Factors. *Molecular Cell*. **1**, 183–192
191. Rosenberger, G., Jantke, I., Gal, A., and Kutsche, K. (2003) Interaction of alphaPIX (ARHGEF6) with beta-parvin (PARVB) suggests an involvement of alphaPIX in integrin-mediated signaling. *Hum Mol Genet*. **12**, 155–167
192. Kutsche, K., Yntema, H., Brandt, A., Jantke, I., Nothwang, H. G., Orth, U., Boavida, M. G., David, D., Chelly, J., Fryns, J. P., Moraine, C., Ropers, H. H., Hamel, B. C., van Bokhoven, H., and Gal, A. (2000) Mutations in ARHGEF6, encoding a guanine nucleotide exchange factor for Rho GTPases, in patients with X-linked mental retardation. *Nat Genet*. **26**, 247–250
193. Manser, E., Huang, H. Y., Loo, T. H., Chen, X. Q., Dong, J. M., Leung, T., and Lim, L. (1997) Expression of constitutively active alpha-PAK reveals effects of the kinase on actin and focal complexes. *Mol Cell Biol*. **17**, 1129–1143
194. ten Klooster, J. P., Jaffer, Z. M., Chernoff, J., and Hordijk, P. L. (2006) Targeting and activation of Rac1 are mediated by the exchange factor beta-Pix. *J Cell Biol*. **172**, 759–769
195. Schmidt, M. H. H., Husnjak, K., Szymkiewicz, I., Haglund, K., and Dikic, I. (2006) Cbl escapes Cdc42-mediated inhibition by downregulation of the adaptor molecule betaPix. *Oncogene*. **25**, 3071–3078
196. Hoffman, G. R., and Cerione, R. A. (2002) Signaling to the Rho GTPases: networking with the DH domain. *FEBS Lett*. **513**, 85–91
197. Lemmon, M. A., and Ferguson, K. M. (2000) Signal-dependent membrane targeting by pleckstrin homology (PH) domains. *Biochem J*. **350 Pt 1**, 1–18
198. Manabe, R., Kovalenko, M., Webb, D. J., and Horwitz, A. R. (2002) GIT1 functions in a motile, multi-molecular signaling complex that regulates protrusive activity and cell migration. *J Cell Sci*. **115**, 1497–1510
199. Rosenberger, G., and Kutsche, K. (2006) AlphaPIX and betaPIX and their role in focal adhesion formation. *Eur J Cell Biol*. **85**, 265–274
200. Kim, S., Lee, S. H., and Park, D. (2001) Leucine zipper-mediated homodimerization of the p21-activated kinase-interacting factor, beta Pix. Implication for a role in cytoskeletal reorganization. *J Biol Chem*. **276**, 10581–10584
201. Koh, C. G., Manser, E., Zhao, Z. S., Ng, C. P., and Lim, L. (2001) Beta1PIX, the PAK-interacting exchange factor, requires localization via a coiled-coil region to promote microvillus-like structures and membrane ruffles. *J Cell Sci*. **114**, 4239–4251
202. Chahdi, A., and Sorokin, A. (2008) Protein kinase A-dependent phosphorylation modulates beta1Pix guanine nucleotide exchange factor activity through 14-3-3beta binding. *Mol Cell Biol*. **28**, 1679–1687

203. Missy, K., Hu, B., Schilling, K., Harenberg, A., Sakk, V., Kuchenbecker, K., Kutsche, K., and Fischer, K.-D. (2008) AlphaPIX Rho GTPase guanine nucleotide exchange factor regulates lymphocyte functions and antigen receptor signaling. *Mol Cell Biol*. **28**, 3776–3789
204. Omelchenko, T., Rabadan, M. A., Hernández-Martínez, R., Grego-Bessa, J., Anderson, K. V., and Hall, A. (2014) β-Pix directs collective migration of anterior visceral endoderm cells in the early mouse embryo. *Genes Dev*. **28**, 2764–2777
205. Matsuda, J., Maier, M., Aoudjit, L., Baldwin, C., and Takano, T. (2020) ARHGEF7 (β-PIX) Is Required for the Maintenance of Podocyte Architecture and Glomerular Function. *J Am Soc Nephrol*. **31**, 996–1008
206. Kutys, M. L., and Yamada, K. M. (2014) An extracellular-matrix-specific GEF-GAP interaction regulates Rho GTPase crosstalk for 3D collagen migration. *Nat Cell Biol*. **16**, 909–917
207. Saleem, M. A., O'Hare, M. J., Reiser, J., Coward, R. J., Inward, C. D., Farren, T., Xing, C. Y., Ni, L., Mathieson, P. W., and Mundel, P. (2002) A conditionally immortalized human podocyte cell line demonstrating nephrin and podocin expression. *J Am Soc Nephrol*. **13**, 630–638
208. Wormer, D., Deakin, N. O., and Turner, C. E. (2012) CdGAP regulates cell migration and adhesion dynamics in two-and three-dimensional matrix environments. *Cytoskeleton (Hoboken)*. **69**, 644–658
209. Shin, E.-Y., Woo, K.-N., Lee, C.-S., Koo, S.-H., Kim, Y. G., Kim, W.-J., Bae, C.-D., Chang, S.-I., and Kim, E.-G. (2004) Basic fibroblast growth factor stimulates activation of Rac1 through a p85 betaPIX phosphorylation-dependent pathway. *J Biol Chem*. **279**, 1994–2004
210. Wormer, D. B., Davis, K. A., Henderson, J. H., and Turner, C. E. (2014) The focal adhesion-localized CdGAP regulates matrix rigidity sensing and durotaxis. *PLoS One*. **9**, e91815
211. Kazanietz, M. G., and Caloca, M. J. (2017) The Rac GTPase in Cancer: From Old Concepts to New Paradigms. *Cancer Research*. **77**, 5445–5451
212. Yu, H. W., Chen, Y.-Q., Huang, C.-M., Liu, C.-Y., Chiou, A., Wang, Y.-K., Tang, M.-J., and Kuo, J.-C. (2015) β-PIX controls intracellular viscoelasticity to regulate lung cancer cell migration. *J Cell Mol Med*. **19**, 934–947
213. Müller, P. M., Rademacher, J., Bagshaw, R. D., Wortmann, C., Barth, C., van Unen, J., Alp, K. M., Giudice, G., Eccles, R. L., Heinrich, L. E., Pascual-Vargas, P., Sanchez-Castro, M., Brandenburg, L., Mbamalu, G., Tucholska, M., Spatt, L., Czajkowski, M. T., Welke, R.-W., Zhang, S., Nguyen, V., Rrustemi, T., Trnka, P., Freitag, K., Larsen, B., Popp, O., Mertins, P., Gingras, A.-C., Roth, F. P., Colwill, K., Bakal, C., Pertz, O., Pawson, T., Petsalaki, E., and Rocks, O. (2020) Systems analysis of RhoGEF and RhoGAP regulatory proteins reveals spatially organized RAC1 signalling from integrin adhesions. *Nat Cell Biol*. **22**, 498–511