Guanine Nucleotide Exchange Factors Activate Rac1 in Podocytes

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ABSTRACT

Focal segmental glomerulosclerosis (FSGS) is a histopathological lesion characterized by podocyte injury, during which the podocyte’s actin-rich projections, called foot processes, retract (i.e. efface) back into the cell body. RhoGTPases, such as Rac1, contribute to the organization of the actin cytoskeleton and both in vivo animal studies and human genetics studies suggest that Rac1 hyperactivity leads to foot process effacement, proteinuria (the leakage of protein from blood into the urine), and FSGS. While Rho guanine nucleotide exchange factors (GEFs) are typically known for activating RhoGTPases, it remains unclear if any GEFs are responsible for Rac1 activity in FSGS. This study investigates this by using two methods: a candidate and an unbiased approach. Candidate GEFs were discovered using RNA-sequencing on two lines of cultured human podocytes, followed by analysis of which GEFs have upregulated mRNA in humans with FSGS compared to healthy control; this led us to investigate Trio. Trio knockout (KO) in cultured human podocytes decreased Rac1 activity, cell size, attachment, and motility. Furthermore, while the profibrotic cytokine that contributes to glomerulosclerosis progression, transforming growth factor β1 (TGFβ1), increases Rac1 activity in control cells, it decreases Rac1 activity in Trio KOs. This may due to simultaneous activation of a Rac1-GTPase activation protein (GAP) called Arhgap31. Additionally, the unbiased approach, which consisted of the proximity-based BioID assay, led us to study β-PIX. TGFβ1 increases the amount of β-PIX isolated by BioID and by pulldown with Rac1. Thus, in conclusion, we have found that two GEFs, Trio and β-PIX, contribute to the Rac1 activation in podocytes.
RÉSUMÉ

La glomérulosclérose segmentaire focale (FSGS) est une maladie rénale caractérisée par une lésion des podocytes, au cours de laquelle les projections riches en actine du podocyte, appelées processus du pied, se rétractent (c'est-à-dire s'effacent) dans le corps cellulaire. La famille des petites protéines Rho-GTPases, comme Rac1, joue un rôle déterminant dans l’organisation du cytosquelette d'actine et des études animales in vivo et des études sur la génétique humaine suggèrent que l'hyperactivité Rac1 entraîne un effacement du pied, une protéinurie (fuite du sang dans l'urine) et une FSGS. Alors que les protéines guanine facteurs d'échange nucléotidique (GEFs) sont connues pour activer les protéines Rho-GTPases, il n'est pas clair si des GEFs sont responsables de l'activité Rac1 dans FSGS. Cette présente examine cela en utilisant deux méthodes: un candidat et une approche impartiale. Les GEF candidats ont été découverts en utilisant le séquençage de l'ARN sur deux lignées de podocytes humains cultivés, suivi par l'analyse des ARNm de GEFs qui sont régulés à la hausse chez les humains avec la FSGS; cela nous a conduit à enquêter sur Trio. Le knockout du trio (KO) dans les podocytes humains cultivés a diminué l'activité de Rac1, la taille des cellules, l'attachement et la motilité. En outre, alors que la cytokine profibrotique qui contribue à la progression de la glomérulosclérose, facteur de croissance transformant β1 (TGFβ1), augmente l'activité Rac1 dans les cellules témoins, elle diminue l'activité Rac1 chez les Trio KO. Ceci est dû à l'activation simultanée d'une protéine d'activation Rac1-GTPase (GAP) appelée Arhgap31. De plus, l'approche non biaisée, qui consistait en un dosage BioID basé sur la proximité, nous a conduit à étudier β-PIX. TGFβ1 augmente la quantité de β-PIX isolé par BioID et par pulldown avec Rac1. Ainsi, en conclusion, nous avons trouvé que deux GEF, Trio et β-PIX, contribuent à l'activation de Rac1 dans les podocytes.
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CHAPTER 1: LITERATURE REVIEW AND OBJECTIVES
1.1 INTRODUCTION TO THE GLOMERULUS AND THE PODOCYTE

1.1.1 The Glomerulus

The kidneys are two organs found dorsally-located in the abdominal cavity. The kidneys have a plethora of functions, including hormone production, maintenance of acid-base homeostasis, blood pressure regulation. However, their most well-known function is the filtration of blood, leading to the formation of urine. On average, a pair of human kidneys produce 1 liter of urine per day (1). The nephron is the basic structural and functional unit of the kidney. The nephron has two components: glomerulus and renal tubules.

The glomerulus is a capillary tuft found at the proximal end of the nephron; it is the basic filtration unit of the kidney. The average human kidney contains 1 million glomeruli, which generate 180 liters of filtrate per day (2). The glomerulus consists of four types of cells: glomerular endothelial cells, specialized visceral epithelial cells (known as podocytes), mesangial cells, and parietal epithelial cells (1). The glomerular endothelial cells form the capillary lumen; these capillaries are unique because, unlike others, they are not supported by interstitial tissue. Podocytes sit atop of these endothelial cells, wrapped around the capillaries, and help maintain the structure of the glomerular capillaries (3). These capillaries are also supported by mesangial cells and the extracellular matrix (ECM) these cells produce. The mesangial cells and their ECM together form the mesangium (1). It was long believed that intraglomerular mesangial cells use their contractile properties to help regulate blood flow through the glomerular capillaries. However, this was concluded from studies performed in vitro; the in vivo validity remains debated (4). Finally, parietal cells make up the Bowman’s capsule, a cup-like structure around the glomerulus. Between the Bowman’s capsule and the glomerulus is the urinary space.

The glomerulus carries out the first step of blood filtration. Blood enters the glomerulus, via the afferent arteriole, where it is filtered and the filtrate is pushed by hydrostatic pressure into the urinary space. From there, the filtrate moves into the renal tubules, where tubular cells secrete and reabsorb molecules to fine-tune what is lost in the glomerular filtrate.

The glomerulus must strike a balance—it must excrete toxins and waste while retaining proteins and blood cells. It does this by forming a size- and charge-selective filter, known as the glomerular filtration barrier, which will retain large particles (such as red blood cells and
albumin) while allowing small particles (such as urea and drug metabolites) to sieve through. Negatively charged particles are also hindered from leaving the plasma (3). The glomerular filtration barrier is made up of three components: fenestrated endothelial cells, an acellular basement membrane, and podocytes. These components interact with one another. For example, during kidney development, podocytes produce vascular endothelial growth factor (VEGF)-A, which attracts glomerular endothelial cells from the metanephric mesenchyme to the developing glomerulus (3). Podocytes also make components of the glomerular basement membrane (GBM) (5).

The endothelial cells’ fenestrae are 70-100nm in diameter (3) and make up 20-50% of the capillary surface area (6). These fenestrae are large enough for proteins, such as albumin, to leak through. However, on the apical surface of these endothelial cells is a layer of glycocalyx, which helps impede proteins from leaving the plasma.

Although all three components of the glomerular filtration barrier contribute to the plasma filtration, the podocyte appears to be the most important part (6).

1.1.2 The Podocyte

Podocytes are epithelial cells derived from the metanephric mesenchyme (3). In a fully-developed kidney, these terminally differentiated cells are wrapped around the glomerular capillaries, facing the urinary space. The podocyte structure is unique—it has a cell body, primary processes, and smaller secondary processes, also known as foot processes. The foot processes from neighboring podocytes interdigitate and form a zipper-like structure. Between foot processes, there are filtration slits, where the last step of glomerular filtration occurs.

These filtration slits are bridged by the slit diaphragm, a modified adherens junction (3). During the comma and S-shape stages of glomerular development, podocyte precursor cells form tight junctions near the apical surface. However, as the cells differentiate, the junctions move towards the basolateral sides while adherens junction and neuronal junction (e.g. nephrin and Neph1) proteins enter the junction (7). The fully-developed slit diaphragm is made up of transmembrane proteins from adjacent foot processes that interact with one another in the small gaps between foot processes.

The mature slit diaphragm consists of multiple protein complexes. One is made up of nephrin, Neph1, and podocin (8). Nephrin is a long transmembrane protein whose extracellular
domain can span across the 40nm space between foot processes and interact in trans with other nephrin molecules from adjacent podocytes. The intracellular domain of nephrin has sites that are phosphorylated by tyrosine protein kinases, such as Fyn, Yes, and phosphatidylinositol-3 kinase (PI3K). Neph1 is homologous to nephrin, but has a shorter extracellular domain. Neph1 is too short to cross the distance between foot processes, but it may interact with nephrin in cis and trans configurations. Neph1 can recruit scaffolding proteins (ex. ZO-1) (7) and adaptor proteins (ex. Grb2) (8). Podocin is also found within the plasma membrane at the slit diaphragm, but both its C- and N-termini are in the cytoplasm. It recruits other slit diaphragm proteins, including nephrin and Neph1, to cholesterol-rich micro-domains of the plasma membrane (7, 8).

Initially, the slit diaphragm was thought of as a simple sieve—the proteins interacting between neighboring podocytes form pores that do not allow proteins through. However, it is now known that the slit diaphragm has multiple functions: macromolecule filtration, signaling, anchoring the podocyte to the GBM, and connecting the slit diaphragm to the cytoskeleton (7).

Slit diaphragm proteins transduce extracellular cues into intracellular signaling pathways. For example, one way podocytes can sense and react to capillary pressure is via its transient receptor potential canonical (TRPC) 6. TRPC6 is a calcium-permeable channel in the slit diaphragm; it regulates the intracellular Ca²⁺ concentration. TRPC6, interacts with podocin, which is thought to be mechanosensitive (7). Thus, it is postulated that TRPC6-mediated changes in Ca²⁺ concentration allow the cytoskeleton to rearrange in response to its environment (9).

Indeed, podocyte foot processes must be dynamic structures in order to respond to changes in capillary pressure (8) and other stresses (5). This movement is controlled by the podocyte cytoskeleton. Microtubules and intermediate filaments are found in the cell body and primary processes, while foot processes are made up of parallel contractile actin bundles (1). When podocytes are injured, their foot processes retract back into the cell body, a process known as foot process effacement. These effaced foot processes fail to form a proper sieve, thereby allowing the leakage of protein (proteinuria) or blood cells (hematuria) from the blood into the urine. While foot processes must be dynamic to adapt to stressors, too much motility may be reflective of foot process effacement (10). For this reason, much research has focused on the podocyte’s actin cytoskeleton.

As mentioned earlier, signaling from slit diaphragm proteins can reorganize the actin cytoskeleton. For example, when nephrin is tyrosine phosphorylated by Fyn, a Src family protein
kinase, it can bind to Nck adaptor proteins. Nck proteins recruit protein complexes, such as neural Wiskott-Aldrich-syndrome protein (N-WASP) and Arp2/3, that will regulate actin polymerization (8). In addition to recruitment, Nck can also allosterically activate N-WASP (11). Nck expression is required for both podocyte development and maintenance. In transgenic mice with podocyte-specific Nck knock out, foot processes are deformed and glomeruli are sclerotic by 3.5 weeks after birth (12). Similarly, inducible podocyte-specific knock out of Nck in transgenic mice resulted in podocyte effacement and proteinuria (13). This demonstrates the importance of signaling between the slit diaphragm and the actin cytoskeleton for podocyte function. The podocyte’s actin cytoskeleton controls podocyte shape, motility, and adhesion to the GBM.

1.1.3 The Glomerular Basement Membrane

The glomerular basement membrane (GBM) is a network of extracellular matrix proteins found between the podocytes and the endothelial cells. The GBM is made up of two fused membranes, one coming from podocytes and the other from the endothelial cells of the capillary during glomerular development (2). It has a number of functions, including providing structural integrity to the glomerulus, contributing to the plasma filtration (via its negative charge), and providing a scaffold onto which endothelial cells and podocytes can attach securely.

The GBM has over 73 components (2), but mostly consists of type IV collagens, laminins, and proteoglycans (6). Type IV collagens come together in heterotrimeric made of three alpha chains. Developing glomeruli are predominately made of α1α1α1 while mature glomeruli consist of α3α4α5. The sulfimine bond (-S=N-) formed between adjacent collagen IV heterotrimers makes the GBM strong. In patients with mutations of type IV collagen α3, α4, or α5, the GBM is not able to withstand as much mechanical strain and will split. This is known as Alport’s syndrome and patients present with hematuria, proteinuria, and renal failure (2).

Laminins are heterotrimeric glycoproteins made up of α, β, and γ chains. These chains are arranged in a t-shape, with the short arm binding other laminins and the long arm binding cell surface receptors. During kidney development, the major laminin is laminin α5β1γ1 (laminin-511); however, this changes and the mature glomerulus contains mostly laminin -521 (2). Unlike type IV collagen, laminin-521 is required for the proper functioning of the GBM. Mutations in
LAMB2, the gene encoding laminin $\beta_2$, causes Pierson syndrome in humans (14) and glomerular dysfunction in mice (2, 15).

The GBM anchors the podocyte via focal adhesions; loss of focal adhesion proteins allows podocyte detachment from the GBM, leading to proteinuria. The focal adhesion complex consists of many proteins, including GBM-binding proteins, actin-binding proteins, signaling proteins, and adaptor proteins (16); thus, focal adhesions also act as a signaling hub. Integrins are heterodimeric transmembrane proteins that bind to laminin and/or collagen IV in the GBM and are linked (via adaptor proteins such as vinculin) to the actin cytoskeleton; they act as receptors for cell adhesion. When integrins bind GBM components, they cluster and recruit adaptor and effector proteins, thereby forming the focal adhesion complex. The types of proteins that are recruited are cell- and context-dependent. In podocytes, the laminin-binding $\alpha_3\beta_1$-integrin has the highest expression and is considered the most important (2). In mice, podocyte-specific knockout of Itgb1, which encodes $\beta_1$-integrin, leads to severe proteinuria at birth and renal failure by 3 weeks (2, 17). Levels of $\alpha_3\beta_1$-integrin are lower in patients with focal segmental glomerulosclerosis (FSGS), a renal condition discussed in the next section (18).

The structure of the glomerulus and the glomerular filtration barrier are visualized in figure 1.1.

1.2 FOCAL SEGMENTAL GLOMERULOSCLEROSIS

1.2.1 Introduction to FSGS

FSGS is both a disease (caused by podocyte injury) and a histological pattern of scarring (secondary to multiple kidney diseases) (19). FSGS is diagnosed by biopsy followed by light microscopy. The histological pattern is well-explained by the name: the “sclerosis” describes the scarring tissue; the “segmental” refers to only part of a glomerulus having sclerosis; and the “focal” defines that only some, not all, glomeruli are affected. However, there are variations in the exact glomerular morphology, FSGS can be classified as collapsing, tip, cellular perihilar, or not otherwise specified. The clinical significance of these morphological differences is still an area of investigation (20-22).

In adults, FSGS is responsible for 4% of end-stage renal disease (ERSD) cases and is the most common cause of nephrotic syndrome (19), which is characterized by proteinuria,
decreased levels of albumin in the blood, and edema. FSGS can be characterized as either primary (often caused by genetic mutations that result in podocyte damage—i.e. podocytopathy), idiopathic, or secondary to other conditions, such as viral infections, drug toxicity, or a previous loss of nephrons (19). In both primary and secondary FSGS, symptoms of podocytopathy include the retraction of foot processes into the cell body (i.e. effacement), podocyte detachment from the GBM, and loss of podocytes into the urine.

FSGS is a heterogeneous disease and may be caused by a circulating factor or a genetic mutation. FSGS recurrence occurs in 30-40% of renal transplant recipients (19). As FSGS recurrence can sometimes be treated with plasmapheresis, this led researchers to postulate that there is a molecule in the plasma that may cause FSGS. The most compelling evidence of this came in 2012, when a 27-year old man with primary FSGS received a kidney transplant but presented with proteinuria 2 days post-transplant and foot process effacement 6 days post-transplant. By day 14 post-transplant, the kidney was removed and donated to a 66-year old with ESRD secondary to diabetes mellitus type II. Biopsies at 8 and 25 days post-transplant, showed reversal of foot process effacement and the kidney showed normal functioning in the second patient 8 months post-transplant (24). While the circulating factor responsible for recurrent FSGS remains debated, researchers have identified a number of genetic mutations resulting in FSGS.

Another cause of nephrotic syndrome is minimal change disease (MCD). By light microscopy, MCD shows normal glomeruli but, by electron microscopy, foot process effacement becomes evident. Furthermore, MCD is sensitive to steroid treatment and foot process effacement is reversible (23). However, whether MCD is a separate disease from FSGS or simply a less progressed form of FSGS is currently a point of debate (23).

1.2.2 Genetic Causes

There are a number of genetic mutations that result in FSGS; they are extensively reviewed elsewhere (19, 25, 26). This manuscript will focus on a select few to highlight the importance of the GBM, the podocyte, its slit diaphragm, and its actin cytoskeleton in FSGS.

Mutations in genes encoding GBM components, such as laminin β2, result in FSGS. LAMB2 encodes laminin β2, which is required in the formation of the laminin-521 found in mature glomeruli; mutations in LAMB2 cause Pierson syndrome (defined by congenital nephrotic
syndrome, ocular abnormalities, and neurodevelopmental defects) (27). Without dialysis, patients with Pierson syndrome develop early-onset ESRD and die within a number of weeks after birth (28). Similarly, a homozygous missense mutation (R1281W) in ITGB4 (encodes β4-integrin) results in decreased expression of β4-integrin in podocytes and is associated with FSGS (29). Thus, FSGS is associated with genetic mutations that affect proper GBM formation.

Mutations in genes required for podocyte differentiation also cause FSGS. Wilms’ Tumor 1 (WT1) is a nuclear transcription factor and acts as a tumor suppressor. During kidney development, it is present in multiple developing kidney structures; however, in the mature kidney, it is only found in the podocyte (30). Dominant mutations in WT1 result in Denys–Drash syndrome (DDS), characterized by Wilms’ tumor (a nephroblastoma), nephropathy, and male pseudohermaphroditism. Heterogeneous transgenic mice that have the Wt1<sup>tmT396</sup> mutation develop DDS and their podocytes show decreased levels of nephrin and synaptopodin (a podocyte-specific protein) (31), suggesting that these podocytes are de-differentiating. Similarly, in an inducible model, decreased WT1 expression led to downregulation of podocalyxin (another podocyte-specific protein) (32). This can be explained by the fact that WT1 binds the podocalyxin gene promoter and promotes transcriptional activation (33). WT1 missense mutations have been found to cause FSGS in humans (34, 35). Together, these studies implicate podocytes in the pathogenesis of FSGS.

Nephrin, podocin, and TRPC6 are found at the slit diaphragm and are encoded by NPHS1, NPHS2, and TRPC6, respectively. NPHS1 mutations result in congenital nephrotic syndrome of the Finnish type, an autosomal recessive disease. Proteinuria can start in utero and it was originally believed that NPHS1 mutations were only in children who presented with nephrotic syndrome by 3 months of age. However, it was later found that NPHS1-associated FSGS can be either early or late-onset (36). At the slit diaphragm, nephrin directly binds podocin, which is also mutated in autosomal recessive nephrotic syndrome (37, 38). Researchers found that 5 disease-causing NPHS2 missense mutations interfered with proper nephrin localization to the membrane in human embryonic kidney (HEK) cells (39). Multiple cases of FSGS are caused by mutations in TRPC6, which encodes the Ca<sup>2+</sup> channel that interacts with podocin described earlier (40-42). Patch-clamp analysis show that gain-of-function TRPC6 mutations allow increased Ca<sup>2+</sup> current through the channel (9, 41). This likely leads to cytoskeletal changes, resulting in podocyte effacement (43).
Defects in the cytoskeleton-related genes also result in FSGS. For example, mutations in ACTN4 (encodes α-actinin-4) causes familial FSGS (44, 45). α-actinin-4 is an actin-bundling protein thought to cross-link actin filaments. Podocyte-specific mutation (K256E, analogous to K228E found in humans) of α-actinin-4 in mice caused proteinuria, podocyte effacement, and segmental sclerosis in 8 out of 18 mice (due to incomplete penetrance also seen in humans). Furthermore, the proteinuric mice had decreased mRNA and protein levels of nephrin (46). In vitro, exogenously expressed K256E α-actinin-4 shows mislocalization away from the cell periphery and results in decreased cell spreading, migration, and actin-based projections compared to wild-type (WT) α-actinin-4 (47). Similarly, mutations of CD2AP (encodes CD2-associated protein) are associated with both autosomal dominant and autosomal recessive sporadic adult-onset FSGS (19, 48, 49). CD2AP is an adaptor protein that directly binds nephrin and anchors it to the cytoskeleton; it also plays a role in actin cytoskeleton regulation (7, 26). Global knock out of CD2AP in mice leads to podocyte failure, proteinuria, and death caused by renal failure by 6 to 7 weeks (50). Furthermore, even heterozygous mice show histological changes in the kidney by 9 months of age. While these haploinsufficient mice did not naturally develop proteinuria within a year, they had more severe and persistent proteinuria in response to nephrotoxic antibodies than their WT counterparts (48).

Finally, defects in proteins that regulate the actin cytoskeleton (ex. Rho GDP-dissociation inhibitor α and RhoGTPase activating protein 24) also cause FSGS in humans. These proteins play a role in actin organization via their effect on RhoGTPases, discussed in section 1.3.

1.2.3 TGFβ1 in FSGS

Transforming growth factor β1 (TGFβ1) is a profibrotic cytokine that contributes to the fibrosis of the glomerulus (51). Elevated serum and urine TGFβ1 is a biomarker of chronic kidney disease (51) and polymorphisms in the TGFB1 gene (which encodes TGFβ1) are associated with chronic kidney disease risk (52). Moreover, there is mounting evidence, in both humans and animal studies, that TGFβ1 plays a role in FSGS progression. TGFβ1 is highly expressed in podocytes from human patients with idiopathic FSGS (53). Both mesangial cells, and possibly podocytes, produce the TGFβ1 that is found in the glomerulus (54).
Animal studies have helped reveal the mechanisms by which TGFβ1 promotes FSGS. In the adriamycin-induced nephropathy, podocyte depletion, glomerular sclerosis, nephrin reduction, and podocyte effacement were all associated with increased TGFβ1 glomerular expression (55). In transgenic mice, doxycyclin-inducible podocyte-specific expression of a CA TGFβ1 receptor 1 (TGFβR1) increased TGFβ1 signaling specifically in podocytes. This also led to progressive FSGS, foot process effacement, and albuminuria. Doxycyclin withdrawal after 7 or 10 days abolished the albuminuria and the aberrant glomerular histology; however, mice that had doxycyclin withdrawal after 14 days continued to have severe albuminuria and advanced glomerulosclerosis. These transgenic mice also had increased podocyte apoptosis, as shown by TUNEL staining (56). A similar transgenic mouse was created by another group and led to similar results. Mice expressing a constitutively active form of TGFβ1 in the liver have increased levels of plasma TGFβ1 and develop proteinuria by 5 weeks (57). By two weeks, these mice had decreased podocytes per glomeruli and increased podocyte apoptosis (58). In addition to promoting apoptosis, TGFβ1 in cultured podocytes is also responsible for decreased proliferation (59), cytoskeletal changes, and decreased cell adhesion (54). The decreased cell adhesion can be explained by the fact that TGFβ1 stimulation decreases expression of α3β1-integrin (60).

1D11 is a mouse monoclonal antibody that neutralizes all three forms of TGFβ. Injection of 1D11 decreases renal injury in multiple models of kidney disease, including Dahl salt-sensitive hypertensive rats (61), STZ-induced diabetic rats (62-64), and 5/6 nephrectomy rats (65). However, all of these models induce hypertension, and the studies that measured blood pressure (61-63, 65) found that 1D11 alleviated hypertension. To establish whether 1D11 can decrease fibrosis independent of its hypertension-alleviating mechanism, Liang et al. studied its effects on adriamycin-induced nephropathy and NEP25 podocyte loss nephropathy. They found that 1D11 prevents fibrosis in both models. In the NEP25 model, 1D11 did decrease podocyte loss but, surprisingly, did not decrease proteinuria or podocyte effacement. The authors concluded that there are distinct mechanisms underlying proteinuria and fibrosis and 1D11 will prevent podocyte loss and fibrosis, but not proteinuria (66).

Similarly, studies of a monoclonal antibody that neutralizes all three forms of TGFβ is being studied in humans. This antibody, named fresolimumab, is in the G4 subclass (IgG4) and has passed phase I clinical trials, reported in 2011 by Trachtman et al. They administered a single dose of fresolimumab (at doses ranging from 0.3mg/kg to 4mg/kg) to 16 patients with
treatment-resistant biopsy-proven idiopathic FSGS. The study was carried out at nine centers in the USA, Germany, Italy, and United Kingdom. The patients were followed for 112 days and side effects were mild to moderate. The results showed that proteinuria fluctuated but urine protein to creatinine ratio had a median decrease of 1.2 mg/mg (67). Currently, a multicenter double-blind, randomized, placebo-controlled phase II clinical trial sponsored by Genzyme is underway (NCT01665391). While the study is complete, the results are yet to be posted.

1.3 THE ROLE OF RHOGTPASES IN PODOCYTES

1.3.1 Introduction to RhoGTPases

The Rho family of guanosine triphosphatase (GTPases) are ubiquitously expressed small (20-30 kilo-Daltons) signaling proteins that act as molecular switches (68). There are around 20 GTPases encoded for within the human genome (69) and they alter between their active and inactive conformations, which are bound to GDP and GTP respectively. There are three prototypical RhoGTPases—Rac1, RhoA, and Cdc42. Their activity levels are routinely measured by pulldown methods. p21 activated kinase 1 (PAK1) is an effector protein of Rac1 and Cdc42 with a Cdc42/Rac Interactive Binding (CRIB) region (70). This CRIB region can be fused to glutathione S-transferase (GST) and then coupled to glutathione-tagged agarose beads and used to pull down active Rac1 and Cdc42. Similarly, active RhoA is isolated by fusing the Rho-binding domain of Rhotekin (a downstream effector protein of RhoA) to GST (71).

Their activity levels are tightly regulated by three groups of proteins: guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs). RhoGTPases are activated by GEFs, which remove the GDP and exchange it with GTP. In contrast, GAPs promote the RhoGTPases to hydrolyze the GTP into GDP, rendering them inactive. Finally, GDIs sequester the RhoGTPases in the cytosol and stop them from being proteosomally degraded but also stop them from releasing GDP, thereby keeping them inactive (72). See figure 1.2 for more information.

RhoGTPases are involved in a plethora of cellular processes, such as proliferation, protein secretion, cell polarity, gene transcription, enzyme activation, and microtubule organization (69, 73). Importantly, RhoGTPases also help control the actin cytoskeleton, thereby regulating cell shape, motility, adhesion, contraction, phagocytosis, and axon guidance (73).
three prototypical RhoGTPases—Rac1, RhoA, and Cdc42—were traditionally thought to regulate the formation of lamellipodia (membrane ruffles often found at the leading edge of a moving cell), stress fibers (bundles of actin), and filopodia (short cellular protrusions), respectively (72, 74). See figure 1.3 for more details. We now know that their roles are more complex and there is considerable crosstalk among these proteins. As the actin cytoskeleton plays such an important role in the maintenance of podocytes, a lot of research has focused on the role of RhoGTPases in podocytes.

1.3.2 Roles of Rac1

Rac1 activity is tightly regulated during podocyte differentiation both in vitro and in vivo. One week of differentiation increased levels of Rac1 activity in cultured murine podocytes; however, this decreased to undifferentiated levels by 2 weeks (75). This decrease may be explained by the increased levels of Arhgap24 (a Rac1 GAP) mRNA and protein found after 7-14 days of cultured murine podocyte differentiation (76). Similarly, in vivo, the podocyte has more active Rac1 during early glomerular development than during the later stages. Rac1 activity is higher at E13.5 (during the comma-shaped development stage) than at E16.5 (at the capillary loop developmental stage) in podocytes (77). In laser-captured microdissected mouse glomeruli, Arhgap24 mRNA levels are low at E12.5 (during the renal vesicle stage of development) but increase at E15.5 (S-shaped body stage) and remain high at E15.5 (mature renal glomerulus) (76). Finally, the levels of GDIα are also increased at E16.5 compared to E13.5 (77), possibly contributing to the decreased levels of Rac1 activity in podocytes.

This fine regulation of Rac1 activity during glomerular development has led researchers to believe that Rac1 plays a role in podocyte differentiation (72). Indeed, when murine podocytes (stably transfected with nephrin) differentiate, they become larger and have more lamellipodia than their undifferentiated counterparts. However, transient transfection of these undifferentiated murine podocytes with constitutively active (CA)-Rac1 will phenocopy differentiated cells (72, 75). Furthermore, stable transfection of both mouse and rat podocytes with nephrin increases Rac1 activity (75, 78). Rac1 activity is also increased in response to antibody-mediated crosslinking of nephrin (used to simulate homotypic nephrin interaction in the slit diaphragm, thereby promoting intracellular nephrin phosphorylation) (75).
Rac1 plays an important role in modulating the actin cytoskeleton. Active Rac1 is generally found at the leading edge of a moving cell, where it promotes actin polymerization and focal complex formation, thereby increasing motility (73). Focal complexes are protein complexes that connect the extracellular matrix to the actin cytoskeleton. In contrast to focal adhesions, which are induced by RhoA, focal complexes are associated with lamellipodia and filopodia (74). In cultured murine podocytes, transfection with constitutively active (CA)-Rac1 promoted lamellipodium formation and increased cell size (79).

Since the actin cytoskeleton is vital for proper podocyte functioning, researchers began investigating the role of Rac1 in podocyte injury. Surprisingly, transgenic mice with podocyte-specific Rac1 knockout have no glomerular phenotype by 3 months (80). However, when mice were treated with uninephrectomy and deoxycorticosterone acetate—high salt—induced hypertension (a model of chronic progressive glomerular damage), podocyte-specific Rac1 deletion increased glomerulosclerosis and albuminuria (81). Similarly, in streptozotocin-induced diabetic mice, podocyte-specific deletion of Rac1 increased albuminuria and cleaved-caspase 3 positive cells in the glomerulus while it decreased podocin protein levels and WT1 positive cells (suggesting loss of podocytes) (82). Together, these studies suggest that Rac1 activity is important for the maintenance of podocytes.

However, there is a plethora of opposing evidence, which suggests that increased Rac1 activity contributes to podocyte injury rather than health. Podocyte-specific Rac1 knockout mice treated with protamine sulfate (used as a model of podocyte injury) did not develop foot process effacement, whereas WT mice did (81). Similarly, researchers found that 5/6 nephrectomy (an experimental model of chronic kidney disease) resulted in glomerulosclerosis and loss of WT1 and podocin, possibly reflecting the loss of podocytes. However, these symptoms were alleviated by Rac1 inhibition (83).

Further corroborating the evidence for Rac1’s role in podocyte injury are in vivo studies of CA-Rac1-expressing podocytes. Transgenic mice with inducible, podocyte-specific expression of CA-Rac1 developed proteinuria 2 days after induction. Interestingly, this proteinuria peaked after 4 days and then began decreasing after 7 days; 3 months of induction failed to develop glomerular sclerosis or kidney failure (79). However, this is likely due to the uneven expression of the transgene by all podocytes. Indeed, when the researchers made transgenic mice using a different reverse tetracycline transactivator (rtTA) that led to increased
transgene expression, these mice had a faster onset of more severe proteinuria. However, the transgene was still not expressed by all podocytes and the proteinuria still began decreasing after 4 days (79). Despite the lack of sclerosis, super-resolution microscopy found that podocytes expressing CA-Rac1 had effaced foot processes. The number of podocytes expressing CA-Rac1 by 4 weeks was very low. The authors attributed this to the CA-Rac1 expression, which will cause the podocytes to detach from the GBM and be lost in the urine (79). Similarly, another group made transgenic zebrafish with CA-Rac1 expressed specifically in podocytes found in the pronephric glomerulus; these zebrafish did not present any abnormalities up to 5 days post-fertilization. However, it was also found that the transgene was not ubiquitously expressed by podocytes; thus, the researchers used a stronger driver line that tripled expression of CA-Rac1. These fish developed proteinuria, dilated Bowman’s capsule, foot process effacement, and decreased slit diaphragm density. Ultimately, these fish had progressive edema and died between 12 and 31 days post-fertilization (84). Together, these studies suggest that Rac1 activity is detrimental to podocytes in a dosage-dependent manner.

Hyperactive Rac1 has also been linked to TGFβ signaling. 5/6 nephrectomy causes hypertension and albuminuria; the hypertension is believed to be the main driver of renal failure in this model. However, while Rac1 inhibition had no effect on blood pressure, it did decrease albuminuria, glomerulosclerosis, and podocyte loss. Interestingly, Rac1 inhibition also decreased mRNA expression of TGFβ in the renal cortex. In cultured mouse podocytes, static mechanical stretch (which simulates glomerular hypertension) activates Rac1 and increases mRNA and protein expression of TGFβ. However, Rac1 inhibition abolished the stretch-induced increase in TGFβ expression and attenuated podocytes apoptosis induced by recombinant TGFβ (83). This suggests that Rac1 activity induces TGFβ, which then mediates apoptosis by activating Rac1.

The notion of Rac1 hyperactivity’s detrimental effects is further supported by human FSGS patients with genetic mutations resulting in elevated Rac1 activation. Loss-of-function mutations in ARHGDIA (encodes GD1α) (85, 86) and ARHGAP24 (77, 85, 86) increase Rac1 activity and are associated with FSGS. This will be further discussed in section 1.4. Thus, in conclusion, the overwhelming majority of evidence suggests that Rac1 hyperactivity causes aberrations in the podocyte cytoskeleton, leading to injury and possible loss of podocytes.

As Rac1 activation appears to be detrimental to podocytes, some researchers have attempted to find what proteins activate Rac1. Slit diaphragm proteins, such as nephrin
(described earlier) and TRPC5, elevate Rac1 activity. In response to angiotension II, TRPC5 conducts a Ca\(^{2+}\) current, which activates Rac1 and leads to the disassembly of stress fiber and increased motility. TRPC5 and Rac1 co-immunoprecipitated in HEK cells and were co-localized at the podocyte periphery, suggesting that they are in a complex (87). This may explain why TRPC5 knockout and inhibition protects the kidney lipopolysaccharide (LPS)-induced damage. In mice, LPS injection induces foot process effacement, proteinuria, and elevated Rac1 activity levels. However, both knockout and inhibition of TRPC5 prevented these changes from occurring (88).

Similarly, \(\alpha_\beta_3\)-integrin also seems to activate Rac1 in podocytes. An inhibitor of \(\alpha_\beta_3\)-integrin abolished LPS-induced foot process effacement, proteinuria, and elevated Rac1 activity levels (89). Another group found that treatment of \textit{in vitro} podocytes with angiopoietin-like 3 leads to increased formations of lamellipodia and filopodia along with increased Rac1 activity. Pre-incubation with an inhibitor of \(\alpha_\beta_3\)-integrin abrogated the changes in cell morphology and change in Rac1 activity (90).

Thus, a number of Rac1-modulating proteins have already been established; however, to date, it is unclear what, if any, GEFs activate Rac1 in FSGS. This will be further discussed in section 1.4.

1.3.3 Roles of RhoA

In general, Rac1 is thought to be antagonized by RhoA. For example, Rac1 activates p190RhoGAP (a RhoA-GAP) (91) and inactivates NET1 (a RhoA-GEF) (92), while RhoA activates ARHGAP24 (93). RhoA and Rac1 have opposing effects on the actin cytoskeleton; this antagonistic relationship creates polarity in a moving cell such that directional movement can occur (94). RhoA activation is classically associated with formation of stress fibers, inhibited motility, and maturation of focal adhesions (73, 95). In cultured podocytes, kymograph analysis showed that transfection with CA-RhoA resulted in less membrane motility (79). However, the coordination between active RhoGTPases in a moving cell is complex. Despite its association with decreased motility, active RhoA is found at the leading edge of a cell, 2\(\mu\)m ahead of active Rac1 and Cdc42 (96).

Similar to Rac1, there is evidence that RhoA activity may contribute to podocyte injury. 7 days after rats are treated with puromycin, the levels of proteinuria peak and RhoA activity is
increased (75). Rho-associated protein kinase (ROCK) is found downstream of RhoA and is responsible for many of RhoA’s effects, including the formation of stress fibers and focal adhesions (97). Similar to Rac1 inhibition, ROCK inhibition also stops glomerulosclerosis, albuminuria, podocyte loss, and increased TGFβ expression from developing following 5/6 nephrectomy (83). Furthermore, transgenic mice with doxycycline-inducible podocyte-specific expression of CA-RhoA developed albuminuria by 4 weeks. Again, the level of transgene expression determined the level of podocyte injury and the level of CA-RhoA expression was directly correlated with the albuminuria severity. In mice with low CA-RhoA expression (low responders), this albuminuria was reversible after 1-2 weeks of doxycycline withdrawal. High responders continued to have high levels of CA-RhoA expression 2 weeks after doxycycline withdrawal and, while the albuminuria did decrease, it remained high. Accordingly, low expression of CA-RhoA led to segmental foot process effacement but lacked histological changes; whereas high expressing mice effaced foot processes along with histologically-evident FSGS (98). Another group (Wang et al.) confirmed that inducible expression of CA-RhoA in podocytes increases proteinuria and foot process effacement (99). Furthermore, they found that murine podocytes transfected with CA-RhoA had increased actin polymerization and reduced apoptosis (99).

However, other evidence suggests that RhoA activity is essential for maintaining podocyte function. In the same paper as the CA-RhoA, Wang et al. also found that inducible expression of dominant negative (DN)-RhoA in the podocyte results in albuminuria and foot process effacement. However, they hypothesized that underlying mechanism causing these renal abnormalities were different in the two mice. In vitro mouse podocytes transfected with DN-RhoA showed disassembly of stress fibers but no change in apoptosis. Thus, the researchers concluded that RhoA activity is required under basal conditions, but it becomes detrimental if it is too high (99). Another group also found that RhoA knockdown in cultured podocytes decreased stress fibers; however, the RhoA knockdown increased apoptosis (100). In contrast, a podocyte-specific knockout of RhoA in mice fails to produce a glomerular defect by 3 months (80).

As researchers are trying to find the consequences of RhoA inactivation/activation, they are also investigating which proteins regulate it. In contrast to TRPC5 (which activates Rac1), angiotension II-induced TRPC6 current activates RhoA and inactivates Rac1, leading to the
formation of stress fibers, decreased motility. TRPC6 co-immunoprecipitates with RhoA and immunofluorescence studies show that RhoA-TRPC6 complexes are found at the plasma membrane, but do not co-localize with the Rac1-TRPC5 complexes (87). TRPC6’s ability to activate RhoA may explain why gain-of-function mutations in TRPC6 cause FSGS (40-42). Furthermore, GEF-H1 activates RhoA under health and proteinuric conditions (101). This will be further discussed in section 1.4.

1.3.3 Roles of Cdc42

Of all the RhoGTPases, Cdc42 appears to be the least studied in podocytes. Cdc42 is responsible for formation of filopodia, can increase Rac1 activity (74, 94), and is associated with a migratory phenotype (102). Unlike RhoA and Rac1, podocyte-specific loss of Cdc42 resulted in albuminuria at birth along with foot process effacement and glomerular dilation (seen in congenital nephropathy). By postnatal day 5, the albuminuria increased and glomerulosclerosis was evident. Both at birth and 5 days postnatally, nephrin and podocin expression was decreased in Cdc42 knockout mice, suggesting that Cdc42 plays a role in maintenance of the slit diaphragm. The knockout mice developed renal failure and died within 2 weeks (80). These results were confirmed by a separate group, Blattner et al., who also developed a podocyte-specific Cdc42 knockout mouse. These transgenic mice also had progressive proteinuria, glomerulosclerosis, foot process effacement, and early death (81). This can be explained by Cdc42’s anti-apoptotic effects. Mice treated with high glucose, lipopolysaccharide, or adriamycin, all have increased podocyte apoptosis (as seen by TUNEL and WT1 co-staining) and decreased Cdc42 expression. In vivo, specific Cdc42 inhibition increased podocyte apoptosis. Cdc42 inhibits death through increasing mRNA and protein levels of the anti-apoptotic yes-associated protein (YAP) (103). Thus, Cdc42 is important for podocyte survival.

However, Cdc42 hyperactivation also results in kidney damage. It was found that synaptopodin, a podocyte-specific actin-associated protein, binds IRSp53 (an adaptor protein) and inhibits the formation of Cdc42:IRSp53:Mena complex. This Cdc42:IRSp53:Mena complex promotes filopodial formation. In cultured podocytes with synaptopodin knockdown, Mena inhibition stops aberrant filipodia from forming. In vivo, Mena inhibition stopped lipopolysaccharide-treated mice from developing proteinuria. This suggests that, in response to synaptopodin knockdown, Cdc42 is activated, leading to podocyte injury (104).
In conclusion, the three typical RhoGTPases, Rac1, RhoA, and Cdc42, appear to play an important role in regulating the actin cytoskeleton and podocyte morphology, motility, and injury. Thus, researchers are investigating which GDIs, GAPs, and GEFs regulate these proteins.

1.4 REGULATION OF RHO-GTPASES

1.4.1 RhoGDIs

There are 3 isoforms of GDIs: 1 (also known as α), 2 and 3. GDIα is expressed at most and is ubiquitous. GDIs bind inactive RhoGTPases and keep them sequestered in the cytosol, thereby regulating their localization (72). This binding also stops RhoGTPases from proteosomal degradation (105). Thus, GDIs create a pool of RhoGTPases that, upon stimulation, can be rapidly released, activated, and translocated to the membrane (72). Global knockout of GDIα results in severe proteinuria and, within 12 months, death due to renal failure (106). Three loss-of-function mutations (ΔD185, R120X, and G173V) in GDIα have been found in FSGS patients (85, 86). In mouse podocytes with endogenous GDIα knocked down and replaced with exogenous mutant GDIα, Rac1 activity was increased (77). Knockdown of GDIα also makes cultured mouse podocytes more susceptible for lipopolysaccharide (LPS)-induced injury (107). The proteinuria and podocyte damage caused by global knockout of GDIα is reversed upon Rac1 inhibition (108). Knockdown of the zebrafish ortholog of ARHGDIA (the gene encoding GDIα) resulted in periorbital and body edema (reflective of nephrosis); this was partially alleviated by Rac1 inhibition (86).

1.4.2 RhoGAPs

The human genome sequencing project estimates that there are around 67 GAPs (109). One method of identifying GAPs is using a pulldown method initially developed in 2006 by Garica-Mata et al (110). This pulldown method consists of a constitutively active RhoGTPase mutant fused to GST and coupled to glutathione-tagged agarose beads. The CA-RhoGTPases were designed based on their analogous Ras mutant and they lack the ability to hydrolyze GTP or activate their downstream effectors. Thus, the CA-RhoGTPases bind GAPs and downstream effectors of the RhoGTPase at a high affinity (110).
Thus far, two GAPs known to play a role in podocytes are ARHGAP24 and p190RhoGAP, which inactivate Rac1 and RhoA, respectively. As mentioned earlier, ARHGAP24 encodes a Rac1 GAP upregulated in differentiating podocytes in vitro and in vivo. In cultured differentiated mouse podocytes, knockdown of ARHGAP24 resulted in increased Rac1 activation, increased membrane ruffling, and faster wound closure. Exon sequencing of ARHGAP24 revealed nonsynonymous variant (Q158R) in a patient with biopsy-confirmed FSGS but not in controls. This variant was associated with FSGS in the patient’s family; his sister had the same mutation and progressed to ESRD by age 12 while his mother had late-onset FSGS and passed away at age 29 from renal failure. While transfection of mouse podocytes with WT ARHGAP24 decreased Rac1 activity, transfection with the corresponding mouse variant, Q156R, actually increased the level of active Rac1. This is likely explained by the Q156R variant heterodimerizing with the WT (76).

Similarly, p190RhoGAP is a RhoA-specific GAP that has been found to inactivate RhoA in cultured podocytes. Podocytes are one of the primary cells infected by human immunodeficiency virus, type 1 (HIV-1). This leads to HIV-1-associated nephropathy (HIVAN), which is associated with severe proteinuria and FSGS. Nef is an HIV-1 accessory protein that induces podocyte de-differentiation in vitro (111); transgenic mice with podocyte-specific Nef expression develop glomerulosclerosis (112). In vitro, Nef induces phosphorylation of p190RhoGAP, which then inactivates RhoA, resulting in decreased stress fibers (113). However, these results await confirmation in vivo.

1.4.3 RhoGEFs

In mammals, 85 GEFs have been identified (69). They are encoded by different gene families: the Dbl family and the DOCK family. The Dbl family is much larger, encoding 69 GEFs in the human, while the DOCK family is made up of 11 members. Proteins in the Dbl family have a Dbl homology (DH) catalytic domain followed by a pleckstrin homology (PH) domain towards the C-terminus. Generally, a protein cannot catalyze GDP removal without both of these domains. GEFs usually contain other domains that help regulate their localization, catalytic activity, protein interactions, and lipid interactions. Unlike Dbl proteins, DOCK proteins can only activate Rac1 and/or Cdc42 but not RhoA. They have a catalytic Dock Homology Region 2 (DHR2) domain and a phospholipid-binding domain (DHR1). The DHR1
helps target the DOCK proteins to the lipid membrane (114). Some GEFs can only activate one RhoGTPase whereas others can activate multiple. Since there are many more GEFs than there are RhoGTPases, one RhoGTPase can be activated by multiple GEFs. Therefore, there may be some redundancy or compensation (114).

Similar to the pulldown method that isolates GAPs, GEFs can also be pulled down using a GST-tagged nucleotide-free RhoGTPase mutant coupled to a glutathione-tagged agarose bead. A dominant negative form of Ras (RasG15A) is unable to bind to either GDP or GTP and therefore remains nucleotide-free. From this, Arthur et al. hypothesized that the analogous mutant in Rac1 would also decrease nucleotide binding. While they claimed the RacG15A mutant decreased incorporation of GTP, the data was not shown (115). Next, Garcia-Mata et al. (110) suggested that this RacG15A would bind GEFs with high affinity because the nucleotide-free form of Ras binds Cdc25 with high affinity (116). While they were able to pulldown GEFs using GST-RacG15A, they never compared it to pulldown with wild-type Rac1 (110). Nevertheless, the use of GST-RacG15A has become an increasingly popular method of pulling down Rac1 GEFs (117-119) and is commercially available. Using these methods, among others, researchers are now beginning to investigate which GEFs are important in podocytes.

In podocytes, GEF-H1 has been found to activate RhoA, while Vav2 and Vav1 may activate Rac1. Passive Heymann nephritis (PHN) is an experimental rat model of membranous nephropathy. GEF-H1 (a Rac1 and RhoA GEF) was found to be activated in glomeruli of PHN rats. In vitro, complement C5b-9 induces GEF-H1 and RhoA activation. Knockdown of GEF-H1 decreased basal and C5b-9-induced RhoA activation, but increased C5b-9-induced cytolysis. Thus, in PNH, C5b-9 may induce GEF-H1 to activate RhoA, which decreases C5b-9-induced cytolysis (101).

Thus far, the only Rac1-GEFs found to play a role in podocytes are Vav2 and Vav1. In vitro, Nef induces the phosphorylation of Vav2, which goes on to activate Rac1 and form lamellipodia (113). A recent study suggests that Vav1 activates Rac1 in podocytes. The authors used an interleukin-13 (IL-13) overexpression rat model of minimal change-like nephropathy and found that these rats had upregulated expression of Vav1. Vav1 is expressed in vivo in rat podocytes (as seen by its colocalization with synaptopodin), but the phosphorylated form is only present in IL-13 overexpressing rats. In vitro, stimulation of human podocytes with IL-13 induces Rac1 activation and cytoskeletal changes; however, this was abrogated when Vav1 was
knocked down (120). Thus, Vav1 may play a role in activating Rac1 in podocytes under minimal change-like nephropathy.

*In vitro* studies showed that Dock1 (a Rac1 GEF) is recruited to the membrane in response to nephrin cross-linking. Dock1, and the closely related Dock5 are expressed in podocytes *in vivo*. However, podocyte-specific knockout of Dock1 or Dock5 or both did not result in podocyte abnormalities or kidney damage (121). This was to be expected since podocyte-specific Rac1 knockout did not produce any renal phenotypes either (80). Surprisingly though, podocyte-specific deletion of Dock1 did not protect mice from LPS-induced foot process effacement and proteinuria (121). This suggests that Dock1 and Dock5 do not play an important role in activating Rac1 in podocytes under either basal or disease conditions.

Thus, as of now, it remains unknown whether there are any GEFs responsible for the Rac1 hyperactivity seen in pathological FSGS conditions. To address this, my lab used both a candidate and unbiased approach to find GEFs that hyperactivate Rac1 in FSGS. The candidate approach gave rise to our investigation of Trio, while the unbiased approach led us to study β-PIX. A literature review of these proteins will be discussed in the following sections.

### 1.5 Trio

#### 1.5.1 Structure and Isoforms

Trio is a large (350 kiloDalton) protein in the Dbl family of GEFs. It was discovered in 1996 by Debant *et al.* in a yeast two-hybrid assay where it interacted with a transmembrane tyrosine phosphatase, LAR (122, 123). Northern blot analysis found that Trio RNA was expressed in human heart, brain, placenta, lung, liver, skeletal muscle, kidneys, and pancreas (122). Trio is named after the fact that it has three enzymatic domains: GEFD1, GEFD2, and a serine/threonine kinase domain. It also has an Ig domain, spectrin-like repeats, two src homology 3 (SHR3) domains, and a Sec14 (also known as the CRAL-Trio) domain (122-124).

The GEFD1 region activates Rac1 and RhoG, whereas the GEFD2 regions activates RhoA (122, 123, 125). The GEFD1 domain consists of the DH1 domain (which catalyzes Rac1/RhoG activation) and the PH1 domain (which contributes to the activation of the RhoGTPases and localization). The PH1 domain binds to Filamin A, an actin cross-linker, thereby localizing Trio to the actin cytoskeleton (123). The GEFD2 domain consists of a DH2...
(which activates RhoA) and PH2 domain. The PH2 domain hinders the DH2 domain from activating RhoA (123). It was a surprise to find that a GEF can activate both Rac1 and RhoA, since these two RhoGTPases usually antagonize each other. However, researchers hypothesize that this helps the temporal timing of RhoGTPase activity (124). For example, at the leading edge of a cell, RhoA is activated, followed by Rac1. In this case, it may be helpful to have Trio at the leading edge as it can activate each protein sequentially (124). However, this idea has yet to be tested.

The other domains of Trio are still under investigation. Pulldown assays in HeLa cells with exogenously expressed peptides found that the SH3 domain of the GEFD1 region interacts with the C-terminus of Rac1 but not to that area of RhoG. Surprisingly though, this interaction between the SH3 domain and Rac1 is not required for Trio to activate Rac1 or RhoG (126). The Ig-like domain also mediates protein-protein interactions; it allows RhoA binding to Trio, which relocalizes exogenously-expressed Trio to punctate-like structures (127). Another domain that may regulate Trio localization is the Sec14 domain. In Kalirin and Dbs (two other GEFs), the Sec14 region is thought to mediate interactions with phospholipids, thereby bringing the GEF to the membrane and regulating local activity (124). However, the role of this domain in Trio remains unclear. Finally, the spectrin-like repeats are believed to auto-inhibit Trio’s Rac1 GEF activity. In vitro, the amino half of the spectrin-like repeats co-immunoprecipitate with the GEFD1 region of Trio. Furthermore, while the GEFD1 region can bind Rac1, this interaction decreases when cells express the amino half of the spectrin-like repeats (128). Other proteins may bind these spectrin-like repeats, thereby hindering them from auto-inhibiting Trio and increasing Trio activity (128). The structure of Trio is drawn in figure 1.4.

Due to alternative splicing, there are 6 main isoforms of Trio: full length (FL), D, A, C/Solo, B, and E. There is a 7th oncogenic isoform of Trio, called Tgat, that will be discussed further in section 1.5.4. FL and isoforms A-D differ by their N-terminal truncation; whereas isoform E differs from FL by missing a large portion of the C-terminus (123). Isoforms D and A contain both GEFD regions, while isoform C/Solo and B only have the GEFD1 region and isoform E only contains the GEFD2 domain (123). Two separate studies found that, in contrast to the ubiquitous FL, the Trio isoforms are expressed specifically in the brain and at a higher expression level than the FL (129, 130).
1.5.2 Function

Due to the specificity of expression in the brain, a lot of Trio research has been conducted in neurons. Indeed, Trio has a wide variety of functions of neurons, including neuronal development (131, 132), axon guidance (133, 134), axon outgrowth (133, 135). Systemic Trio knockout in mice results in death between E15.5 and birth or soon after. The biggest abnormalities seen in these mice are in the skeletal muscles and brain. Histochemical studies showed abnormal cellular organization in the olfactory bulb and hippocampus (131). Four patients have been found with loss-of-function mutations of Trio; these individuals suffer from mild to borderline intellectual disability (132).

In addition to studies done in neurons, in vitro studies of exogenously expressed Trio have shown that Trio plays a role in regulating the actin cytoskeleton and lamellipodia formation. In HeLa cells, exogenous expression of the GEFD2 region increased the levels of active RhoA and the formation of stress fibers. In contrast, exogenous expression of FL Trio increased lamellipodia formation and the actin cytoskeleton became concentrated at the periphery (126). The same was seen in HeLa (126), NIH 3T3 mouse embryonic fibroblasts (136), and hamster CCL39 fibroblasts (125) with exogenously expressed GEFD1. Thus, overexpression of FL Trio mimicked the phenotypes seen by GEFD1 but not by GEFD2 expression (124). Accordingly, kymographic analysis showed that HeLa cells with Trio knockdown generated unstable membrane protrusions, but this was rescued in a Rac1-dependent manner with expression of the GEFD1 region (126). Thus, Trio likely activates Rac1, inducing lamellipodia formation.

Trio also plays a role in cell spreading and migration. Trio knockdown in glioblastoma cells decreased migration and invasion (137). Similarly, HeLa cells spreading on fibronectin have increased Rac1 activity; knockdown of Trio inhibits both the spreading and the Rac1 activity, as well as migration. From this, the researchers hypothesized that, during cell spreading, β1-integrin activates Trio; however, this was never confirmed (124, 126). Expression of the GEFD1 region in the Trio knockdown HeLa cells rescued the cell spreading and migration (126). Similarly, GEFD1 overexpression in NIH 3T3 fibroblasts increased the rate of cell spreading and motility (136). In contrast, RhoA activity was high in stationary cells (in suspension before spreading) but decreased upon spreading; Trio knockdown did not affect this (126). Therefore,
the Trio’s pro-motility effect is likely due to its activation of Rac1. Trio also promotes leukocytes transendothelial migration (138).

Trio is also required for junction formation and cell-cell adhesion; it is activated upon interaction with adhesive receptors such as cadherins and β2-integrin (123). Knockdown of Trio in human umbilical vein endothelial cells (HUVEC) results in decreased neutrophil binding to these HUVEC; this effect was rescued by expression of the GEFD1 region coupled to the spectrin-like repeats. This suggests that Trio’s GEFD1 region promotes neutrophil adhesion to HUVEC (138). In epidermal keratinocytes, Trio interacts with RhoA, filamin A (an actin-binding protein), calcium-sensing receptor (CaR), and epithelial (E)-cadherin at cell-cell junctions in response to increased extracellular Ca^{2+}. Trio knockdown or expression of dominant negative Trio peptides (that compete with Trio’s ability to interact with filamin) inhibit the Ca^{2+}-induced formation of these adherens junctions; this indicates that Trio is required for adherens junction formation. It is worth noting that increased extracellular Ca^{2+} also induced Rac1 activity and this effect was also abrogated by expression of dominant negative Trio peptides (139). However, the relevance of Rac1 activation in the formation of the adherens junction was not investigated further. In endothelial cells, Trio interacts with vascular endothelial (VE)-cadherin and activates Rac1 at adherens junctions between cells. During nascent cell-cell junction assembly, the interaction between Trio and VE-cadherin increased, and endothelial cells with Trio knockdown are unable to reform cell-cell junctions. This demonstrates the importance of Trio in the formation of cell-cell junctions (140).

In addition to cell-cell adhesion, Trio also plays a role in cell-matrix adhesion. Trio was originally discovered as an interactor of the LAR transmembrane protein tyrosine phosphatase, a transmembrane protein found at the focal adhesions (122). In contrast to untransfected COS-7 cells (fibroblast-like mouse cells derived from the kidney), which have focal contacts at the basal membrane, COS-7 cells overexpressing FL Trio show focal contacts at the cell periphery (136). Furthermore, in COS-7 and HeLa overexpression systems, Trio co-immunoprecipitates and colocalizes with focal adhesion kinase (FAK—a tyrosine kinase found at focal adhesions) at the membrane. Co-expression of these proteins increases the level of Trio found in the detergent-insoluble fraction, suggesting that it is relocalized to the cell membrane. FAK phosphorylates the tyrosine kinase domain of Trio at Tyr^{2737} and Trio increases FAK autophosphorylation (141).
The result of these phosphorylations remained uninvestigated; however, they suggest that Trio may play a role in the focal adhesion complex.

1.5.3 Regulation

In addition to FAK, Abl and Fyn also phosphorylate Trio. In *Drosophila*, Abelson tyrosine kinase (Abl) interacts with and may phosphorylate Trio; together, these proteins regulate axon outgrowth. However, the effect of Abl’s phosphorylation of Trio remains unestablished (142, 143). Trio can also be phosphorylated by Fyn, a Src family tyrosine kinase. In cultured murine neuroblastoma N1E-115 cells, co-transfection of Trio and Fyn increases Trio phosphorylation at Tyr2622. While this phosphorylation is not required for Trio to activate Rac1 under basal conditions, it is required to increase Rac1 activity in response to netrin-1 (a guidance cue important in neuronal development). This phosphorylation was required for netrin-1 to promote rat embryonic cortical axon outgrowth (135).

Netrin-1-induced Rac1 activation and axon outgrowth is also dependent on Hsc70, a constitutively and ubiquitously expressed ATP-dependent chaperone. Co-immunoprecipitations studies on E17.5 rat cortical tissue extracts revealed that Trio interacts with Hsc70, but not the highly homologous Hsp70; this interaction increased after 5 minutes of netrin-1 stimulation. Co-transfection of Trio and Hsc70 in HEK293 cells increased Rac1 activity compared to cells transfected with just Trio. However, co-transfection of Trio and an ATPase-deficient Hsc70 failed to activate Rac1, showing that Hsc70 must have intact chaperone activity in order to regulate Trio’s activation of Rac1 (134).

Finally, Trio activity has also been found to be regulated by DISC1, Kidins220/ARMS, and Tara. Studies in *C. elegans* found that disrupted-in-schizophrenia 1 (DISC1) binds to the spectrin-like repeats and alleviates Trio of their auto-inhibition, thereby increasing Rac1 activity. This was confirmed in mammalian COS cells (128). Similarly, in PC12 cells (pheochromocytoma cells derived from the rat adrenal medulla) and E18 hippocampal primary neurons, Trio’s spectrin-like repeats are bound by Kidins220/ARMS (an integral membrane protein). This interaction localizes Trio to the membrane and promotes it’s activation of Rac1 (144). Finally, Trio-associated repeat on actin (Tara), an F-actin binding protein, also regulates Trio activity. Tara was discovered as an interactor of Trio at the GEFD1 region and found to regulate the actin cytoskeleton (145). In Madin-Darby Canine Kidney (MDCK) epithelial cells,
knockdown of Tara increases Rac1 activity; however, this hyperactivity is abolished by ITX3, a specific inhibitor of Trio. This suggests that Tara inhibits Trio-Rac1 signaling (146). A summary of proteins that regulate Trio can be found in Table 1.2.

1.5.4 In disease

Trio has been found to play a role in multiple types of cancer, including oral, lung, breast, cervical, urinary bladder, soft tissue sarcoma, and glioblastoma (a highly malignant and invasive brain tumor) (123, 147). These diseases are associated with Trio somatic mutations, overexpression, or abnormal alternative splicing (123). Reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemical studies showed that Trio expression is increased in glioblastoma compared to low-grade gliomas or non-neoplastic brain tissue. Furthermore, analysis of brain tumor expression database revealed that high Trio expression is correlated with decreased survival (137). In cultured glioblastoma cells, siRNA knockdown of Trio decreased proliferation (137).

Trio’s oncogenic role is partially due to its ability to activate Rac1. In glioblastoma, TNF-like weak inducer of apoptosis (TWEAK) activates Rac1, leading to increased invasion, as seen by both a radial cell migration assay and an organotypic brain slice invasion assay. TWEAK stimulation increases the binding of Trio to GST-RacG15A and knockdown of Trio abolished the TWEAK-induced Rac1 activation. Trio knockdown in glioma cells decreased migration and lamellipodia formation (117). In 3T3 fibroblasts, expression of GEFD1 or CA-Rac1 allowed cells to grow and form colonies on soft agar; this anchorage-independent growth was not dependent on the SH3 domain of the GEFD1 region (136). Furthermore, NIH 3T3 cells overexpressing GEFD1 grew to a higher saturation density, suggesting that Trio plays a role in cell contact inhibition (136). Thus, Trio’s ability to activate Rac1 and induce invasion and anchorage-independent growth while decreasing cell contact inhibition, likely contributes to its oncogenic properties.

Finally, an alternatively spliced isoform of Trio (Tgat) was discovered in adult T-cell leukemia (ATL). Tgat only contains the GEFD2 domain fused to a unique C-terminal 15 amino acid sequence made up of exon 58. Tgat expression in NIH 3T3 cells led to increased RhoA activation, stress fiber formation, and focal adhesion complexes. These transfected fibroblasts also showed decreased cell contact inhibition, increased anchorage-independent growth,
increased invasion, and tumorigenicity when injected into nude mice. While Tgat missing the unique C-terminal sequence can activate RhoA, it is unable to promote anchorage-independent growth or develop tumors in mice. However, treatment of Tgat-expressing NIH 3T3 cells with a ROCK inhibitor also decreased the transformation ability, as measured by a focus formation assay. Thus, both the RhoA activation and the C-terminal 15 amino acid sequence are important for transformation (148).

1.6 β-PIX

1.6.1 Structure and Isoforms

β-PIX (also known as Arhgef7 or Cool-1) is a Dbl-family protein that was first discovered in 1998 as an interactor of p21-activated protein kinase (PAK) 1 (149). PAK1-3 are directly activated by Rac1 and Cdc42, and act as downstream effectors. β-PIX is an activator of Rac1 and Cdc42, but not RhoA. Similar to Trio, β-PIX has a number of different domains. Embryonic β-PIX contains an N-terminal calponin homology (CH) domain that is no longer found in adult β-PIX. Instead, the N-terminus of adult β-PIX contains an SH3 domain that binds to the proline-rich sequences in PAK1-3 (150). In COS-7, HEK293, and MDCKII cells, it was found that this SH3 domain also binds the proline stretch in the C-terminus of Rac1 in a nucleotide-independent manner (151). As all Dbl-family proteins, β-PIX has a DH and PH domain responsible for RhoGTPase activation. It also has a G protein-coupled receptor kinase-interacting target (GIT)-binding domain (GBD), which will bind to the Spa2-homology domain (SHD) of GIT1 and GIT2. The GIT proteins are GAPs for ADP-ribosylation factor (Arf) small GTP-binding proteins. There is a coiled-coil domain which allows β-PIX trimerization and localization to the cell periphery (150, 152). Finally, the C-terminus contains a PDZ target motif that binds to PDZ-domain proteins (150).

The only other member in the PIX family of proteins is α-PIX. α-PIX is expressed mainly in hematopoetic and muscle cells whereas β-PIX is ubiquitous. Structurally, the two PIX proteins are similar with three main differences: (a) α-PIX contains the CH domain that is missing in adult β-PIX, (b) α-PIX is missing the C-terminal PDZ binding domain found in β-PIX, and (c) α-PIX is missing a T1 insert present in β-PIX. This T1 insert in β-PIX inhibits
constitutive GEF activity, making β-PIX’s nucleotide exchange activity very low under basal condition in vitro (153). In contrast, α-PIX-mediated activation of Rac1 and Cdc42 is more robust. Src can phosphorylate Y442 on β-PIX which decreases the T1 insert’s autoinhibition but only allows for activation of Cdc42, not Rac1 (153).

There is only one isoform of α-PIX, whereas β-PIX has a number of isoforms which have not been fully characterized yet. Alternative transcription start sites are responsible for the difference between adult and embryonic β-PIX. Alternative splicing gives rise to β1-PIX and β2-PIX variants found in rats and cultured human cells. β2-PIX is missing the PDZ target motif and the coiled-coil domain; instead it has a serine-rich C-terminus. β2-PIX is the dominant isoform in the brain whereas β1-PIX was dominant in HeLa, COS-7, and NIH 3T3 cells (152). Using a mouse brain cDNA library, β1-PIX was found to have different variants, β-PIX-a, β-PIX-b, β-PIX-bL, β-PIX-c, and β-PIX-d. Both β-PIX-b and β-PIX-c have an inserted sequence of 59 amino acids between the GBD and the coiled-coil domain, but β-PIX-c is also missing 75 amino acids between the PH domain and the GBD domain (154). Furthermore, an internal ribosome entry site (IRES)-mediated mechanism gives rise to alternative translation, forming the β-PIX-bL isoform. β-PIX-bL is similar to the β-PIX-b isoform but contains an extra 105 amino acids in the CH domain and has a serine-rich sequence at the N-terminus. This isoform is highly expressed during embryonic development in the rat brain (155). Finally, β-PIX-d is missing a leucine zipper domain, has an inserted sequence of 11 amino acids at the C-terminus, and a distinct 3′-UTR. This isoform is also expressed mainly in the central nervous system of rat embryos (156).

1.6.2 Function

Similar to Trio, β-PIX has a number of different functions, including cell migration (157-160), formation of focal adhesions (158, 159), actin cytoskeleton organization (159), and synaptic vesicle transport (161). Overexpression systems suggest that β-PIX plays a role in activating Rac1 and regulating the actin cytoskeleton. β-PIX overexpression in HeLa cells causes membrane ruffling but, surprisingly, no filopodia formation. HeLa cells co-expressing dominant negative Rac1 along with β-PIX or expressing a β-PIX mutant missing the DH domain do not
have any morphological differences from control cells. Together, this suggests that the morphological changes are due to β-PIX’s ability to activate Rac1 (149).

This notion is further supported by in vivo studies. Global knockout of Arhgef7, the gene encoding β-Pix, in mice results in embryonic lethality at E8.5. At E6.5 and E8.5, the knockout embryos are much smaller and less elongated than WT. During embryonic development, anterior visceral endoderm (AVE) cells collectively migrate away from the distal end of the embryo and help create an anterior-posterior axis; Rac1 activity is required for this migration. In WT mice, AVE fragments isolated from E6.5 mice showed β-Pix expression. In β-Pix knockout mice, AVE directional migration was lost and the AVE cells’ protrusions are not parallel, as is seen in WT mice. Thus, the authors concluded that β-Pix controls Rac1 activation at the leading edge of AVE cells to allow for directional movement (160).

One underlying mechanism that allows β-Pix to control motility is its ability to regulate focal adhesions. In 2010, Kuo et al. performed a proteomic analysis on focal adhesions isolated from HFF1 human foreskin fibroblasts (162). One protein that came down in this focal adhesome was β-Pix. β-Pix was found in nascent adhesions at lamellipodia, where it activates Rac1, promotes nascent adhesion turnover, inhibits focal adhesion maturation, and increases migration (162). Furthermore, β-Pix plays a role in signaling at the focal adhesion. In NIH 3T3 cells, it is responsible for the phosphorylation of FAK and motility in response to lysophosphatidic acid (LPA) (163). In return, FAK can also phosphorylate β-Pix, which will increase its binding to Rac1, recruit active Rac1 to focal adhesions, and promote cell spreading (164).

β-Pix forms a complex with α-Pix, GIT1, and GIT2. These GIT-PIX complexes are found at the focal adhesions, in the cytoplasm, and at the leading edge (165). GIT binds paxillin (an adaptor molecule found at the focal adhesion), and in this way, may be responsible for bringing β-Pix to the focal adhesion (150). In turn, the PIX proteins regulate the interaction between GIT1 and paxillin and lead to focal complex disassembly (166). Furthermore, the GIT-PIX complex is a signaling hub. β-Pix recruits PAK to focal complexes and adhesions (165). PAK is activated downstream of Rac1 and Cdc42, and it can also phosphorylate GIT and PIX. This feedback is thought to amplify the Rac1-Cdc42-PAK signaling; however, this has yet to be proven (150). GIT proteins inactivate Arf and increased Rac1 and/or Cdc42 activation is associated with decreased Arf6 activation (150). However, as of now, the ultimate consequence of these complex signaling mechanisms remains unclear.
FIGURES FOR CHAPTER 1

Figure 1.1 The glomerulus, the podocyte, and the slit diaphragm.

The glomerulus is the basic filtration unit of the kidney. Blood enters it through the afferent arteriole and exits via the efferent arteriole. When in the glomerulus, the blood is filtered through the glomerular basement barrier, which is made up of endothelial cells, the glomerular basement membrane (GBM), and podocytes. The filtrate escapes into the urinary space (which is surrounded by the Bowman’s capsule) and then goes to the proximal tubules.

The foot processes from neighboring podocytes interdigitate and wrap themselves around the capillary. Between foot processes are a modified adherens junction, called the slit diaphragm. There are a number of different proteins (ex. nephrin, NEPH1, podocin, Fat1, α-actinin-4, Nck, CD2AP, and TRPC6) in the slit diaphragm, which, together, form a signaling hub. In addition to podocytes being attached to one another by slit diaphragms, they are also attached to the GBM by a variety of proteins (ex. α3β1-integrin, α-actinin-4, ILK, and CD151).

Abbreviations: ACTN4, alpha-actinin-4; CD2AP, CD2-associated protein; GBM, glomerular basement membrane; ILK, integrin-linked kinase; P, podocin; TRPC6, transient receptor potential cation channel 6.

Figure 1.1

a

Afferent arteriole
Bowman's capsule
Glomerular capillary
Urinary space
Proximal tubule

b

Podocyte
GBM
Foot processes
Endothelium

b

F-actin
Slit diaphragm
Podocyte foot process

a3β1
a3β1
ACTN4
NCK
NEPH1
Fat1
CD2AP
Nephrin
NCK
NEPH1
TRPC6
NCK

a3β1
GBM
a3β1

Fenestrated endothelium
RhoGTPases act as molecular switches by alternating between their active and inactive states, which are GTP- and GDP-bound, respectively. When active, they contribute to a number of cellular processes, including organization of the actin cytoskeleton. Due to their interaction with the cytoskeleton, they can regulate cell shape, motility, adhesion, contraction, phagocytosis, and axon guidance (69, 73). RhoGTPases are activated by guanine nucleotide exchange factors (GEFs). GEFs will bind the inactive GDP-bound form of a RhoGTPase, remove the GDP, and replace it with a GTP. As the name implies, GTPase activating proteins (GAPs) activate the RhoGTPase’s intrinsic GTPase activity, thereby rendering them inactive. Finally, guanine nucleotide inhibitors (GDIs) will bind inactive RhoGTPases and sequester them in the cytosol. This will prevent their proteosomal degradation, thereby creating a pool of inactive RhoGTPases that can be quickly mobilized when they are needed (72).
**Figure 1.3 Rac1, RhoA, and Cdc42 in a moving cell.**

In a migrating cell, active Cdc42 is found in filopodia (area 1). Active Rac1 is found at the leading edge (area 2), where it promotes the formation of new adhesions (area 3). In contrast, RhoA is found throughout the body of the cell, where it promotes maturation of focal adhesions (area 4) and formation of stress fiber (area 6). RhoA will also inhibit Rac1 activation on the lateral sides (area 5), such that the cell moves in only one direction.

The following image is reprinted and slightly modified from (94) Trends in Cell Biology, 21, Christophe Guilluy, Rafael Garcia-Mata, Keith Burridge, Rho protein crosstalk: another social network? 718-726, 2011 with permission from Elsevier.
**Figure 1.4 Trio domains and isoforms.**

Trio is a large protein with three enzymatic domains: GEFD1, GEFD2, and a serine/threonine kinase domain. It also has an Ig domain, spectrin-like repeats, two src homology 3 (SHR3) domains, and a Sec14 (also known as the CRAL-Trio) domain (122-124).

The GEFD1 region activates Rac1 and RhoG, whereas the GEFD2 regions activates RhoA (122, 123, 125). Each GEF domain has a DH domain (which catalyzes RhoGTPase activation) and a PH domain. While the PH1 domain helps activate Rac1/RhoG, the PH2 domain inhibits the DH2 domain from activating RhoA (123). The SH3 domain of GEFD1 interacts with the C-terminus of Rac1, but is not required for Trio’s activation of Rac1 (126). The Ig-like domain also mediates protein-protein interactions and localization (127). The Sec14 domain is also predicted to modulate Trio localization (124). The spectrin-like repeats are believed to auto-inhibit Trio’s Rac1 GEF activity (128); this inhibition may be relieved by other proteins binding the spectrin-like repeats (128).

This image was adapted from (123) with permission
### TABLES FOR CHAPTER 1

#### Table 1.1 Regulation of Rac1 in Podocytes

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RATIONALE AND OBJECTIVES

Rationale

Proteinuria (protein leakage from the blood into the urine) is a hallmark of chronic kidney disease, which leads to end stage renal disease and loss of kidney function. Proteinuria occurs when there is a defect in the glomerulus, the basic filtration unit of the kidney. Part of this glomerular filtration barrier is made up by specialized epithelial cells called podocytes. Morphological changes in podocytes allow proteins to leak through the glomerular capillaries into the urine, resulting in proteinuria. Rho family small GTPases (RhoGTPases) are regulators of the actin cytoskeleton in podocytes; therefore, they play a critical role in determining podocyte morphology. There is a plethora of evidence that hyperactivation of a prototypical RhoGTPase, Rac1, in the podocyte results in actin cytoskeletal changes, detachment, and apoptosis, ultimately leading to proteinuria and glomerular sclerosis. In general, GTPase-activating proteins cause RhoGTPases to go into their inactive forms whereas guanine nucleotide exchange factors (GEFs) push RhoGTPases into their active state. There are approximately 80 known GEFs; however, it remains unclear if any are responsible for Rac1 activation in podocytes under basal conditions or during injury.

Objectives

1. Identify potential Rac1-GEFs in podocytes using candidate and unbiased approaches.
2. Verify expression of these Rac1-GEFs in cultured podocytes and in mouse glomeruli.
3. Study the functional effects of knocking out a Rac1-GEF in podocytes.
4. Find an extracellular stimulus that promotes Rac1 activity and study if this activation is mediated by the identified Rac1-GEF.
Chapter 2:

Guanine Nucleotide Exchange Factors Activate Rac1 in Podocytes

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A modified version will be submitted to International Journal of Molecular Sciences
PREFACE TO CHAPTER 2

We and others have previously shown that activation of Rac1 is detrimental to podocyte health both in vitro and in vivo. Therefore, Rac1 hyperactivity is associated with foot process effacement, proteinuria, and FSGS. While a number of proteins (such as TRPC5 and nephrin) have been implicated in activating Rac1, the direct activator(s) of Rac1 in the context of FSGS has not been discovered. We predicted that a guanine nucleotide exchange factor (GEF) contributes to the activation of Rac1, leading to podocyte abnormalities and FSGS. Here, we use a candidate and an unbiased approach to find Rac1-GEFs. The candidate approach led us to investigate Trio, while the unbiased approach has encouraged us to study β-PIX.
CONTRIBUTION OF AUTHORS

RNA-sequencing of human podocytes and immunofluorescence of paraffin-embedded mouse glomeruli was performed by Lamine Aoudjit. Cindy Baldwin performed (a) the first immunoblot of Trio on cultured human podocytes and HEK cells transfected with full-length Trio, (b) created the Trio KO and control Cas9 cell lines, (c) the MTT assay on control Cas9 and Trio KOs, and (d) created human podocytes expressing myc-BirA and myc-BirA-RacG15A. Mirela Maier (the candidate) did all other experiments in chapter two, and wrote the manuscript with the advice and help of Dr. Tomoko Takano. A modified version of this manuscript will be submitted to the International Journal of Molecular Sciences.
Guanine Nucleotide Exchange Factors Activate Rac1 in Podocytes

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A modified version of this will be submitted to the International Journal of Molecular Sciences

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Abstract

Focal segmental glomerulosclerosis (FSGS) is a histopathological lesion characterized by podocyte injury, during which the podocyte’s actin-rich projections, called foot processes, retract (i.e. efface) back into the cell body. RhoGTPases, such as Rac1, contribute to the organization of the actin cytoskeleton and both in vivo animal studies and human genetics studies suggest that Rac1 hyperactivity leads to foot process effacement, proteinuria (the leakage of protein from blood into the urine), and FSGS. While Rho guanine nucleotide exchange factors (GEFs) are typically known for activating RhoGTPases, it remains unclear if any GEFs are responsible for Rac1 activity in FSGS. This study investigates this by using two methods: a candidate and an unbiased approach. Candidate GEFs were discovered using RNA-sequencing on two lines of cultured human podocytes, followed by analysis of which GEFs have upregulated mRNA in humans with FSGS compared to healthy control; this led us to investigate Trio. Trio knockout (KO) in cultured human podocytes decreased Rac1 activity, cell size, attachment, and motility. Furthermore, while the profibrotic cytokine that contributes to glomerulosclerosis progression, transforming growth factor β1 (TGFβ1), increases Rac1 activity in control cells, it decreases Rac1 activity in Trio KOs. This may due to simultaneous activation of a Rac1-GTPase activation protein (GAP) called Arhgap31. Additionally, the unbiased approach, which consisted of the proximity-based BioID assay, led us to study β-PIX. TGFβ1 increases the amount of β-PIX isolated by BioID and by pulldown with Rac1. Thus, in conclusion, we have found that two GEFs, Trio and β-PIX, contribute to the Rac1 activation in podocytes.
Introduction

Nephrotic syndrome is characterized by proteinuria (leakage of protein from the blood into the urine), hypoalbuminemia (decreased levels of albumin in the blood), and edema; it often results in end-stage renal disease (ERSD). Focal segmental glomerulosclerosis (FSGS) is the most common cause of nephrotic syndrome and accounts for 4% of ESRD cases in adults (19). Minimal change disease (MCD) is another cause of nephrotic syndrome; whether MCD is a separate disease from FSGS or simply a less progressed form of FSGS is currently a point of debate (23). Both MCD and FSGS are characterized by injury to the podocyte, a specialized epithelial cell found in the glomerulus. The glomerulus is the kidney’s basic filtration unit and carries out the first step of urine formation. The glomerulus is a capillary tuft in which blood is pushed through the glomerular filtration barrier, which is made up of fenestrated endothelial cells, an acellular basement membrane, and podocytes. This filtration barrier must allow the excretion of metabolic wastes while retaining blood cells and proteins. Podocyte injury compromises this filtration barrier, allowing proteins and sometimes red blood cells (hematuria) to be lost in the urine.

The podocyte has a unique cell morphology—coming out of the cell body are microtubule-rich primary processes, which then divide into smaller actin-rich secondary processes, also called foot processes. Foot processes from neighboring podocytes interdigitate and are connected by a modified adherens junction, known as the slit diaphragm. This forms a sieve through which the blood can be filtered. Proteinuria is caused by retraction of these actin-rich foot processes retract, a process known as foot process effacement. Indeed, genetic mutations in genes encoding proteins that mediate actin cytoskeleton organization cause FSGS. For example, mutations in ACTN4 (encodes α-actinin-4, an actin-bundling protein) (44-47) or CD2AP (encodes CD2-associated protein, an adaptor protein that anchors the slit diaphragm to the actin cytoskeleton) (48-50) have been found to cause FSGS.

The actin cytoskeleton is also regulated by the Rho family of GTPases (RhoGTPases). RhoGTPases act as a molecular switch, alternating between the active, GTP-bound form, and the inactive, GDP-bound form. They are regulated by three groups of proteins—guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs). GEFs remove the GDP off an inactive RhoGTPase and replace it with a GTP, thereby activating the protein. GAPs promote RhoGTPases to hydrolyze GTP into
GDP, thereby rendering them inactive. Finally, GDIs bind the inactive RhoGTPases and keep them sequestered in the cytosol, protected from proteosomal degradation. The three prototypical RhoGTPases—Rac1, RhoA, and Cdc42—were traditionally associated with the formation of lamellipodia (membrane ruffles often found at the leading edge of a migrating cell), stress fibers (bundles of actin filament), and filopodia (short spike-like membrane protrusions), respectively (74).

Rac1 hyperactivity has been implicated in podocyte injury and FSGS. Transgenic mice with doxycyclin-inducible podocyte-specific expression of constitutively active (CA)-Rac1 develop proteinuria 2 days after induction. Higher transgene expression results in more severe proteinuria and foot process effacement, suggesting that podocyte damage depends on the level of Rac1 hyperactivity (79). Similarly, transgenic zebrafish with low expression of CA-Rac1 specifically in podocytes found in the pronephric glomerulus did not present any abnormalities up to 5 days post-fertilization. However, increased expression of CA-Rac1 resulted in proteinuria, foot process effacement, decreased slit diaphragm density, and ultimately, progressive edema and death (84). This also supports the notion that Rac1 activity is detrimental to podocytes in a dosage-dependent manner.

Furthermore, mutations in RhoGTPase-regulating proteins lead to increased Rac1 activity and have been found in FSGS patients. Three loss-of-function mutations (ΔD185, R120X, and G173V) in ARHGDIA (which encodes GDIα) have been found in FSGS patients (85, 86). In cultured murine podocytes, replacing endogenous GDIα with mutant GDIα increased Rac1 activity (77). The proteinuria and podocyte damage caused by global knockout of GDIα in mice is reversed upon Rac1 inhibition (108). Similarly, a mutation in ARHGAP24 (which encodes a Rac1-GAP) was found to be associated with familial FSGS. In murine podocytes, transfection with this mutant ARHGAP24 elevated Rac1 activity (76).

As Rac1 hyperactivity appears to be injurious to podocytes, researchers have investigated which proteins may activate it. Thus far, the only Rac1-GEFs found to play a role in podocytes are Vav2 and Vav1. Vav2 was found to activate Rac1 in response to stimulation with Nef, a human immunodeficiency virus, type 1 (HIV-1) accessory protein associated with HIV-1-associated nephropathy (HIVAN), severe proteinuria, and FSGS. In vitro, Nef induces the phosphorylation of Vav2, which goes on to activate Rac1 and form lamellipodia (113). However, these results await in vivo validation. A recent study used an interleukin-13 (IL-13)
overexpression rat model of minimal change-like nephropathy and found that these rats had upregulated expression of Vav1. *In vitro*, treating human podocytes with IL-13 increases Rac1 activity and induces cytoskeletal reorganization; however, these changes were abolished by Vav1 knockdown (120). Thus, Vav1 may play a role in activating Rac1 in podocytes under minimal change-like nephropathy. Another study investigated the role of two closely related GEFs, Dock1 and Dock5, in podocytes. While they were expressed in podocytes *in vivo*, their knockout in the podocyte neither resulted in kidney abnormalities nor protected mice from lipopolysaccharide (LPS)-induced foot process effacement and proteinuria (121). This suggests that Dock1 and Dock5 do not play an important role in activating Rac1 in podocytes.

Thus, as of now, the activator of Rac1 underlying FSGS pathogenesis remains unknown. To address this, my lab used both a candidate and unbiased approach to find GEFs that hyperactivate Rac1 in FSGS. The candidate approach gave rise to our investigation of Trio, while the unbiased approach led us to study β-PIX.
Materials and Methods

Reagents

All tissue culture media, blasticidin, and puromycin were from Wisent (Saint-Bruno, Quebec). Interferon-γ and 5-2-1 laminin were from Cedarlane (Burlington, ON). TGFβ1 was from Peprotech (Dollard des Ormeaux, QC). BioRad assay dye and electrophoresis reagents were from BioRad Laboratories (Mississauga, ON). Normal goat serum and lipofectamine 2000, were from Invitrogen (Burlington, ON). Aqua Mount and enhanced chemiluminescent (ECL) were from Thermo Scientific. Glutathione-Sepharose 4B beads were from GE Healthcare (Baie-D'Urfe, QC). Complete Mini Protease Inhibitor Cocktail was from Roche Diagnostics (Montreal, QC). The Nucleofector Kit for Basic Mammalian Epithelial Cells was bought from Lonza (Mississauga, ON). Male Sprague-Dawley rats were from Charles River Canada (Saint-Constant, QC).

pGEX2t-GST-CA-Rac1, pGEX2t-GST-RacWT and pGEX2t-GST-alone were from generated by subcloning (167) CA-Rac1 and Rac1WT into pGEX2t from GE Healthcare Life Science (Chicago, IL, USA). The pLenti CMV rtTA3 Blast (w756-1) was a gift from Eric Campeau (Addgene plasmid # 26429). pMD2.G (Addgene plasmid # 12259) and psPAX2 (Addgene plasmid # 12260) were gifts from Didier Trono. The lentiviral Cas9 and sgRNA Trio were from Genecopoeia (Thorold, ON). The pTRE2-GFP-alone and gTRE2-GFP-Trio-GEFD1 were kind gifts from Dr. Nathalie Lamarche-Vane (McGill University) (134).

Rhodamine-conjugated phallolidin was from PromoKine (Heidelberg, Germany). Rabbit anti-p-p38, anti-rabbit IgG Alexa fluor 488 and anti-mouse IgG Alexa flour 555 were from Cell Signalling (Beverly, MA). Mouse anti-α-tubulin, mouse anti-synaptopodin, rabbit anti-p38, HRP-conjugated streptavidin, HRP-conjugated anti-rabbit and anti-mouse antibodies were from Abcam (Cambridge, MA). Rabbit anti-Trio antibodies were from Cedarlane (Burlington, ON) or Santa Cruz (Dallas, TX). Mouse anti-Rac1 was from Millipore (Etobicoke, Ontario). Mouse anti-Hsc70, mouse anti-WT-1, mouse anti-myc were from Santa Cruz as well. Rabbit anti-calnexin was from Stressgen (San Diego, CA). IRDye LiCor secondary antibodies were from LiCor (Burlington, ON).

Cell culture and transfection
Two lines of conditionally immortalized human podocytes (HP and LY) were kind gifts from Dr. Moin Saleem (University of Bristol) (168). Briefly, HP were cultured in RPMI 1640, 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S) and kept at 33°C. LY cells were used for the RNA-sequencing; for all other experiments, only HP were used. HP were used for experiments between passages 18 and 45. Conditionally immortalized mouse podocyte (SMP) were received from Dr. Stuart Shankland at University of Washington, and cultured in DMEM, 10% FBS, and 1% P/S and interferon γ (10 U/mL) at 37°C, as previously established (169). HEK293 were cultured in DMEM, 10% FBS, and 1% P/S at 37°C.

Transfection, Transduction with Lentiviral Particles, and Nucleofection

HEK293 were transiently transfected with lentiviral Cas9, psPAX2 (packaging plasmid), pMDG.2 (envelope plasmid) at a ratio of 4:3:1 with Lipofectamine 2000 transfection reagent. The cells were incubated in this for 18 hours, after which the medium was replaced. 24 and 48 hours after replacing medium, the virus-containing medium was collected and filtered through a 0.45 μm filter. HP cells were incubated with 1 mL of this lentiviral particle-containing medium for 24 hours. Cells were then selected for using blasticidin (8 μg/mL) to create the control Cas9 HP. The process was then repeated on the control Cas9 cells using sgRNA targeting Trio to create Trio KOs, which were selected for by blasticidin and puromycin (0.2 μg/mL). Trio KOs underwent a limiting dilution to create monoclonal cell lines.

To create the HP myc-BirA and HP myc-BirA-RacG15A cells, HP cells were first stably transfected with pLenti CMV rtTA3 (using the transduction method described above) and selected for with blasticidin. Next, they were transfected with pLenti CMV-myc-BirA or pLenti CMV-myc-BirA-RacG15A and selected for with blasticidin and puromycin. Finally, cells underwent a limiting dilution and the cells with the highest expression of the transgene were selected for use.

For the nucleofection experiment, 50x10⁴ HP rtTA3 cells were mixed with 100 μL of Nucleofector Solution mix (as per instructions from Amaxa Basic Nucleofector Kit Primary Mammalian Epithelial Cells). This mixture was then added to 1 μg of DNA (pTRE2-GFP-Trio-GEFD1 or pTRE2-GFP alone), nucleofected (using the Amaxa Biosystems Nucleofector I, program S-05), and added to 5-2-1 laminin-covered coverslips. After 8 hours, doxycyclin (1 μg/mL) was added overnight to induce expression.
RNA-sequencing

Two human podocyte lines (LY and HP) were differentiated by a temperature switch to 37°C for 1 week and RNA was prepared using RNeasy mini kit (QIAGEN). RNA-seq was performed at the McGill University and Genome Quebec Innovation Centre for Eukaryotic protein coding transcripts.

Nephromine / Nephroseq Analysis

Our original search for candidate GEF was conducted on Nephromine using the “Ju Podocyte” dataset, which consisted of 436 glomerular and tubulointerstitial samples. This led us to investigate Trio. In the meantime, Nephromine has been updated into Nephroseq (www.nephroseq.org) and samples from the “Ju podocyte” dataset have been incorporated into the “Ju CKD Glom”, “Ju CKD TubInt” and “Ju CKD TubInt 2” datasets. Our search for β-PIX and Hsc70 expression was then conducted on the updated Nephroseq, using the Ju CKD Glom dataset, which is made up of microdissected glomeruli from 199 CKD patients or healthy living donors from the European renal cDNA Biobank (ERCB).

RT-PCR

Total RNA was isolated from HP using the Qiagen RNeasy MiniKit (Qiagen, Montreal, QC). Reverse transcription of 1 μg of RNA was done using the QuantiTect Reverse Transcription Kit (Qiagen, Montreal, QC). PCR was performed using DNA Polymerase (ZmTech Scientifique Montreal, QC) with 34 cycles of the following: 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 3 minutes. Bands were extracted using the UltraClean 15 DNA Purification Kit (MoBio, Carlsbad, CA).

Immunofluorescence of Cultured Podocytes

HP cells are plated on sterilized coverslips coated in 5-2-1 laminin (0.25 μg/μm²). Cells attached to coverslip overnight and were treated the next day. After treatment, cells were fixed in 4% paraformaldehyde for 15 minutes at room temperature, permeabilized with 0.5% Triton X-100 (in PBS), and blocked in 3% BSA (in PBS) for 20 minutes. Rabbit anti-Trio (1.5 hours, 1:50), mouse anti-vinculin (1.5 hours, 1:40), and rhodamine-conjugated rabbit phalloidin (20
minutes, 1:50) were used to stain Trio, vinculin, and actin, respectively. Samples stained for Trio and vinculin were then incubated with anti-rabbit IgG2 Fab2 Alexa Flour 488 and anti-mouse IgG Fab2 Alexa Flour 555, respectively, at 1:1000 dilution in 3% BSA for 1 hour. Cells were visualized using the AxioObserver-100 microscope (Zeiss).

ImageJ was used to quantify cell size, vinculin number, and peripheral vinculin localization. The actin cytoskeleton was used to determine cell size. Particles between 1-8 μm² were quantified for vinculin number and then normalized to cell area. For peripheral vinculin localization, lines were randomly drawn across the cell and the fluorescence intensity in the outer 10 μm was normalized to the fluorescence intensity in the next 20 μm.

**Immunofluorescence of Paraffin-Embedded Kidney Sections**

Kidneys were removed from healthy mice, fixed in 100% methanol for 30 minutes at −20°C, paraffin embedded, and sliced into 4 μm-thick sections. Slides were covered in xylene for 10 minutes two times. This was followed by a 10 minutes wash with ethanol, a 5 minute wash with ethanol, a 5 minute wash with 95% ethanol (in PBS). They were then washed for 3 minutes in decreasing concentrations of ethanol (70%, 50%, and 30%) and finally in pure PBS for 5 minutes 3 times. Citrate antigen retrieval consisted of incubating slides in boiling 10mM citrate buffer (pH 6.0) for 9 minutes. Slides were treated in 1% Triton X-100 (in PBS) for 30 minutes, and then blocked in 10% normal goat serum (NGS), 0.3% Triton X-100, and PBS for 1 hour at room temperature. Rabbit anti-Trio, mouse anti-WT1, and mouse anti-podocalyxin antibodies were diluted 1:50 in 5% NGS, 0.3% Triton X-100 overnight at 4°C. Slides were washed 3 times (5 minutes) in PBS, and then incubated in secondary antibody diluted (1:1000) in 0.3% Triton X-100 (in PBS) at room temperature for 40-45 minutes. The secondary antibodies used were anti-rabbit IgG2 Fab2 Alexa Flour 488 and anti-mouse IgG Fab2 Alexa Flour 555. This was followed by three 10 minute washes before mounting the coverslip with AquaMount.

**SDS-PAGE and Immunoblotting**

HP or SMP were washed once with PBS and then 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 NaCl, 10 mM sodium pyrophosphate, 25 mM NaF, 2 mM sodium orthovanadate, 1% Triton X-100, and protease cocktail inhibitor cocktail]. Lysates were briefly (4 seconds) sonicated at a low power (10) and
then spun down (13,000 rpm, 30 seconds, 4°C). The concentration of supernatant was determined by Bradford assay. 20-30 μg were used for observing total cell lysate, and various amounts were used for different pulldowns (see next sections). SDS-PAGE separated proteins, which were then transferred to nitrocellulose membranes.

Two systems of immunoblotting visualization were used: BioRad and LiCor. The membranes were handled differently depending on the visualization method. For the BioRad system, membranes were blocked with 5% skim milk (in Tris-buffered saline-Tween) at room temperature and then incubated in primary antibody overnight at 4°C. The following day, membranes were washed twice (30 minutes each wash), incubated with secondary antibodies conjugated with horseradish peroxidase (dilution 1:1,000 – 1:2,000) for 1 hour at room temperature. Two 30-minutes washes proceeded, and then proteins were visualized using ECL. The only exception to this was the use of streptavidin antibody, which is already conjugated to HRP. For this, the membrane was blocked in 5% bovine serum albumin (BSA), incubated in streptavidin:HRP for 40 minutes at room temperature, washed once (5 minutes), and then visualized using ECL.

When using the LiCor system, membranes were blocked for 1 hour at room temperature, and then incubated in primary antibodies overnight at 4°C. The membranes were then washed four times (5 minutes each wash), incubated with fluorescent secondary antibodies (dilution 1:20,000 – 1:10,000) for 1 hour at room temperature, and then washed 4 times for 5 minutes again. After the addition of the secondary antibodies, the membranes were kept away from light. Proteins were visualized using the LiCor machine.

Densitometric analysis was performed using the BioRad or LiCor imaging systems. The following antibody dilutions were used: rabbit anti-Trio 1:100 – 1:1,000; mouse anti-Rac1: 1:1,000; mouse anti-p-p38 1:1,000; mouse anti-p38 1:1,000; rabbit anti-Arghap31 1:1,000; rabbit anti-β-PIX 1:1,000; mouse anti-tubulin 1:5,000; rabbit anti-calnexin 1:1,000, streptavidin:HRP 1:20,000.

**CRIB Pulldown Assay for Active Rac1**

Active Rac1 was pulled down as previously described (167). Briefly, the Cdc42-Rac1 Interactive Binding domain fused to GST beads (GST-CRIB) were then coupled to glutathione-agarose beads. Treated HP cells were lysed on ice (as described above) and 100-150 μg was
incubated with 15 μg GST-CRIB beads for 1 hour at 4°C. Pulldown samples were washed three times and proteins were separated on SDS-PAGE gel (12.5%). Densitometric analysis was performed using the BioRad imaging system and values were normalized to tubulin loading and then to unstimulated cells.

**GEF and GAP Pulldown Assays**

GEFs were pulled down using either wild type Rac1 fused to GST or RacG15A fused to GST. GAPs were pulled down by constitutively-active Rac1 fused to GST. The GST-RacWT GST-RacG15A, and GST-CA-Rac1 were all coupled to glutathione-agarose beads. Treated cells were lysed on ice, sonicated, and equal amounts of total cell lysate (550-1,000 μg) was incubated with beads for 2 hours at 4°C. Pulldown samples were three times and separated by SDS-PAGE (5%). Densitometric analysis was performed using the LiCor imaging system. Normalization occurred to either total cell lysate tubulin, Arhgap31, or β-PIX and then to untreated cells.

**MTT Assay**

2x10^4 cells were plated in 5-2-1 laminin (0.25 μg/μm^2) -covered wells in a 96-well plate and incubated at 33°C for 24 hours to allow cells to attach. At 24 or 48 hours, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to the RPMI to a final concentration 1mg/mL. Cells were incubated at 33°C for 4 hours, and then the cells were incubated with acidified isopropanol at room temperature for 15 minutes to dissolve the absorbed MTT. The dissolved MTT was measured using a wavelength of 550 nm by a microplate reader (ELX808, Bio-Tek Instruments) using KC Junior software (Bio-Tek). Experiments were done in triplicates, and the proliferation rate was determined by normalizing the number of viable cells at 48 hours to that at 24 hours.

**Wound Healing Assay**

Cells were plated on 35-mm plates and left to attach overnight. The following day, the wound was created using a 10 μL pipette tip. Scraped off cells were washed off using PBS and then RPMI medium was replaced. Cells were visualized at 5x magnification using the AxioObserver-100 microscope (Zeiss) immediately after the wound was created and again after 5 hours. Wound size was measured using ImageJ; wound closure at 5 hours was normalized to
the original wound size and then wound closure rate of Trio KOs was normalized to that of control Cas9.

**Attachment Assay**

10^4 cells were added to a 5-2-1 laminin (0.25 μg/μm²)-covered well in a 96-well plate and allowed to attach for 1 hour 15 minutes at 33°C. Unadhered cells were washed away with PBS, and the adherent cells were fixed with 4% paraformaldehyde for 15 minutes. Fixed cells were washed and then incubated with 0.1% crystal violet dissolved in 200mM 3-(N-morpholino)propanesulfonic acid (MOPS) for 15 minutes at room temperature. Cells were washed 3 times with PBS and then incubated for 15 minutes with 10% acetic acid. The dissolved crystal violet was measured using a wavelength of 550 nm by a microplate reader (ELX808, Bio-Tek Instruments) using KC Junior software (Bio-Tek). All experiments were done in triplicates and repeated 3-4 times.

**BioID Experiment**

The BioID experiment was performed as described before (170). Briefly, cells were incubated overnight in doxycyclin (myc-BirA: 5 μg/mL; myc-BirA-RacG15A: 1 μg/mL) and biotin (50 μM). After treatment with TGFβ1 (10ng/mL), cells were collected by scraping in ice-cold PBS, pelleted at 800 rpm for 5 minutes at 4°C, and flash frozen on dry ice. The pellets were incubated for 1 hour at 4°C with RIPA buffer [50mM Tris (pH 7.4), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1mM EDTA] supplemented with PMSF (1mM) and protease inhibitor cocktail. These lysed cells were then sonicated and centrifuged (25-30 minutes, 14,000 rpm, 4°C). The supernatant was incubated with Dynabeads MyOne Streptavidin C1 (Life Technologies, Burlington, ON) for 3 hours at 4°C. Finally, the beads were rinsed 5 times in RIPA followed by 4 washes in low detergent buffer [25mM Tris (pH7.4), 100mM NaCl, 0.025% SDS]. Samples were then sent to mass spectroscopy for analysis. Results are normalized to the expression level of the myc-BirA or myc-BirA-RacG15A.

**Statistics**

All data is presented as mean ± SEM. A two-tailed t-test is used to determine statistical differences between untreated and cells treated at a certain time point.
Results

Identification of Trio as a Candidate Rac1-GEF in Podocytes

85 GEFs have been identified in mammals (69). The first step of finding a candidate GEF was investigating which GEFs are present in podocytes. To do this, we performed RNA-sequencing (RNA-seq) on immortalized cultured human podocytes. RNA-seq is a high-throughput technology that directly sequences the mRNA transcripts, thereby giving a more precise estimate of absolute transcript levels than microarrays. RNA-seq was performed on two different types of cultured human podocytes—LY and HP. The LY samples were done in duplicates, while the HP samples were done singly (Figure 1a). The mRNA expression levels of GEFs were similar in the two cell lines. Trio mRNA was among the most highly expressed in both cell types.

Overexpression of full-length (FL) Trio leads to lamellipodia formation and cortical actin (126); these phenotypes were mimicked by overexpression of the GEFD1 region of Trio (which is responsible for Rac1 activation) but not by overexpression of the GEFD2 (activates RhoA) domain (125, 126, 136). This suggests that Trio has a preferential GEF activity towards Rac1 over RhoA (124, 126). Trio was initially discovered as an interactor of the LAR transmembrane protein tyrosine phosphatase, a transmembrane protein found at the focal adhesions (122). As Rac1 also plays a role in regulating focal adhesion formation, we decided to investigate Trio further.

Next, we looked a nephromine (now called nephroseq, found at www.nephroseq.com), a web-based database that compiles renal gene expression data, to find which GEFs are upregulated in human patients suffering from FSGS or MCD vs. healthy controls. We used the “Ju podocyte” dataset, which consisted of 436 total glomerular and tubulointerstitial samples. In this dataset, gene expression was used to predict podocyte-specific transcripts “by support vector machine based in silico nano-dissection”. Although many of the GEFs only had a small degree of overexpression in the proteinuric diseases, it was statistically significant (figure 1b). Trio was
overexpressed in both FSGS (1.24-fold, \( p = 0.004 \)) and MCD (1.18-fold, \( p = 0.006 \)) compared to healthy controls.

Finally, we combined the RNA-seq data with the nephromine data to get three candidate GEFs—Trio, Arhgef10, and Net1 (figure 1c). We decided to pursue Trio because (1) out of the three candidates, it was the most highly expressed in both podocyte cell lines (2) its preferential activation of Rac1 over RhoA (124, 126) and (3) its role in cell adhesion.

### 3 Isoforms of Trio are Expressed in Podocytes

Next, we verified that Trio was expressed at the protein level. We verified the specificity of the antibody using HEK293 cells transfected with full-length Trio or vector alone. In both transfected HEK293 and HP cells, we found three Trio isoforms, full-length (334 kDa), isoform D (303 kDa), and isoform A (254 kDa) (figure 2a) (123). Trio was also found in immortalized mouse podocytes (SMP) and glomeruli isolated from mice (figure 2b). Finally, in vivo podocyte expression of Trio was confirmed using immunofluorescence on mouse glomeruli. Trio colocalizes with Wilms’ tumor-1 (WT1), a nuclear transcription factor only found in the podocyte in the mature glomerulus. Furthermore, it is found bordering podocalyxin, a protein found at the apical side of podocytes and in endothelial cells (figure 2c). We thus established that the GEF Trio was expressed at both the mRNA and protein level in vitro and in vivo.

### Trio Contributes to Basal Rac1 Activity and Cell Size

Next, we sought to determine whether Trio played any role in controlling Rac1 activity in podocytes. To do this, we first created a Trio knockout (KO) human podocyte cell line (Trio KO) using the CRISP/Cas9 system. As control, Cas9 cells (control Cas9) without the sgRNA plasmid were established. Cells underwent a limiting dilution to create two clonally-derived cell lines (Trio KO #13 and #14). Although the expression of Trio was not completely abolished, it was greatly reduced (figure 3a). Next, a Cdc42/Rac Interactive Binding (CRIB) assay was used to detect active Rac1 in control Cas9 and Trio KOs under basal, non-stimulated conditions. Both Trio KO #13 had lower Rac1 activity than control Cas9 (figure 3b, c).

As an increase in Rac1 activity is associated with increased cell size (75) and transfection of mouse podocytes with constitutively active (CA)-Rac1 made cells larger, we measured the size of our Trio KOs. Immunofluorescence with phalloidin staining revealed that, compared to
control Cas9, Trio KOs are smaller (figure 3e,f). Conversely, we generated HP cells expressing a GFP-tagged constitutively active form of Trio, which only contains the Rac1/RhoG GEF domain (called the Trio GEFD1 domain), without the inhibitory spectrin-like repeats or the RhoA GEF domain (the GEFD2 domain). Compared to HP expressing GFP-alone, the HP with GFP-Trio-GEFD1 were larger (figure 3g,h). Together, these results suggest that Trio plays a role in activating Rac1, thereby increasing cell size through the GEFD1 domain.

Finally, Trio knockdown in cultured glioblastoma cells decreased proliferation (137). Thus, an MTT assay was performed to compare proliferation. Between 24 and 48 hours after plating, the Trio KOs had similar proliferation rate as the Cas9 cells. However, it is possible that a difference in proliferation would be observed if the time frame was longer.

**TGFβ1 Increases Trio and Rac1 Activity**

We next sought to find an extracellular ligand that could increase Trio’s activity towards Rac1. The first step of this was finding an activator of Rac1. We decided to investigate transforming growth factor β1 (TGFβ1), a profibrotic cytokine that has been heavily implicated in the pathogenesis of FSGS and is highly expressed in podocytes from human patients with idiopathic FSGS (51, 53). Furthermore, TGFβ1 has been shown to increases Rac1 activity in mesangial cells, also found in the glomerulus (171). HP cells were stimulated with TGFβ1 over a time course, and lysates were subjected to a CRIB pulldown (PD). Rac1 activity was increased by 60 minutes of stimulation, but not at 30 or 45 minutes (figure 4a,b). We also looked at p38 mitogen active protein kinase (MAPK), which is activated partially by Rac1 in response to LPS (107) and is a marker of podocyte injury (172). By immunoblot analysis, we saw that TGFβ1 stimulation increases phosphorylation of p38 (p-p38) on Thr^{180}/Tyr^{182}, which represents its active form (figure 4a,c).

Next, we investigated whether Trio contributed to this TGFβ1-induced Rac1 activation. Rac1-GEFs can be pulled down using a GST-tagged nucleotide-free Rac1 mutant (RacG15A) coupled to a glutathione-tagged agarose bead (110). To our surprise GST-RacG15A did not pull-down Trio from SMP cells treated with TGFβ1. However, Trio was pulled down (under both basal and TGFβ1 stimulated conditions) using an assay of a GST-tagged wild type Rac1 (GST-RacWT) (Supplemental figure 1). In cultured human podocytes, a time course of TGFβ1 showed
that Trio is pulled down most by GST-RacWT after 45 minutes of stimulation (figure 4d,e). This suggests that TGFβ1-induced Trio to bind, and subsequently activate, Rac1.

To ascertain that Trio does indeed contribute to TGFβ1-induced Rac1 activation, a CRIB PD was done on control Cas9 and Trio KOs. To our surprise, while TGFβ1 increased Rac1 activity in the control Cas9, it actually decreased Rac1 activity in Trio KOs (figure 5a,b). This led us to hypothesize that TGFβ1 was simultaneously activating a Rac1-GAP. In mammary tumor explants expressing activated Neu/ ErbB-2 (Neu-NT) receptor, Arhgap31 is required for TGFβ1 to induce migration and invasion (173). Furthermore, our RNA-seq data showed that Arhgap31 is expressed in both LY and HP cells. Thus, we set to determine if Arhgap31 is activated by TGFβ1. To do so, we used an assay that specifically pulls down Rac1-GAPs, which consists of GST-tagged constitutively-active (CA)-Rac1 (GST-CA-Rac) (110), followed by immunoblotting for Arhgap31. A time course of TGFβ1 treatment showed that Arhgap31 is activated and binds Rac1 at 30, 45, and 60 minutes (figure 5c,d). The results indicate that TGFβ1 induces both Trio and Arhgap31 activation, which have opposing effects on Rac1 activity. Thus, we propose, that in wild type HP, Trio wins the tug-of-war and leads to increased Rac1 activation in response to TGFβ1.

Finally, we continued our investigation of p38 activation. Compared to control Cas9, Trio KOs have decreased levels of p-p38 (fig 5e,f), consistent with the decreased levels of Rac1 activity in these cells. Considering that TGFβ1 decreases Rac1 activity in Trio KOs, and p38 activation occurs partially through Rac1, we hypothesized that TGFβ1 would not result in a great increase in p-p38 in Trio KOs. However, to our surprise, we found that TGFβ1 induces a greater increase in p38 activation in Trio KOs than in control Cas9 (figure 5e,g). This suggests that Rac1 is not the only regulator of p-p38.

Trio Affects Motility, Attachment, and Vinculin Localization

Active Rac1 is generally found at the leading edge of a moving cell, where it promotes actin polymerization and focal complex formation, thereby increasing motility (73). Trio knockdown in glioblastoma cells (137) or HeLa cells (126) decreased migration. This led us to explore Trio’s role in migration. First, we performed immunofluorescence studies on HP stimulated with TGFβ1. Cells were fixed after TGFβ1 treatment and stained for Trio. Within 30
minutes of TGFβ1 treatment, Trio was relocalized to the periphery of the cell, at the lamellipodia
(figure 6a), where active Rac1 is also typically found in a moving cell (73). A wound healing
assay revealed that Trio KOs have a significant migration impairment, with decreased wound
closure by 5 hours (figure 6b,c). Together, these results suggest that Trio promotes podocyte
motility, possibly through its ability to activate Rac1.

Trio is present at focal adhesion complexes (141) and Rac1 activity is hypothesized to
cause podocyte detachment from the glomerular basement membrane (79). Thus, Trio’s effect on
podocyte attachment was explored. Despite the decreased Rac1 activity found in Trio KOs, these
cells also had decreased attachment (figure 6d). We hypothesized that this was due to Trio’s
interaction with the focal adhesion complex. Immunofluorescence staining of vinculin (a
component of the focal adhesion complex) revealed that control Cas9 cells had vinculin spread
throughout the cell. In contrast, Trio KOs had vinculin concentrated more around the periphery
of the cell (figure 6e,f). This vinculin relocalization may be responsible for the decreased
attachment seen in Trio KOs.

BioID Identifies β-PIX as another potential Rac1-GEF

Finally, we supplemented our candidate approach of finding a Rac1-GEF in podocytes
with an unbiased proteomics approach. For this, we used the BioID system, which entailed
creating a fusion between Escherichia coli biotin protein ligase (BirA) and Rac1G15A. When
GEFs bind Rac1G15A, the BirA ligase induces proximity-dependent biotinylation (174).
Cultured HP were stably transfected with this doxycyclin-inducible myc-tagged fusion protein
(myc-BirA-RacG15A) or myc-BirA without a bait protein as a negative control. Cells were
treated with doxycyclin and biotin overnight and then stimulated with one hour of TGFβ1.
Biotinylated proteins were isolated by affinity capture and identified by mass spectrometry.
Since we used the RacG15A construct (and not RacWT), it was not surprising to find that Trio
was not greatly pulled down. However, a number of other GEFs, such as β-PIX, Dock9 and
Fdg6, were isolated. β-PIX (i.e. Arhgef7) was isolated at the highest concentration (figure 7a).
Nephroseq revealed that, in the Ju CKD dataset, β-PIX mRNA is increased in glomeruli from
FSGS patients as compared to healthy donors (1.07-fold, p = 0.011). The level of β-PIX isolated
was slightly increased by TGFβ1 stimulation (figure 7a). We verified this using the GST-RacWT
PD, and found that TGFβ1 increases β-PIX binding to Rac1 by 45 minutes (figure 7b,c).
Discussion

We have identified Trio as a Rac1 activator in podocytes. Trio is among the most highly expressed GEF at the mRNA level in both LY and HP cells. It is expressed at the protein level in cultured HP and mouse podocytes, as seen by immunoblotting and immunofluorescence. In cultured HP, immunoblotting and RT-PCR confirmed the presence of three isoforms (full-length, A, and D). All of these isoforms contain the GEFD1 region of Trio, which is responsible for activating Rac1, and differ by their C-terminal (123). This is novel, as expression of the D and A isoforms was previously thought to be specific to the nervous system (123). Using the CRISPR/Cas9 system, we decreased Trio expression in HP. Knocking out Trio in cultured podocytes decreases Rac1 activity, cell size, and motility. In contrast, nucleofection of HP with the GEFD1 domain of Trio, which is responsible for Rac1 and RhoG activation, increased cell size. Together, this suggests that Trio is partially responsible for Rac1 activation, likely through its GEFD1 domain, in podocytes under unstimulated conditions.

Furthermore, we have elucidated an additional mechanism by which TGFβ1 may contribute to FSGS pathogenesis. TGFβ1 causes podocyte effacement, apoptosis, decreased proliferation, and decreased cell adhesion (54-56, 58, 59). Monoclonal antibodies neutralizing all three forms of TGFβ have shown promise in animal models (61-66) and have passed phase I clinical trials in patients with steroid-resistant idiopathic FSGS (67). Our results suggest that TGFβ1 causes Rac1 hyperactivation partially via Trio and/or β-PIX. As Rac1 hyperactivation has been implicated in podocytopathy (79, 84), this may explain the underlying mechanism of TGFβ1-induced podocyte injury. Interestingly, TGFβ1 simultaneously promotes Trio, β-PIX, and Arhgap31 activation. This suggests that when Trio is present, Rac1 will ultimately be activated by Trio and β-PIX more than it is deactivated by Arhgap31. In contrast, when Trio is knocked out, TGFβ1 decreases Rac1 activity. This suggests that β-PIX (which is normally activated by TGFβ1 by 45 minutes) cannot activate Rac1 in the absence of Trio. This may be due to the effect of p21-activated protein kinases (PAKs). β-Pix forms a complex with α-Pix, GIT1, and GIT2; these GIT-PIX complexes are found at the focal adhesions, in the cytoplasm, and at the leading edge (165). PAK is activated downstream of Rac1 and Cdc42, and it can also phosphorylate GIT and PIX. This feedback is thought to amplify the Rac1-Cdc42-PAK signaling (150). Thus, it is possible that Trio activates Rac1, which activates PAK, leading to the phosphorylation and subsequent activation of β-Pix.
The mechanism by which TGFβ1 activates Trio remains unanswered. Trio’s ability to activate Rac1 has been shown to be regulated by a number of proteins, including Fyn (a Src family tyrosine kinase) (135), disrupted-in-schizophrenia 1 (DISC1) (128), Kidins220/ARMS (an integral membrane protein) (144), Trio-associated repeat on actin (Tara) (an F-actin binding protein) (145, 146), and Hsc70 (a constitutively and ubiquitously expressed ATP-dependent chaperone) (134). All of these proteins were expressed in our cultured LY and HP cells at the mRNA level, as seen by the RNA-seq data (Supplemental table 1). In Nephroseq, Hsc70 is increased 1.15-fold (p = 0.002) in FSGS vs. healthy living donor (in the Ju CKD dataset). Interestingly, TGFβ1 has been previously reported to increase Hsc70 expression in cultured chicken embryo cells (175). Although we did not find Hsc70 expression to be increased within 60 minutes of TGFβ1 stimulation of HP (data not shown), TGFβ1 may increase Hsc70’s interaction with Trio, thereby activating Trio’s Rac1-GEF activity.

We have also elucidated a possible flaw with the RacG15A PD assay. The use of GST-RacG15A has become an increasingly popular method of pulling down Rac1 GEFs (117-119) and is commercially available. To our knowledge, this is the first time a GST-RacG15A PD is compared to GST-RacWT. GST-RacG15A failed to isolate active Trio. This may be due to the fact that β-PIX can bind Rac1 independent of its nucleotide status (151). Thus, it is possible that β-PIX can bind to the nucleotide-free RacG15A mutant at a high enough affinity so as to inhibit Trio’s binding to it. In contrast, GST-RacWT pulled down Trio activated by TGFβ1. The co-immunoprecipitation of a GEF and Rac1 has been previously used as a marker of GEF activation (176). Thus, we showed that the gold-standard GST-RacG15A assay may not bind all active Rac1-GEFs.

Unfortunately, we were unable to make HP cells stably transfected with myc-BirA-RacWT. This is likely due to the fact that overexpression of Rac1 induces podocytes apoptosis (79). Even under the doxycyclin-inducible reverse tetracycline transactivator (rtTA) promoter, there was Rac1 overexpression in cells before treatment with doxycyclin. Thus, we performed a BioID experiment using HP stably transfected with doxycyclin-inducible myc-BirA-RacG15A or myc-BirA (as a negative control). We isolated β-PIX, which was also increased upon TGFβ1 stimulation. This was verified using the GST-RacWT PD. Unlike conventional proteomics methods, the BioID system can identify insoluble and membrane-associated proteins and detect weak and/or transient interactions (174). Indeed, when we tried to pulldown GEFs with GST-
RacG15A and analyze the samples with proteomics, we did not find any GEFs pulled down (data not shown). This confirms that the BioID system is a more sensitive assay of finding interactions.

In conclusion, we found that Trio and β-PIX are GEFs that play a role in activating Rac1 in podocytes, thereby possibly contributing to the Rac1 hyperactivation seen to cause foot process effacement and proteinuria. Trio regulates podocyte size, attachment, and migration, implying that it may be essential for podocyte maintenance. TGFβ1 induces Trio, β-PIX and Arhgap31 activity, but ultimately leads to Rac1 activation. This Rac1 hyperactivity may be the underlying mechanism by which TGFβ1 causes FSGS and foot podocyte effacement.
FIGURES FOR CHAPTER 2

Figure 1: Trio mRNA is expressed in cultured podocytes and is upregulated in human proteinuric diseases

To find candidate GEFs in podocytes, we first (a) RNA-seq on cultured human podocytes called LY cells (duplicates—labelled (1) and (2)) and compared the results to a separate line of human podocytes called HP. In both cell lines, we found that Trio mRNA was highly expressed. (b) Nephromine was utilized to find which GEFs are upregulated in FSGS or MCD. We analyzed the Ju podocyte data set and plotted the fold-change between FSGS or MCD versus healthy controls. (c) We combined the RNA-seq data with the data-mining results. We found three candidate GEFs—Trio, Arhgef10, and Net1—that were highly expressed in our cultured podocytes and overexpressed in both FSGS and MCD as compared to control.
Figure 1:

**a.** RNA-Seq in Cultured Human Podocytes

**b.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>RNA-seq Value (FPKM)</th>
<th>Fold Change FSGS vs. Healthy</th>
<th>p-value of FSGS</th>
<th>Fold Change MCD vs. Healthy</th>
<th>p-value of MCD</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAR1P</td>
<td>15.131</td>
<td>1.152493</td>
<td>0.010602846</td>
<td>1.044891</td>
<td>0.254458001</td>
</tr>
<tr>
<td>TRIO</td>
<td>9.089855</td>
<td>1.2411289</td>
<td>0.003816891</td>
<td>1.1757832</td>
<td>0.006472929</td>
</tr>
<tr>
<td>PLEKHG3</td>
<td>8.49107</td>
<td>-1.1832892</td>
<td>0.901809245</td>
<td>1.1255884</td>
<td>0.055123</td>
</tr>
<tr>
<td>ARHGEF10</td>
<td>3.823455</td>
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<td>0.067488175</td>
<td>1.0756325</td>
<td>0.0659562</td>
</tr>
<tr>
<td>NET1</td>
<td>3.54417</td>
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<td>0.001970526</td>
<td>1.2709979</td>
<td>0.0757334</td>
</tr>
<tr>
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<td>0.220520958</td>
<td>1.3059467</td>
<td>0.016017925</td>
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<td>0.94903006</td>
<td>1.1804833</td>
<td>0.037117939</td>
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<td>ITSN1</td>
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<td>0.032480587</td>
<td>1.135989</td>
<td>0.077896989</td>
</tr>
<tr>
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<td>1.1885197</td>
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<td>1.0830041</td>
<td>0.207571016</td>
</tr>
<tr>
<td>DOCK2</td>
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<td>-1.0656044</td>
<td>0.726118107</td>
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<tr>
<td>DOCK4</td>
<td>0.92833</td>
<td>1.2178122</td>
<td>0.043188832</td>
<td>1.0659411</td>
<td>0.282756434</td>
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<tr>
<td>DOCK6</td>
<td>0.8069085</td>
<td>1.1808064</td>
<td>0.02005367</td>
<td>1.1422396</td>
<td>0.083840572</td>
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<tr>
<td>TIAM1</td>
<td>0.6229045</td>
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<td>0.005511826</td>
<td>1.0283202</td>
<td>0.455340544</td>
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<tr>
<td>ARHGEF6</td>
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<td>0.0000484</td>
<td>1.2962024</td>
<td>0.023181035</td>
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</tbody>
</table>

**c.** RNA Sequencing Gene Expression Set (FSGS) includes FAR1P, TRIO, PLEKHG3, ARHGEF10, NET1, ARHGEF12, VAV2, PLEKHG3, and ARHGEF18.

Molecular Complex Dynamics (MCD) set includes FAR1P, TRIO, PLEKHG3, ARHGEF10, NET1, ARHGEF12, VAV2, PLEKHG3, and ARHGEF18.
**Figure 2: Trio is expressed in podocytes in vitro and in vivo**

(a) HEK293 cells were transfected with full-length GFP-tagged Trio or vector alone and lysed one day after transfection, alongside cultured HP cells. The LiCor system (see methods) was used to detect GFP-Trio. A red secondary antibody was used for GFP detection, while a green secondary antibody was used to detect Trio. The result were three (full-length, D, and A) yellow bands (detecting both Trio and GFP) in transfected HEK293, but no signal in untransfected HEK293. Correspondingly, three isoforms were detected in HP cells. (b) Immunoblotting revealed Trio expression in cultured mouse podocytes (left) and mouse glomerular lysate (right). (c) Within the mouse glomerulus, podocyte expression of Trio was shown using immunofluorescence staining. Top: Trio (red) surrounded WT1-positive nuclei with some projections (arrows). Bottom: Trio staining showed partial overlap with podocalyxin (arrow heads), which is expressed on the apical membrane of podocyte foot processes.
Figure 2:

a. Human podocytes

HEK293T (transfection)

- FL
- D
- A

250 kDa

IB: Tubulin

b. Mouse Glomerular

MP

Lysate

250kDa

250kDa

c. Podocyte marker

Trio

Merge

WT1 (podocyte nucleus marker)

Podocalyxin (also expressed in Endo)
Figure 3: Trio Contributes to Basal Rac1 Activity and Cell Size

(a) The CRISPR/Cas9 system was used to create Trio KO human podocytes. Monoclonal populations were selected and tested for Trio expression using immunoblotting. Although the KO was not complete, it decreased Trio expression by 60% (Trio KO #13) or 58% (Trio KO #14) when normalized to calnexin and then to control Cas9. (b,c) CRIB pulldown assays revealed that both Trio KO #13 (0.39 ± 0.10, n = 3, **p < 0.01 compared to control Cas9) and Trio KO #14 (0.24 ± 0.06, n = 3, ***p < 0.001 vs. control Cas9) had decreased Rac1 activity compared to control Cas9. Quantification was done using densitometric analysis, and results were normalized to tubulin and then to Rac1 activity in control Cas9 cells. (d) However, an MTT assay showed that the proliferation rate of Trio KOs did not differ from that of control Cas9 cells between 24 hours and 48 hours after plating. Trio KOs #9 and #19 also had decreased Trio expression (not shown) and were used for the MTT assay. (e,f) Control Cas9 and Trio KOs were fixed and stained for phalloidin. Trio KOs (2349 ± 114 μm², n = 89 cells over two experiments, ***p< 0.001 vs. control Cas9) were clearly smaller than control Cas9 (2349 ± 140 μm², n = 81 over two experiments) (f) 40x magnification is shown. (g,h) Finally, HP were nucleofected with GFP-alone or GFP-Trio-GEFD1 and stained for phalloidin and induced with doxycylin. Cells not stimulated with doxycylin did not show GFP expression (data not shown). GFP-Trio-GEFD1 cells were larger (3223 ± 131 μm², n = 65 over two experiments, ***p<0.001 vs. GFP-alone) than GFP-alone expressing cells (2327 ± 142 μm², n = 61 over two experiments).
Figure 3:

a. Cas9, Trio KO #13, Trio KO #14

b. Rac1 Activity of Control vs. Trio KOs

<table>
<thead>
<tr>
<th></th>
<th>Control Cas9</th>
<th>Trio KO #13</th>
<th>Trio KO #14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rac1</td>
<td>1</td>
<td>**</td>
<td>***</td>
</tr>
</tbody>
</table>

Active Rac1, Total Rac1, Total Tubulin

c. Control Cas9, Trio KO #13, Trio KO #14

d. Proliferation Rate

<table>
<thead>
<tr>
<th></th>
<th>Control Cas9</th>
<th>Trio KO #9</th>
<th>Trio KO #19</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fold Increase in Number of Cells</td>
<td>1.5</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Active Rac1, Total Rac1, Total Tubulin

e. Cas9, Trio KO #14

f. Cell Size of Trio KOs

<table>
<thead>
<tr>
<th></th>
<th>Control Cas9</th>
<th>Trio KO #14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area (um²)</td>
<td>3000</td>
<td><strong>3000</strong></td>
</tr>
</tbody>
</table>

GFP, Phalloidin, Merge

g. GFP-alone, GFP-Trio-GEFD1

h. Cell Size of Control vs. Trio-GEFD1

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Trio-GEFD1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area (um²)</td>
<td>2500</td>
<td><strong>3000</strong></td>
</tr>
</tbody>
</table>
Figure 4: TGFβ1 Increases Rac1 Activity and Trio Binding to RacWT
(a,b,c) HP cells were stimulated with 10ng/mL of TGFβ1 for 0, 30, 45 or 60 minutes and then subjected to a CRIB PD. Precipitates and total lysates were immunoblotted for Rac1, p-p38, and p38. (b,c) Results were normalized to tubulin and then to the highest Rac1 activity or p-p38 level (found at 60 minutes). (d) HP cells were subjected to the same time course of TGFβ1 (10ng/mL) and then subjected to a PD using GST-RacWT. The PD samples and total lysates were blotted for Trio and tubulin. Two negative controls were used: (i) the GST-RacWT beads were not incubated with cell lysate and run on a gel alone (left lane labelled – ) and (ii) a mixture of the lysates were PD with GST-alone, lacking the RacWT construct (rightmost lane). No signal was found in either of these lanes, suggesting that the PD and antibody was specific. (e) Quantification was done normalizing to tubulin and then to Trio activity at 0 minutes of stimulation. Trio binding to RacWT increased 1.82-fold ± 0.29, n = 3-4, *p<0.05 vs. unstimulated.
Figure 4:

(a) TGFβ1 (min):

- Active Rac1
- Total Rac1 (10% input)
- p-p38 (10% input)
- Total p38 (10% input)
- Tubulin (10% input)

(b) Rac1 Activity

(c) p-p38

(d) TGFβ1 (min):

- PD: GST-RacWT
- PD: GST-alone

- Trio PD
- 250kDa

- Trio 23% input
- 250kDa

- Tubulin 23% input

(e) TGFβ1 Increases Trio Binding

- RacWT

- Fold Increase in Trio Activity (a.u.)
Figure 5: TGFβ1 Decreases Rac1 activity in Trio KOs, While Increasing Arhgap31 Activity in Control Cells

(a) Control Cas9 and Trio KOs were incubated with 10ng/mL of TGFβ1 for 60 minutes. This was followed by a CRIB PD and immunoblotting for Rac1 and tubulin. In control Cas9 cells, TGFβ1 increased Rac1 activity 1.72-fold ± 0.20, n = 4; whereas in Trio KOs, TGFβ1 decreased Rac1 activity 0.59-fold ± 0.13, n = 4. (b) Results were first normalized to tubulin and then fold increase of +TGFβ1 over - TGFβ1. **p < 0.01 vs. control Cas9. (c) HP were treated with TGFβ1 over a time course and pulled down with GST-CA-Rac1. Precipitates and lysates were blotted for Arhgap31 and tubulin. (d) Levels of active Arhgap31 were normalized to Arhgap31 levels in the total lysate (i.e. input) and then to 0 minutes. Arhgap31 activity was increased at 30 minutes (1.55-fold ± 0.16, *p < 0.05 vs. unstimulated), 45 minutes (1.56-fold ± 0.21, *p < 0.01 vs. unstimulated), and 60 minutes (1.53-fold ± 0.12, **p < 0.05 vs. unstimulated), n = 3-4. (e) Control Cas9 cells and Trio KOs were stimulated with TGFβ1 (10 ng/mL, 60 minutes) and lysates were blotted for p-p38 and total p-38. (f) Basal (unstimulated) levels of p-p38 were normalized to tubulin and then to control Cas9 levels. Trio KOs had 0.55 ± 0.28 fold decrease, n = 4, *p<0.05 versus control Cas9. (g) Samples were normalized to tubulin and then fold increase of +TGFβ1 over - TGFβ1. TGFβ1 increased p-p38 more in Trio KOs than in control Cas9 (control Cas9: 1.54 ± 0.45 fold, Trio KO: 2.71 ± 0.70 fold, n = 4, *p < 0.05 vs. control Cas9).
Figure 5:

a. TGFB1
Active Rac1
Total Rac1
Total Tubulin

b. TGFB1-Induced Change in Rac1 Activity

Fold Increase in Rac1 Activity (a.u.)

Control Cas9 | Trio KO #14
---|---

![Bar chart showing fold increase in Rac1 activity for Control Cas9 and Trio KO #14.]


c. PD: GST-CA-Rac
Arhgap31 PD
Arhgap31 250kDa
Tubulin 27.5% input

PD: GST-alone

TGFβ1 (min): 0 | 30 | 45 | 60 | pool

Fold Increase in GAP31 Activity (a.u.)

Minutes of TGFβ1 Stimulation

0 | 30 | 45 | 60

** | ** | ***

![Bar chart showing fold increase in GAP31 activity for different time points of TGFβ1 stimulation.]

d. TGFβ1 Increases Arhgap31 Binding RacWT

![Bar chart showing fold increase in GAP31 activity for different conditions.]

e. Control Cas9 | Trio KO #14
TGFβ1
p-p38
Total p38
Tubulin

![Image showing Western blots for p-p38 and Total p38.]

f. Basal Level p-p38

Fold Increase in p-p38 (a.u.)

Control Cas9 | Trio KO14
---|---

![Bar chart showing fold increase in p-p38 for Control Cas9 and Trio KO14.]

g. TGFB1-Induced Increase in p-p38

TGFB1-induced fold increase in p-p38 (a.u.)

Control Cas9 | Trio KO14
---|---

![Bar chart showing TGFB1-induced increase in p-p38 for Control Cas9 and Trio KO14.]

Figure 6: Trio Affects Motility, Attachment, and Vinculin Localization

(a) HP cells were plated overnight on 5-2-1 laminin and then stimulated with TGFβ1 (10ng/mL). Cells were fixed and stained for Trio. TGFβ1 promotes Trio re-localization to the periphery (white arrows). 40x magnification is shown. (b) Control Cas9 and Trio KOs were allowed to grow to confluency, serum starved in medium without FBS overnight, and then a wound was created. Pictures were taken at wound formation and 5 hours after. 5x magnification is shown. (c) The wound closure at 5 hours was normalized to that at 0 hours. Control Cas9 cells had a greater wound closure than Trio KOs by 5 hours (control Cas9: 21.4 ± 0.55, Trio KOs: 17.3 ± 0.99, n = 4, *p < 0.05 vs. control Cas9). (d) The same number of control Cas9 and Trio KOs were plated on 5-2-1 laminin and allowed to attach for 1.25 hours. Afterwards, attached cells were fixed and visualized using crystal violet. The absorbance at 550nm is reflective of the number of cells. Control Cas9 cells had more attachment than Trio KOs (control Cas9: 0.17 ± 0.01, Trio KO: 0.13 ± 0.01, n = 3-4, *p < 0.05 vs. control Cas9). (e,f) Control Cas9 and Trio KOs were plated overnight on 5-2-1 laminin, fixed, and stained for phalloidin and vinculin. 40x magnification is shown. The amount of vinculin at the periphery was quantified (e) in ImageJ by drawing random lines across the cell visualized for vinculin and measuring the intensity in the outer 10μm normalized to the intensity in the next 20μm. Trio KOs had more peripheral vinculin than control Cas9 (control Cas9: 0.55 ± 0.02, n = 21 cells counted over two experiments; Trio KO: 0.72 ± 0.04, n = 19 cells counted over two experiments, ***p < 0.001 vs. control Cas9).
Figure 6:

(a) Images showing wound closure over time with different treatments. The graphs below show the results of motility, attachment, and vinculin localization.

(c) Motility: % wound closure at 5 hours for Control Cas9 and Trio KO #13.

(d) Attachment: Absorbance at 550 nm for Control Cas9 and Trio KO #14.

(e) Peripheral Vinculin Localization: Peripheral vs. central vinculin for Control Cas9 and Trio KO #14.

(f) Immunofluorescence images of Vinculin, Phalloidin, and Merge for Control Cas9 and Trio KO #14.
Figure 7: BioID Identifies β-PIX as another potential Rac1-GEF

(a) HP cells were stably transfected with myc-BirA-RacG15A or myc-BirA (negative control). Cells were stimulated with doxycyclin and biotin overnight and treated with TGFβ1 (60 minutes, 10ng/mL) before being lysed and pulled down with magnetic streptavidin beads. Beads were then washed and sent to proteomics. β-PIX (i.e. Arhgef7), Dock9, and Fdg6 were isolated. β-PIX (i.e. Arhgef7) was isolated at the highest quantity. It slightly increased with TGFβ1 stimulation.

(b) To further investigate whether TGFβ1 increases β-PIX activation, TGFβ1-treated HP cells were subjected to a GST-RacWT PD. The PD samples and total lysates were immunoblotted for β-PIX and tubulin. (c) The pulled down β-PIX was normalized to the input β-PIX and then to 0 minutes of stimulation. β-PIX binding to Rac1 increases by 45 minutes of TGFβ1 stimulation (1.46 ± 0.37 fold, n = 4-5, *p < 0.05 vs. unstimulated).
Figure 7:

(a) Graph showing normalized file area (Quantity) x 10000000 for different samples labeled as BirA - TGFβ1, BirA + TGFβ1, BirA-RacG15A - TGFβ1, and BirA-RacG15A + TGFβ1.

(b) Western blot analysis of TGFβ1 min: 0, 30, 45, 60, with PD: GST-RacWT and PD: GST-alone. Image shows bands at 75kDa and 23% input.

(c) Bar graph showing fold increase in β-PIX activity (a.u.) with minutes of TGFβ1 stimulation. Significant increase marked with asterisk.
Supplemental Figure 1: Trio is pulled down by GST-RacWT but not GST-RacG15A or GST-alone

SMP were treated with TGFβ1 (60 minutes, 10ng/mL) pulled down with GST-RacWT, GST-RacG15A, or GST-alone. Precipitates and lysates were blotted for Trio and tubulin. Three isoforms were pulled down by GST-RacWT (and increased with TGFβ1 stimulation), but no signal was found in the GST-RacG15A or GST-alone pull downs.
Supplemental Table 1: RNA-seq Data of Trio-Regulating Proteins

Below are the FPKM values from our RNA seq data done on cultured LY cells (in duplicates, labelled 1 and 2) and HP cells. We have analyzed the mRNA levels of proteins previously reported to control Trio’s Rac1-GEF activity.

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<th>Protein</th>
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<th>LY (2)</th>
<th>HP</th>
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</thead>
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<td>Tara</td>
<td>5.74</td>
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<td>Hsc70</td>
<td>163.8</td>
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<td>329.7</td>
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CHAPTER 3: CONCLUSIONS AND DISCUSSION
Before this study was done, the only GEFs that were known to activate Rac1 in podocytes were Vav1 (in the context of HIV-associated nephropathy) and Vav2 (in interleukin-13 overexpression rat minimal change-like nephropathy model). However, it was unclear if there are any GEFs that activate Rac1 under basal conditions or during FSGS. This study addressed that by using a candidate and unbiased approach. In the candidate approach, we combined RNA-seq on two lines of immortalized human podocytes with analysis of GEFs upregulated in human FSGS using the Nephromine database. We found three candidate GEFs—Trio, Net1, and Arhgef10—and decided to pursue Trio for three reasons: (1) among the three candidate, it was the most highly expressed in both cell lines, (2) it has a preferential activity towards activating Rac1, and (3) it plays a role in cell adhesion and focal adhesion complexes, which play a vital role in podocytes as they help anchor the cells to the glomerular basement membrane.

Next, we knocked out Trio (Trio KOs) in our cultured human podocytes (HP) using the CRISPR/Cas9 system. Although the knockout was not complete, the level of Trio expression was greatly reduced. As control, we used control Cas9 cells, which are HP expressing only the Cas9 but missing the sgRNA which knocks out Trio. In comparison to control Cas9, Trio KOs have decreased basal, unstimulated Rac1 activity, cell size, motility, attachment, and increased localization of vinculin to the periphery. It is interesting to note that Trio KOs have decreased attachment as expression of constitutively active (CA)-Rac1 in podocytes decreases cell attachment. This suggests that Trio is not contributing to cell adhesion through activation of Rac1, but rather on its own. In other words, in addition to activating Rac1 under basal conditions, Trio may have some Rac1-independent functions. Therefore, I would hypothesize that, in mice, podocyte-specific knockout of Trio would lead to renal phenotypes, possibly including loss of podocytes and proteinuria. This is in contrast to the podocyte-specific deletion of Rac1, which does not lead to any renal abnormalities under basal conditions.

Furthermore, this study discovered an additional mechanism by which TGFβ1 may contribute to FSGS pathogenesis. In glomerular mesangial cells, TGFβ1 increase Rac1 activity, leading to increased expression of type I collagen expression; this likely contributes to glomerulosclerosis. In addition, we found that TGFβ1 directly effects podocytes, by increasing Rac1 activity. Interestingly, we found that TGFβ1 increases both GEF (Trio and β-PIX) activity and GAP (Arhgap31) activity. In HP cells, the GEFs win this battle and there is an increase in Rac1 activity. However, in Trio KOs, TGFβ1 decreases Rac1 activity. This suggests that either
(a) β-PIX cannot activate Rac1 more than Arhgap31 can deactivate it or (b) β-PIX is unable to activate Rac1 in the absence of Trio. One possible explanation for the former would be the role of PAKs. β-PIX activates Rac1, which activates PAKs; in turn, PAKs phosphorylate β-PIX. While the consequence of this PAK-mediated phosphorylation of β-PIX remains unclear, it is thought to increase the β-PIX-Rac1-PAKs signaling mechanism. Therefore, it is possible that Trio stimulates Rac1 activity, which increases PAK activity, leading to the phosphorylation and activation of β-PIX. This may explain why β-PIX fails to activate Rac1 in the absence of Trio.

This study also investigated two assays: the pulldown of GEFs using RacG15A and the use of BioID. Our results show that while Trio was isolated using GST-RacWT, GST-RacG15A or GST-alone failed to pulldown this protein. To our knowledge, there has never been a comparison between the pulldown by RacWT vs. RacG15A. Our results suggest that RacG15A may be unable to pulldown all active GEFs due to competition with GEFs (such as β-PIX) that will bind Rac1 independent of its nucleotide status. The co-immunoprecipitation of a GEF with Rac1 has been previously used as a measure of GEF activity. This further validates our use of GEF binding to RacWT as an assay for GEF activity. In contrast to the RacG15A assay, we validated the use of the BioID assay as more sensitive. We did a GST-RacG15A pulldown assay and sent the samples for analysis by mass spectroscopy; however, we did not get any GEFs that were isolated using this method. In contrast, using the BioID assay with HP expressing myc-BirA-RacG15A, we were able to identify four new candidate GEFs—β-PIX, Dock9, Fdg6, and Farp1. This confirms that BioID is a more sensitive assay and is good for capturing weak and transient interactions with membrane-bound proteins.

Further studies are required to elucidate the role of Trio in vivo. Podocyte-specific Trio knockout mice could be used to analyze (a) life span, (b) proteinuria, (c) histopathological lesions, (d) Rac1 activity levels in glomeruli, and (e) number of podocytes per glomeruli, which would be expected to be low due to decreased adhesion. These mice would also elucidate whether Trio is required for podocyte development. Furthermore, previously established (56) transgenic mice expressing doxycyclin-inducible, podocyte-specific CA-TGFβ1 receptor 1 could be treated with Trio inhibitor, ITX3, to study the TGFβ1–Trio signaling pathway in vivo.

In conclusion, we have identified two GEFs—Trio and β-PIX—which contribute to Rac1 activation in podocytes. These proteins may be contributing to the Rac1 hyperactivity in
response to TGFβ1 and underlie the mechanism by which TGFβ1 promotes proteinuria and FSGS. This may have important implications in the clinic, as treatment options are very limited for patients with primary steroid-resistant FSGS.
REFERENCES


