Regulation of TRPC6 Podocyte Surface Expression by Synaptopodin

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UNIVERSITY OF MIAMI

REGULATION OF TRPC6 PODOCYTE SURFACE EXPRESSION BY SYNAPTOPODIN

By

Hao Yu

A DISSERTATION

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REGULATION OF TRPC6 PODOCYTE SURFACE
EXPRESSION BY SYNAPTOPODIN

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Mutations of classic Transient Receptor Potential Channel 6 (TRPC6) were identified in hereditary FSGS, most of which are gain-of-function. Increased expression of wild-type TRPC6 in glomeruli was observed in several human acquired proteinuric diseases. Synaptopodin, an actin binding protein that is important in maintaining podocyte function, is downregulated in various glomerular diseases. Here, we provided evidence that synaptopodin maintains podocyte function by regulating podocyte surface expression and activity of TRPC6. We show indirect interaction and nonrandom association of synaptopodin and TRPC6 in podocytes. Knockdown of synaptopodin in cultured mouse podocytes increased the expression of TRPC6 at the plasma membrane, whereas overexpression of synaptopodin decreased it. Mechanistically, synaptopodin-dependent TRPC6 surface expression required functional actin and microtubule cytoskeletons. Functionally, overexpression of wild-type TRPC6 or the FSGS-inducing mutant TRPC6^{M131T} in synaptopodin-depleted podocytes enhanced TRPC6-mediated calcium influx and induced apoptosis. In vivo, knockdown of synaptopodin by tail vein injection of siRNAs caused increased podocyte surface expression of TRPC6. Administration of cyclosporine A, which stabilizes synaptopodin, reduced LPS-induced proteinuria significantly in wild-type mice but to a lesser extent in TRPC6 knockout mice.
Furthermore, administration of cyclosporine A reversed the LPS-induced increase in podocyte surface expression of TRPC6 in wild-type mice. Our findings suggest that alteration in synaptopodin levels under disease conditions may modify intracellular TRPC6 channel localization and activity, which further contribute to podocyte dysfunction. Therefore, reducing TRPC6 surface levels may be considered to be a new approach to restoring podocyte function.
DEDICATION

This work is dedicated to my parents Xiaomin Yu, Fengzhi Bian and my husband Dony Maiguel for their understanding and support.
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Chapter 1

Introduction

Kidneys are responsible for filtering the blood. They remove water-soluble wastes, control the body's fluid balance, and regulate the balance of electrolytes. Each human kidney contains approximately 0.8 to 1.5 million nephrons, which are the basic structural and functional units of the kidney (Hall and Guyton, 2011).

1.1 Nephron

The main functions of a nephron are to eliminate wastes and maintain concentrations of electrolytes and metabolites in the blood. A nephron consists of a renal corpuscle and renal tubule. Filtration of the blood that occurs in the renal corpuscle generates a filtrate that is composed of water, ions, glucose and small proteins. Renal tubule reabsorbs desirable solutes and water and excretes the rest as urine. Nephrons also play a role in regulating blood pressure and volume (Hall and Guyton, 2011).

1.1.1 Renal corpuscle

A renal corpuscle consists of a capillary tuft (glomerulus) surrounded by Bowman’s capsule that opens into renal tubule (Figure 1.1). Glomerulus contains three cell types: fenestrated endothelial cells (forming the wall of capillaries), podocytes that are attached to the outer aspect of the glomerular basement membrane (GBM) and mesangial cells that sit between the capillary loops. The endothelial cells, glomerular basement membrane and podocytes form the glomerular filtration barrier (Menon et al., 2012).
Figure 1.1 Simplified schematics demonstrate the location and components of a nephron. (A) Schematic of a nephron and the collecting duct it drains into (“The Excretory System”, http://bio100.class.uic.edu/lecturesf04am/lect21.htm). (B) Schematic of the renal corpuscle showing the glomerulus and its podocytes (Margaret Reece, “Kidney Physiology Basics”, http://www.medicalsciencenavigator.com/kidney-physiology-basics/).

Table 1.1. Selective filtration by glomerular filtration barrier (Pugh-Clarke, 2008). The molecules listed were tested for permeability, calculated by the concentration of the molecule in the glomerular filtrate (fluid collected from Bowman’s capsule) compared to the concentration of the molecule in the plasma.

<table>
<thead>
<tr>
<th>substance</th>
<th>MW (D)</th>
<th>permeability ([filtrate]/[plasma])</th>
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<tr>
<td>water</td>
<td>18</td>
<td>1</td>
</tr>
<tr>
<td>sodium</td>
<td>23</td>
<td>1</td>
</tr>
<tr>
<td>Urea</td>
<td>60</td>
<td>1</td>
</tr>
<tr>
<td>glucose</td>
<td>180</td>
<td>1</td>
</tr>
<tr>
<td>inulin</td>
<td>5,500</td>
<td>1</td>
</tr>
<tr>
<td>myoglobin</td>
<td>17,000</td>
<td>0.75</td>
</tr>
<tr>
<td><strong>albumin</strong></td>
<td><strong>69,000</strong></td>
<td><strong>0.005</strong></td>
</tr>
</tbody>
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The glomerular filtration barrier is freely permeable to water, small ions and small proteins in plasma, yet maintains size and charge selectivity for large proteins and larger
molecules. For example, healthy glomerular filtration barrier is unfilterable to albumin (Table 1.1). When glomerular filtration barrier is damaged, leakage of large protein such as albumin from blood into urine will occur (proteinuria).

1.1.2 Renal tubules

After leaving glomerulus, glomerular filtrate enters renal tubules for further processing. The tubule portion of a nephron consists of proximal tubules, loop of Henle and distal tubules sequentially. They are responsible for reabsorbing water, glucose, amino acids, salts such as sodium, potassium, calcium, and chloride as well as secreting ammonium and hydrogen ions (Hall and Guyton, 2011).

1.1.3 Podocyte

Among the three types of cells in glomeruli, podocyte plays a critical role in maintaining the function of the glomerular filter. Podocyte damage is typically associated with the development of proteinuria which can lead to permanent deterioration of the glomerular filter. Podocyte is a highly specialized cell that consists of a cell body, major processes (MPs) and foot processes (FPs) (Mundel and Kriz, 1995) (Figure 1.2A). Podocyte FPs are anchored to the GBM via integrin. Podocyte FPs form a highly branched interdigitating network with FPs of neighboring podocytes connected by the slit diaphragm (SD) a multiprotein complex similar to adherens junctions which covers the regions between opposing podocyte FPs (Figure 1.2 B). Slit diaphragm serves as the final barrier of urinary protein loss.

The cytoskeleton of major processes predominantly contain microtubules and intermediate filaments while foot processes are characterized by the presence of highly
ordered parallel contractile actin filament. The actin cytoskeleton in podocyte FPs participates in regulating the permeability of the filtration barrier by modulating FP morphology. The function of podocytes is thought to be largely based on their complex cell architecture, particularly the FP structure.

Figure. 1.2. Pictures of glomerular podocytes. (A) scanning electron microgram of podocytes in a glomerulus demonstrates the cell body(CB), major processes(MP), and the finely interdigitating foot processes(FP) (Welsh and Saleem, 2012). (B) Transmission electron microscopy picture of the glomerular filtration barrier shows the podocyte foot processes, slit diaphragm and fenestrated endothelium (“Kidney”, Sally Marshall, http://www.diapedia.org/acute-and-chronic-complications-of-diabetes/71040851155/kidney).

Interference with podocyte's normal physiological activities often results in disruption of the actin cytoskeleton which is characterized by FP effacement (the simplification of the FP structure and the loss of the normal interdigitating pattern). FP effacement is often associated with proteinuria under pathological conditions. The most typical example is minimal change disease (see section 1.2.1). FP effacement is occasionally seen in regions of healthy podocytes which is thought to be an adaptive response in order to perform its regular function (Kriz et al., 2013)
FP effacement requires reorganization of actin filaments. Proteins directly or indirectly regulating the plasticity of the podocyte actin cytoskeleton such as α-actinin-4 (Kaplan et al., 2000), myosin (Mele et al., 2011) and synaptopodin (see section 1.3.4) have been shown to be of crucial importance for the maintenance of glomerular filter function.

Slit diaphragm is comprised of multiple proteins produced by podocytes including nephrin, Neph1, P-Cadherin, FAT, podocin, ZO-1 and TRPC6. A number of studies have shown that mutations affecting slit diaphragm proteins including, CD2AP (Lowik et al., 2007), nephrin (Kestila et al., 1998), PLCE1 (Hinkes et al., 2006), podocin (Boute et al., 2000), and TRPC6 (Reiser et al., 2005; Winn et al., 2005), lead to glomerular disease due to disruption of the filtration barrier (Tryggvason et al., 2006).

1.2 Nephrotic syndrome

Nephrotic syndrome (NS) is a group of symptoms including proteinuria (nephrotic-range proteinuria is 3 grams per day or more. On a single spot urine collection, it is 2 g of protein per gram of urine creatinine), hypoalbuminemia and edema. Nephrotic syndrome may occur when the filtering units of the kidney (glomeruli) are damaged which causes protein normally kept in the plasma to leak into the urine in large amounts and therefore the reduction of the amount of protein in the blood (Hull and Goldsmith, 2008).

Nephrotic syndrome is caused by different disorders that damage kidney glomeruli such as minimal change disease (MCD), focal segmental glomerulosclerosis (FSGS), membranous nephropathy (MN) and hereditary nephropathies. It can also result from systemic disease that affect other organs in addition to kidneys such as diabetes, systemic lupus erythematosus, genetic disorders, viral infections (e.g. human immunodeficiency
virus) and preeclampsia. Kidney diseases that affect tubules and interstitium, such as interstitial nephritis, will not cause nephrotic syndrome (Hull and Goldsmith, 2008).

1.2.1 Minimal change disease

Minimal change disease (MCD) is the most common cause of the nephrotic syndrome (NS) in children and one of the major causes of NS in adults. It accounts for 70 to 90% of the NS in children younger than 10 years old and 10-25% in adults. (Waldman et al., 2007)

In MCD, renal biopsies show no glomerular lesions on light microscopy and negative staining on immunofluorescence microscopy. Podocyte foot process effacement, the only visible change, can only be seen under an electron microscope (EM) (Waldman et al., 2007). Most of the cases are idiopathic. Historically, MCD was considered to be associated with abnormal immune system. T-cell-related mechanisms are implicated in the pathogenesis (Saha and Singh, 2006). Therefore, the treatment for adult MCD is mainly oral glucocorticoids which are immunosuppressive. This treatment leads to remission in over 80% of cases. Patients that relapse can become steroid-resistant, steroid-dependent, or frequently relapsing. Immunosuppressive drugs cyclosporine and tacrolimus help achieve remission in steroid-dependent and steroid-resistant patients (Hogan and Radhakrishnan, 2013).

1.2.2 Focal segmental glomerulosclerosis

Focal segmental glomerulosclerosis (FSGS) accounts for approximately 20% cases of nephrotic syndrome in children and 40% in adults. It is the most common primary glomerular disorder causing end-stage renal disease in the US (D'Agati et al., 2011). "Focal" means that some of the glomeruli become scarred while others remain normal.
"Segmental" means that only part of an individual glomerulus is damaged. FSGS is a podocytopathy characterized by the presence of nephrotic syndrome with FSGS lesion under light microscopy and foot process effacement under EM (Sethi et al., 2015). Although the cause of primary focal segmental glomerulosclerosis is often unknown, it has long been attributed to a circulating permeability factor. Soluble urakinase receptor has been suggested to be a cause of primary FSGS (Wei et al., 2011; Wei et al., 2008). Several viruses (such as HIV) and drugs (such as bisphosphonate pamidronate) have been associated with FSGS. In addition, a number of genetic mutations of proteins of the slit diaphragm (nephrin, podocin and CD2AP), podocyte membrane (β4-integrin, TRPC6), actin cytoskeleton (myosin 1E, α-actinin-4) result in FSGS or nephrotic syndrome (Greka and Mundel, 2012).

The goal of therapy for FSGS patients is to induce a complete or partial remission of proteinuria and preserve renal function (D'Agati et al., 2011). Remission is associated with improved long-term survival (D'Agati et al., 2011). Similar to MCD, FSGS is also historically considered as immunological disease. Treatments for FSGS include corticosteroid and immunosuppressive agents such as the calcineurin inhibitor cyclosporine A (CsA). In addition to the immunosuppressive effect of steroids and cyclosporine, these agents were shown to have direct beneficial effect on podocytes mainly through stabilizing actin cytoskeleton and the slit diaphragm (Faul et al., 2008; Schonenberger et al., 2011). However, 30% - 50% of the adult patients are resistant to steroids. Most of the genetic forms of the disease are also steroid-resistant. Some case reports indicated that CsA is still effective in steroid-resistant nephrotic syndrome or FSGS.
caused by genetic mutations podocin, MYO1E (Mele et al., 2011), TRPC6 (Gigante et al., 2011; Santin et al., 2009) and CoQ6 (Heeringa et al., 2011; Santin et al., 2011).

1.2.3 Membranous nephropathy

Membranous nephropathy (MN) is the most common cause of nephrotic syndrome in adults. MN is caused by immune complex deposition along the glomerular basement membrane. Studies in Heymann nephritis, an experimental model of MN, suggested that deposits locally activate complement to cause podocyte injury, loss of slit diaphragms, and proteinuria (Ma et al., 2013). Immunosuppressive therapies are most commonly used for MN such as calcineurin inhibitors cyclosporine and tacrolimus. For those patients who don’t respond to these immunosuppressive therapies, rituximab which damages B cells has been suggested to be effective (Waldman and Austin, 2012). Interestingly, rituximab was also shown to have a direct protective effect on podocytes in FSGS (Fornoni et al., 2011).

1.3 Podocyte biology

As a major component of the glomerular filtration barrier, podocytes are of critical importance to the normal function of the kidney. Podocyte biology has been under intensive research for the past decade. A number of the studies concern reorganization or disruption of podocyte actin cytoskeleton (Welsh and Saleem, 2012). In this study, we focused on the regulation of calcium in podocytes.

1.3.1 Calcium in podocytes

Numerous studies have focused on the role of calcium and calcium dependent pathways in podocyte function and injury. In an earlier study, cytosolic calcium and PKC were reported to reduce complement-mediated podocyte injury (Cybulsky et al., 1990). It was
proposed by Rudiger et al that an increase of \([\text{Ca}^{2+}]_\text{i}\) might be an early event in the pathogenesis of podocyte FP effacement in polycation-mediated podocyte injury (Rudiger et al., 1999). Protamine sulfate induced proteinuria and podocyte foot process effacement are associated with increased \([\text{Ca}^{2+}]_\text{i}\) in podocytes (Pippin et al., 2009). N-type calcium channel is involved in podocyte injury and proteinuria in spontaneously hypertensive rat/ND mcr-cp (Fan et al., 2010). Calcimimetic R-568 was shown to have anti-apoptotic and actin-stabilizing effects in podocytes (Oh et al., 2011)

Angiotensin II (Ang II) was reported to contribute to the abnormal elevation of TRPC channel mediated calcium influx in isolated glomeruli from diabetic nephropathy rats (Ilatovskaya et al., 2015). In 2005, Winn et al and Reiser et al identified TRPC6 mutations in familial FSGS (Reiser et al., 2005; Winn et al., 2005). Since then, TRPC6 and other TRPC channels emerged as prime candidates for studying calcium in podocyte biology.

1.3.2 TRPC channels

Transient Receptor Potential (TRP) protein superfamily consists of a diverse group of cation channels that play important roles in many non-excitable cells (Montell, 2005). TRP channels are receptor-operated (ROC) \(\text{Ca}^{2+}\)-permeable ion channels and are also candidates for store-operated (SOC) \(\text{Ca}^{2+}\) entry (Montell, 2005). TRPs are classified into TRPC, TRPV, TRPM, TRPP, TRPN, and TRPML subfamilies. They serve diverse functions ranging from thermal, tactile, taste, and osmolar sensing to fluid flow sensing. The initially identified members of TRP superfamily are referred to as canonical or classic TRP channels (TRPC channels). There are seven members in TRPC subfamily in mice (TRPC1-7) and six in human where TRPC2 is a pseudogene and not counted. The seven family members can be further grouped on the basis of their amino acid homology. TRPC1 and
TRPC2 are almost unique, while TRPC4 and TRPC5 share a ~65% homology. TRPC3, 6 and 7 form a structural and functional TRPC3/6/7 subfamily sharing 70-80% homology. All TRPC3, 6, and 7 can be directly activated by DAG (Montell, 2005).

Analysis of the subunit composition of mammalian TRPC channels revealed that four TRPC subunits assemble a homo- or hetero- tetramer as a functional channel. Specifically, all TRPC proteins are able to form homomeric channels. TRPC2 does not interact with other TRPC members but itself. TRPC1 is able to form heteromeric tetramers with TRPC4 and TRPC5. TRPC4/5 or TRPC3/6/7 can assemble into heterotetramers within their subfamilies (Hofmann et al., 2002).

Members of the TRPC family have similar structures: four N-terminal ankyrin repeats, six transmembrane domains (S1–S6), a putative pore region between S5 and S6, and a TRP domain following the sixth transmembrane domain. The intracellular N- and C- termini have multiple potential protein binding sites for potential regulation and trafficking of the channel (Dryer and Reiser, 2010).

1.3.3 TRPC6

Among the 7 TRPC proteins, TRPC6 has been associated with familial nephrotic syndrome. More than 20 mutations in TRPC6 have been linked to familial FSGS. The majority of the mutations are gain-of-function (Gigante et al., 2011; Heeringa et al., 2009; Reiser et al., 2005; Riehle et al., 2016; Winn et al., 2005; Zhu et al., 2009). Glomerular expression of wild-type (wt) TRPC6 was elevated in acquired human glomerular diseases including MCD, FSGS and MN (Moller et al., 2007). Moreover, proteinuria caused by angiotensin II treatment was attenuated in TRPC6 knockout (TRPC6<sup>−/−</sup>) mice (Eckel et al., 2005).
These findings led to the hypothesis that hyperactive mutant forms of the channel or increased channel expression, possibly via increased TRPC6 mediated-calcium signaling, cause podocyte dysfunction and glomerular damage. In support of this hypothesis, Krall et al. showed that transgenic mice with overexpression of WT or mutated TRPC6 channels specifically in kidney podocytes exhibited proteinuria and glomerular lesions that resembled human FSGS (Krall et al., 2010). It is noteworthy that a few newly identified mutations that are associated with FSGS were shown to be loss-of-function (Riehle et al., 2016), providing new insights to the role of TRPC6 in hereditary nephrotic syndrome.

Other TRPC channels are relatively less studied in podocytes. TRPC5 was recently shown to contribute to podocyte injury. Depletion or inhibition of TRPC5 protected podocyte from cytoskeletal remodeling upon injury and reduced proteinuria (Schaldecker et al., 2013). The same group also showed that TRPC6 and TRPC5 have antagonistic effects on AngII receptor (AT1R) overexpressing podocytes upon AngII treatment (Tian et al., 2010). However, there has been no report on the expression pattern or pathological effect of TRPC5 in human kidney disease patients.

The majority of the published studies concerning regulation and functions of TRPC6 in podocytes examined the overall expression levels of the channel (Moller et al., 2007) (Chen et al., 2011; Eckel et al., 2011; Kistler et al., 2013; Liu et al., 2012; Nijenhuis et al., 2011; Sonneveld et al., 2014; Tian et al., 2010; Wang et al., 2009; Zhang et al., 2009)

However, TRPC6 activity has been reported to be at least partially regulated by the channel’s expression levels at the cell surface (Cayouette et al., 2004). Irregularly high surface levels of mutated forms of TRPC6 have been implicated in disease mechanisms.
Limiting surface levels of wild type TRPC6 in cardiomyocytes by Klotho is protective against cardiac hypertrophy (Xie et al., 2012). Studies that focused on cell membrane expression and trafficking of TRPC6 were mostly done in model cell lines such as HEK293 or HEK293T cells under normal conditions (Cayouette et al., 2010; Cayouette et al., 2004; Lussier et al., 2008). Few studies focus on regulation of TRPC6 cell surface expression and its influence on cell function in specialized cell types. Calmodulin and PI3 kinase regulate membrane translocation of TRPC6 and subsequently endothelial migration (Chaudhuri et al., 2016). TRPC6 surface expression in podocytes could be affected by insulin (Kim et al., 2012). Mechanisms underlying modulation of TRPC6 cell surface expression in podocytes under physiological and pathological conditions remains largely unknown.

1.3.4 Synaptopodin

In 1991, a 44KD fragment was identified as an actin-associated protein of differentiated podocytes, where it is part of the actin-based contractile apparatus in the foot processes (Mundel et al., 1991). This fragment was later identified to a proteolytic fragment of the 110KD synaptopodin protein (Mundel et al., 1997a). Expression of synaptopodin was only found in brain and kidney podocytes (Mundel et al., 1997a).

Synaptopodin shows no significant homology to any published proteins. It has high content of proline (~20%) evenly distributed along the protein (Mundel et al., 1997a). Several possible phosphorylation sites spread along the molecule. Phosphorylation of synaptopodin is important for its stability (Faul et al., 2008).

Synaptopodin functions as a regulator of actin dynamics and cell motility. In the brain, synaptopodin is strongly expressed by spine-bearing telencephalic neurons, where it is
associated with the postsynaptic density (Mundel et al., 1997a) and is necessary for formation of the dendritic spine apparatus (Deller et al., 2003). Furthermore, synaptopodin contributes to the regulation of dendritic spine dynamics and synaptic function (Zhang et al., 2013). The essential role of synaptopodin in regulating synaptic plasticity in cultured hippocampal neurons is calcium store-related (Korkotian et al., 2014).

In podocytes, synaptopodin expression appears along the stress-fibers in dotted pattern. Mechanically, synaptopodin protects podocyte by stabilizing its stress fibers. It promotes RhoA signaling by competitive blocking of Smurf1-mediated ubiquitination of RhoA, thereby preventing the targeting of RhoA for proteasomal degradation (Asanuma et al., 2006). Additionally, synaptopodin suppresses Cdc42 signaling by disrupting Cdc42:IRSp53:Mena signaling complexes (Yanagida-Asanuma et al., 2007).

Two isoforms of synaptopodin were identified in humans (Asanuma et al., 2005; Mundel et al., 1997a). Synpo-long (NCBI Reference Sequence: NM_007286.5, 903 amino acids) is expressed in kidney podocytes. Synpo-short (NCBI Reference Sequence: NM_001109974.2, 685 amino acids) is expressed in brain. The first 670 aa are identical to those of the synpo-long isoform and the last 15 aa are distinct (Asanuma et al., 2005). Synpo-long interacts with α-actinin4 in podocytes and synpo-short interacts with α-actinin2 to induce formation of long unbranched actin filaments (Asanuma et al., 2005). In synpo−/− mice (Deller et al., 2003), another isoform synpoT (181 aa, identical to the c-terminus of synpo-long) is up-regulated in podocytes which partially compensates for the loss of synpo-long (Asanuma et al., 2005).

Synaptopodin is widely used as a protein marker for glomerular podocytes in immunohistochemistry. The expression level of synaptopodin is also frequently used as an
indication of glomerular and podocyte health. Decreased synaptopodin expression in glomeruli has been observed in numerous kidney diseases including FSGS and HIV-associated nephropathy (Barisoni et al., 1999), idiopathic nephrotic syndrome of childhood including MCD, diffuse mesangial hypercellularity (Srivastava et al., 2001). In addition, low synaptopodin levels were reported to be associated with poor response to steroid therapy (Hirakawa et al., 2006; Srivastava et al., 2001). Decreased synaptopodin was also found in several in vitro and in vivo kidney disease models including LPS and Angiotensin II mouse models (Faul et al., 2008; Tian et al., 2010) (Marko et al., 2012). Since synaptopodin plays an important role in stabilizing podocyte stress fibers, restoring synaptopodin levels has been considered as a potential approach to improving glomerular functions.

Cyclosporine A (CsA) is a medicine that lowers the body’s natural immunity. It is used to prevent rejection of transplanted organs. CsA is also used in treatment of nephrotic syndrome (such as MCD and FSGS) for its effects of lowering proteinuria and inducing remission (Meyrier, 2005). Originally, the antiproteinuric effect of CsA was attributed to its immunosuppressive action because the immune system response is thought to be involved in nephrotic syndrome. Faul et al demonstrated that CsA is able to reduce proteinuria by maintaining the actin cytoskeleton structure of renal podocytes through preserving synaptopodin. Phosphorylation of synaptopodin is required for its interaction with 14-3-3β. This interaction protects synaptopodin from cathepsin L-mediated degradation. CsA, as a calcineurin inhibitor, blocks the calcineurin-mediated dephosphorylation of synaptopodin, thereby protects synaptopodin from cathepsin L-mediated degradation (Faul et al., 2008).
In this study, we present evidence that TRPC6 cell surface expression in podocytes is regulated by synaptopodin. We observed that down-regulation of synaptopodin resulted in elevated TRPC6 at podocyte cell membrane and increased apoptosis, upon TRPC6 activation. Conversely, overexpression of synaptopodin led to decreased surface TRPC6. The changes in TRPC6 podocyte surface levels were accompanied by correspondent changes in calcium influx mediated by the channel. We also show that in vivo knockdown of synaptopodin led to increased podocyte surface TRPC6. Cyclosporine A was able to alleviate LPS-induced proteinuria in wild-type mice but to a lesser degree in TRPC6−/− mice. In wild-type mice, podocyte surface TRPC6 was elevated upon LPS treatment and was abrogated by administration of CsA.
Chapter 2
Interaction between TRPC6 and synaptopodin

2.1 Methods and materials

Antibodies

The following primary antibodies were used for western blotting and immunocytochemistry: rabbit anti-TRPC6(ACC-017) (Alomone Labs, Israel), goat anti-synaptopodin, rabbit anti-calnexin, rabbit anti-nephrin, goat anti-VE cadherin, (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-HA (Thermo Scientific, IL), mouse anti-pan-cadherin (Sigma-Aldrich, MO), mouse anti-GAPDH (Abcam, MA), mouse anti-α-tubulin, rabbit anti-HSP90, rabbit anti-CaMKII, rabbit anti-CaMKII (pThr286) (Cell Signaling Technology, MA), rabbit anti-PDGFR-β (Millipore, CA), Guinea pig anti-nephrin (Fitzgerald, MA), alexa-fluor 568 phalloidin (Life Technologies, CA). Secondary antibodies used for western blotting (donkey anti-mouse, rabbit, goat) were from LI-COR (Lincoln, NE). Secondary antibodies used for immunocytochemistry were Alexa-fluor donkey anti-rabbit, donkey anti-mouse, donkey anti-goat (Life Technologies, CA).

Cell culture

Mouse wild-type podocytes were cultured as described previously (Mundel et al., 1997b). Briefly, podocytes were plated on collagen I-coated cell culture dishes and propagated at 33°C in RPMI1640 medium (Corning) supplemented with 10% FBS and 100U/mL penicillin/streptomycin and interferon-γ (15U/mL) (Sigma). Podocytes were thermos-shifted to 37°C and maintained in interferon-γ free medium to induce differentiation. HEK 293 cells were grown in DMEM with high glucose, L-glutamin and
sodium pyruvate supplemented with 10% FBS at 37°C. HEK293 cells were transfected with FuGene 6 (Promega) for co-immunoprecipitation and lentivirus production.

**Western blotting**

For the preparation of whole-cell lysates, cells were gently scraped, collected and lysed on ice by the addition of 1% Trinton-100 with 1mM EDTA in TBS plus protease inhibitor cocktail (Roche Diagnostics, Germany) (and phosphatase inhibitors for detecting pThr286 CaMKII). Cell lysates were incubated for 30 min on ice, and centrifuged at 13,000 rpm for 15 min at 4°C. Supernatants were collected to obtain protein extracts. Protein concentration was determined by Pierce BCA protein assay kit (Thermo Scientific, IL). Protein extracts were then added to 1xLDS sample buffer with 1X reducing agent (Life technologies, CA) and heated at 70°C for 10 mins. Western blotting was performed using Xcell Surelock mini-cell electrophoresis system and block module (Life Technologies, CA). Target proteins were probed using corresponding primary antibodies and secondary antibodies and visualized and quantitated with Odyssey CLx infrared imaging system (LI-COR Biosciences, NV)

**Co-immunoprecipitation**

The recombinant mouse GFP-TRPC6 and flag tagged synaptopodin, podocin, nephrin and CD2AP were expressed in HEK293 cells. Flag tagged proteins were immunoprecipitated from cell lysates using anti-flag-M2 beads (Sigma). Eluates were analyzed by western blotting using anti-flag (Sigma), or anti-GFP (Life Technologies) antibodies. Co-immunoprecipitation in cultured mouse podocytes was done as described previously (Kim et al., 2009). Fully differentiated podocytes were lysed in 50 mM Tris-Cl,
pH 7.6, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 2 mM EDTA, 1 mM PMSF, and protease inhibitor mixture (Sigma). Lysates were cleared by centrifugation and the resulting extracts (500 μg of protein) were incubated in the presence of anti-synaptopodin, or IgG (1-2 μg), for 4 h at 4 °C. We then added 20μl of protein A/G agarose (Santa Cruz Biotechnology) to the lysates and incubated for 12 h. Pellets were washed, boiled for 5 min in SDS sample buffer, and analyzed by western blotting. A diluted sample of cell lysate was used as input.

**Far western blotting**

Bait proteins, Synpo-long-flag, Synpo-short-flag, SynpoT-flag (Asanuma et al., 2005), podocin-flag and CD2AP-flag, were expressed in HEK293 cells and purified using anti-flag-M2 beads (Figure 2.1D). The prey protein TRPC6-HA and its negative control empty plasmid were transfected into HEK293 cells. Native western blotting was then performed using NativePAGE Novex Bis-Tris Gel System (Invitrogen, CA). 50 ug of HEK293 lysates containing TRPC6-HA or negative control were loaded per lane. PVDF membrane was incubated with purified bait proteins (total 5 ug, 3.3 ug/mL) for 4 hours at room temperature. Binding of the bait proteins on the membrane was detected by probing the flag tag. Expression and location of TRPC6-HA was detected by probing the HA tag. The NativeMark standards (Thermo Fisher) was used as molecular weight marker.

**Immunogold Electron Microscopy**

Mouse kidneys were harvested after perfusion with 4% PFA in PBS. Kidneys were sectioned and fixed in 4% PFA with 0.001% glutaraldehyde overnight. Kidney tissues were dehydrated and embedded in LR white resin (London Resin Company, England) according
to manufacturer’s instructions. Thin sections were placed on nickel grids for immunostaining. Grids were floated with section side down on top of solution drops during immunostaining. Briefly, free aldehyde groups were blocked with 50mM glycine in PBS for 15 mins. After blocking with blocking solution (Aurion Immunogold Reagents and Accessories, Netherlands) for 30 mins, sections were incubated with primary antibodies overnight at 4°C. After washing with BSA-c buffer (Aurion) three times, sections were incubated with gold particle conjugated secondary antibodies for 1 hour at room temperature. Sections were post fixed in 2% glutaraldehyde in PBS for 5 mins and thoroughly washed in PBS and water. Sections were heavy metal stained by uranyl acetate for 15 mins and then washed with water several times and air dried. Images were taken by Gatan Erlangshen ES1000W camera on the Philips CM10 electron microscope at 80kV accelerating voltage.

2.2 Indirect interaction between TRPC6 and synaptopodin

To explore the association of TRPC6 with important regulators of podocyte function, we performed Co-immunoprecipitation (Co-IP) in HEK293 cells. We first co-transfected HEK293 cells with TRPC6-GFP and flag-tagged synaptopodin (Synpo-flag), or TRPC6-GFP with major slit diaphragm components podocin-flag, nephrin-flag or CD2AP-flag (Figure 2.1 A). We found that TRPC6 interacted with synaptopodin as well as podocin and nephrin but not CD2AP. To confirm the findings, we repeated the Co-IP assays with flag tagged TRPC6 with GFP tagged synaptopodin, podocin, nephrin and CD2AP and obtained the same results (Figure 2.1B).
To verify the interaction in podocytes, we performed co-IP in cultured mouse podocytes. Endogenous TRPC6 was pulled down by antibody against synaptopodin; conversely, endogenous synaptopodin was pulled down by antibody against TRPC6 (Figure 2.1C), confirming the interaction between TRPC6 and synaptopodin.

Far western blotting was performed to further examine whether the interaction detected by co-IP was direct (Wu et al., 2007). If the prey protein (TRPC6) and the bait protein (Synpo-long, Synpo-short, or SynpoT) directly interact, we should be able to detect the bait protein on the blot where the prey protein is. However, we failed to detect Synpo-long, Synpo-short or SynpoT on the blot indicating no direct interaction between Synpo-long, Synpo-short or SynpoT and TRPC6 (Figure 2.1E). Podocin was served as positive control and CD2AP as negative control. A major band appears between 66KD and 146KD and another band above 480KD on the podocin-TRPC6 blot, corresponding to the molecular weights of the TRPC6 monomer (approximately 110 KD) and tetramer (the functional unit of homomeric TRPC6 channel) (approximately 440KD).

2.3 Non-random association of TRPC6 and synaptopodin in podocyte foot processes

To examine the localization of TRPC6 and synaptopodin proteins in podocytes \textit{in vivo}, we performed immunogold double labelling of the two proteins in kidney sections of a male wild-type C57B6/J mouse. The association of the two proteins was determined by Fisher’s exact test (Mayhew and Lucocq, 2011) (Figure 2.2A). The Fisher exact test probability ($P = 0.0002$), together with the odds ratio ($3.394 > 1$) (95% confidence interval: 1.786 to 6.454) (Table 2.1), indicates that there is non-random association between TRPC6 and synaptopodin in foot processes. Isotype control, no-primary antibody control, and
TRPC6<sup>−/−</sup> mouse control (Table 2.2) were performed to confirm the specificity of the double Immunogold labelling.

**Figure 2.1** TRPC6 interacts indirectly with synaptopodin. (A) Co-IP performed in HEK293 cotransfected with TRPC6-GFP and flag-tagged synpo-long, podocin, nephrin, or CD2AP (B) Co-IP performed in HEK293 cotransfected with TRPC6-flag, and GFP-tagged synpo-long, podocin, nephrin or CD2AP. (C) Co-IP performed in cultured mouse podocytes. (D) Purification of the indicated flag-tagged proteins. The purified proteins were used in far western blotting as bait. (E) Far Western blotting shows no direct interaction between the indicated isoforms of synaptopodin and TRPC6.
Figure 2.2. TRPC6 and synaptopodin non-randomly associate in podocyte foot processes. A representative image of Immunogold double labeling of TRPC6 and synaptopodin shows TRPC6 and synaptopodin locating in close vicinity in podocyte foot processes. a and b are magnifications of areas highlighted in A. Scale bar: 500 nm. Gold particle diameter for TRPC6: 15 nm; synaptopodin: 10 nm.

Table 2.1. Quantification of the double Immunogold labeling of TRPC6 and synaptopodin in podocyte foot processes. Number of foot processes that contained synaptopodin and/or TRPC6 gold particles or did not contain any gold particles were counted.

<table>
<thead>
<tr>
<th>No. of foot processes</th>
<th>TRPC6+</th>
<th>TRPC6-</th>
<th>Row totals</th>
<th>Ratios TRPC6+/TRPC6-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synpo+</td>
<td>36</td>
<td>38</td>
<td>74</td>
<td>36/38=0.947</td>
</tr>
<tr>
<td>Synpo-</td>
<td>24</td>
<td>86</td>
<td>110</td>
<td>24/86=0.279</td>
</tr>
<tr>
<td>Column totals</td>
<td>60</td>
<td>124</td>
<td>184</td>
<td>p=0.0002 Odds ratio=3.395</td>
</tr>
</tbody>
</table>

Table 2.2. Three types of negative control for specificity of the double Immunogold labeling experiment.
Chapter 3

Regulation of TRPC6 podocyte surface expression by synaptopodin in vitro

3.1 Methods and materials

Lentiviral transduction of podocytes

For overexpression, C-terminal HA- mouse TRPC6 cDNA and mouse TRPC6<sup>M131T</sup> cDNA (Krall et al., 2010) were subcloned into the VVPW lentiviral expression vector (a point mutation A4G in both cDNAs was repaired during subcloning). C-terminal flag-human synaptopodin (full-length) was subcloned into VVPW. For synaptopodin knockdown, we used the pLKO-TRC2 vector (RNAi Consortium; supplied by Addgene). Four shRNA sequences targeting synpo<sup>T</sup> and full-length synpo mRNA were tested and two most efficient sequences were used in this study (GGACCTTCTTCCTGTGCTAGT and GCATCCTCGGCTACAACATCT)

Lentiviral infection of podocytes was performed as described previously (Kistler et al., 2013). Briefly, 80% confluent HEK 293 cells were transfected in antibiotic-free DMEM, 10% FBS with the VVPW or pLKO-TRC2 plasmid, and the two helper plasmids psPAX2 and pCMV-VSVG (both from Addgene) in a ratio of 3:2:1 (for VVPW) or 10:9:1 (for pLKO-TRC2) using FuGENE 6 (Promega). Control virus was produced using empty VVPW vector or pLKO-TRC2 containing scrambled shRNA (Addgene). The medium was changed to DMEM, 10% FBS, containing penicillin/streptomycin 16h after transfection. Cell culture supernatant that contained lentivirus was collected at 24h and 48h thereafter. The supernatant was centrifuged and filtered through a 0.45μm filter, aliquoted, and stored at -80 °C. Podocytes were transduced with lentivirus containing supernatant 8 –10 days
after induction of differentiation and used for experiments 4-5 days after transduction. Lentivirus was titrated using Lenti-X qRT-PCR titration kit (Clontech, CA) to ensure equal loading of lentivirus of the same type across the experiment repetitions.

**Real-time PCR**

Total RNA was extracted from podocytes with RNeasy Plus Mini kit per the manufacturer’s protocol (QIAGEN, Germany). RNA was quantitated and cDNA synthesis was performed using iScript cDNA Synthesis kit (Bio-Rad, CA). Quantitative PCR was performed using SsoAdvanced SYBR Green Supermix (Bio-Rad) and specific primers for TRPC6 (TCGAGTTGGGGATGCTTTAC and AGCTGGATGGTTGAGGATTG), synaptopodin (CGGACCTTCTTCTGTGCTA and ATCCTCGGAGTGCAGACAGA) and 18s rRNA (GCAATTATTCCCCATGAACG and GGCCTCACTAAACCACATCCAA) as internal control with Bio-Rad CFX Connect Real-time System. Fold expression changes were determined using the comparative CT method for relative quantification with the calculation $2^{-\Delta\Delta CT}$, and data were graphed using Prism (GraphPad) software.

**Deglycosylation**

Mouse podocytes were transduced with wild-type TRPC6–HA or TRPC6$^{M131T}$-HA lentiviruses in parallel with empty lentivirus as control. Five days after transduction, podocytes were lysed and treated with PNGase F (New England Biolabs, MA) according to manufacturer’s instruction, followed by western blot analysis of the expression of TRPC6 and HA.
Cell surface biotinylation

These assays were done using Pierce cell surface protein isolation kit (Thermo Scientific). Briefly, podocytes were washed twice with PBS and incubated with Sulfo-NHS-SS-Biotin for 40 mins at 4°C with gentle rocking. Reaction was stopped by adding Quenching solution. After washing twice with TBS, cells were scraped gently and collected by centrifugation. Cells were then lysed and the biotin labeled proteins were isolated following manufacturer’s instructions. Labelled proteins were eluted with 1X LDS sample buffer with 1X reducing agent (Life Technologies) by incubating at 70°C for 15 mins. Eluates were analyzed by western blotting.

Opera high throughput immunocytochemistry

Podocytes were grown and differentiated in 96-well CellCarrier plate (Perkin Elmer, MA). Cells were transduced by lentiviruses or treated with cytochalasin D or nocodazole as described in this and the next chapter. Cells were then fixed and stained with primary and fluorophore conjugated secondary antibodies as well as HCS CellMask blue stain (Life Technologies) for high throughput imaging and analysis. Images of cells were obtained by Opera High Content Screening System (Perkin Elmer) and analyzed as described in results section by Columbus Image Data Storage and Analysis System (Perkin Elmer).

Confocal immunocytochemistry

Podocytes were seeded and differentiated on collagen I-coated glass coverslips at 37°C. Scrambled and synpo shRNA lentiviruses were applied to the podocytes on day 7 or 8. After 4 days, podocytes were treated with cytochalasin D or nocodazole (Sigma) as indicated in section 3.5. At the end of the treatment, cells were fixed with 4%
paraformaldehyde (PFA) (Electron Microscopy Sciences, PA) for 20 min at room temperature (RT). Cells were washed three times with PBS and permeabilized with 0.3% Triton X-100 for 5 mins. Then cells were incubated with 5% donkey serum + 1% BSA blocking solution for 1 h, followed by incubation with primary antibodies against TRPC6 and tubulin for 2 h at room temperature. Corresponding fluorescent secondary antibodies and phalloidin were applied for 1 h at RT before mounting with Prolong Gold antifade reagent with DAPI (Life Technologies). Images were taken by Zeiss inverted LSM 700 confocal microscope with Zen Black software.

3.2 Total TRPC6 levels in synaptopodin knockdown or overexpressing podocytes

To explore the biological significance of the association of TRPC6 and synaptopodin, we first considered the possibility of synaptopodin regulating TRPC6 overall expression in podocytes. Effective knockdown and overexpression of synaptopodin in differentiated podocytes were achieved by lentiviral expression (Figure 3.1 A-D). We found that neither total TRPC6 mRNA nor total TRPC6 protein levels were affected by down-regulation or overexpression of synaptopodin in podocytes (Figure 3.1 A-D).

3.3 TRPC6 surface expression in synaptopodin knockdown or overexpressing podocytes by surface biotinylation

We then considered if changes in synaptopodin expression levels would affect cell surface levels of TRPC6 in podocytes. We first examined if down-regulation of synaptopodin affected surface TRPC6 levels. Cell surface TRPC6 expression was determined by surface biotinylation (Cell Surface Protein Isolation Kit, Thermo Fisher
Scientific, IL) and was compared between podocytes transduced with scrambled shRNA (SC) and synaptopodin shRNA (Synpo KD) lentiviruses (Figure 3.3A).

**Figure 3.1** Real-time PCR and western blot results show synaptopodin knockdown and overexpression. (A) Significant down-regulation of synpo mRNA in synpo shRNA transduced podocytes was detected by real-time PCR. TRPC6 transcripts levels show no difference. n=6 (B) Representative western blot showing down-regulation of synaptopodin (Synpo) protein in synpo shRNA transduced (Synpo KD) podocytes Quantification of Synpo fold change was based on 3 repeated experiments. TRPC6 and pan-cadherin were also blotted. (C) Significant increase in synpo mRNA levels in synpo-flag transduced podocytes was detected by real-time PCR. TRPC6 transcripts levels show no difference. n=6. (D) Representative western blot showing up-regulation of Synpo protein in synpo-flag transduced podocytes Quantification of Synpo protein fold change was based on 3 repeated experiments. TRPC6 and pan-cadherin were also blotted. Student’s t test was used. **: p<0.01; ***: p<0.001; ****: p<0.0001 graphs represent mean± s.e.m
Figure 3.2. Overexpression of wild-type TRPC6 and mutant TRPC6$^{M131T}$. (A) wtTRPC6-HA, TRPC6$^{M131T}$-HA expression in cultured mouse podocytes by lentiviral infection. Pan-Cadherin expression was not affected by overexpression of wtTRPC6 or TRPC6$^{M131T}$. (B) The additional TRPC6 (HA) bands above the endogenous TRPC6 band caused by lentiviral expression of wtTRPC6-HA and TRPC6M131T–HA were removed by treatment of glycosydase PNGase F.

We also tested the effect of synaptopodin knockdown in the surface expression of overexpressed wtTRPC6 and FSGS-inducing mutant TRPC6$^{M131T}$ (Heeringa et al., 2009) which mimics the pathological conditions found in human patients. Additional bands above the endogenous TRPC6 band in wtTRPC6-HA and TRPC6$^{M131T}$-HA overexpressing podocyte lysates (Figure 3.2 B) were removed by treatment of glycosydase PNGase F indicating that the higher molecular weight forms are glycosylated TRPC6 protein(Kistler et al., 2013; Schlondorff et al., 2009). They were used in quantification of TRPC6 intensity in addition to the endogenous TRPC6 band. pan-Cadherin protein levels remained unchanged in Synpo KD (synaptopodin knockdown) and Synpo OE (synaptopodin overexpression) or wtTRPC6 and TRPC6$^{M131T}$ overexpressing podocytes (Figure 3.1 B, D and 3.2 A), therefore was used as loading control for total and surface fractions. ER protein calnexin served as an indicator of the purity of the cell surface fraction.
Surface HA and TRPC6 were blotted in SC and Synpo KD podocytes that
overexpressed wtTRPC6 (named SC-wtTRPC6, SynpoKD-wtTRPC6) (Figure 3.3 B) or
TRPC6\textsubscript{M131T} (named SC-TRPC6\textsuperscript{M131T}, SynpoKD-TRPC6\textsuperscript{M131T}) (Figure 3.3 C). TRPC6
levels were normalized to pan-Cadherin. Quantification showed an approximately 2.8 fold
increase of surface TRPC6 in SynpoKD compared with SC podocytes (Figure 3.3 A, D),
approximately 2.5 fold in SynpoKD-wtTRPC6 compared with SC-wtTRPC6 podocytes
(Figure 3.3 B, E), and approximately 1.4 fold in SynpoKD-TRPC6\textsuperscript{M131T} compared with
SC- TRPC6\textsuperscript{M131T} podocytes (Figure 3.3 C, F). Note that the glycosylated forms (upper
bands) of TRPC6 appeared more prominent in cell surface fraction, indicating that the
glycosylated TRPC6 was enriched.

Alternatively, we tested whether overexpression of synaptopodin caused decreased
surface expression levels of TRPC6. Surface biotinylation assays were performed in EV
(empty vector) and Synpo OE podocytes and EV, Synpo OE podocytes that overexpressed
wtTRPC6 (named EV-wtTRPC6, SynpoOE-wtTRPC6), or TRPC6\textsuperscript{M131T} (named EV-
TRPC6\textsuperscript{M131T}, SynpoOE-TRPC6\textsuperscript{M131T}). Quantification showed a 28.9% decrease of surface
TRPC6 in Synpo OE compared with EV podocytes (Figure 3.4 A, D), 41.3% in SynpoOE-
wtTRPC6 compared with EV-wtTRPC6 podocytes (Figure 3.4 B, E), and 23.2% in
SynpoOE-TRPC6\textsuperscript{M131T} compared with EV- TRPC6\textsuperscript{M131T} podocytes (Figure 3.4 C,
F). Together, these results showed that down-regulation of synaptopodin caused increased
podocyte surface TRPC6 and overexpression of synaptopodin led to reduced podocyte
surface TRPC6.
Figure 3.3. Surface biotinylation assays demonstrate the effects of synaptopodin knockdown on cell surface TRPC6 levels. (A) to (F) Representative western blots of surface biotinylation assays and quantifications of TRPC6 levels in SC and Synpo KD podocytes (A and D, n=5), SC-wtTRPC6 and SynpoKD-wtTRPC6 podocytes (B and E, n=4), SC-TRPC6$^{M131T}$ and SynpoKD-TRPC6$^{M131T}$ podocytes (C and F, n=4). GraphPad’s multiple t tests were used for statistical analysis. *: p<0.05; **: p<0.01; ***: p<0.001 graphs represent mean± s.e.m
Figure 3.4. Surface biotinylation assays demonstrate the effects of synaptopodin overexpression on cell surface TRPC6 levels. (A) to (F) Representative western blots of surface biotinylation assays and quantifications of TRPC6 levels in EV and Synpo OE podocytes (A and D, n=3), EV-wtTRPC6 and SynpoOE-wtTRPC6 (B and E, n=4), EV-TRPC6<sup>M131T</sup> and SynpoOE-TRPC6<sup>M131T</sup> (C and F, n=4). GraphPad’s multiple t tests were used for statistical analysis. *: p<0.05; ***: p<0.001 graphs represent mean± s.e.m.
3.4 TRPC6 cell membrane region expression in synaptopodin knockdown or overexpressing podocytes by Opera HCS

In addition to surface biotinylation assays, we utilized Opera High Content Screening (HCS) system to quantitatively measure the expression of TRPC6 on plasma membrane in response to up- or down-regulated synaptopodin in podocytes.

Briefly, mouse podocytes were grown and differentiated in 96-well CellCarrier plates. Cells were then transduced with lentiviruses. SC, Synpo KD, SC-wtTRPC6, SynpoKD-wtTRPC6, SC-TRPC6\textsuperscript{M131T} SynpoKD-TRPC6\textsuperscript{M131T} podocytes as well as EV, Synpo OE, EV-wtTRPC6, SynpoOE-wtTRPC6, EV-TRPC6\textsuperscript{M131T} SynpoOE-TRPC6\textsuperscript{M131T} podocytes were stained for TRPC6 and synaptopodin. Confocal images of all cells in the wells of interest were obtained by Opera High Content Screening System (Perkin Elmer) and analyzed by Columbus Image Data Storage and Analysis System (Perkin Elmer). Membrane and cytoplasm regions of individual cells were designated by Columbus software as shown (Figure 3.5 A). Specifically, for each cell, membrane region was defined as ±5% of total cell area along the edge of the cell) and cytoplasm region was defined as the remaining cell area. The intensities of the two regions of each cell were recorded. For a particular well, the sum of mean fluorescence intensity (MFI) of TRPC6 at membrane region and MFI of TRPC6 at cytoplasm region was calculated as total MFI of TRPC6. TRPC6 membrane-to-total ratio = MFI of TRPC6 at membrane/total MFI of TRPC6. In this manner, the fluorescence intensity of TRPC6 at plasma membrane was quantitated based on all the cells in a well (usually between 400 to 600 cells) by an unbiased methodology.
Figure 3.5. TRPC6 expression in cell membrane region is affected by synaptopodin levels in podocytes shown by quantitative immunocytochemistry with Opera High Content Screening system. (A) Membrane and cytoplasm regions of individual cells were designated by Columbus software as shown. Synaptopodin (Synpo) intensity significantly decreased in Synpo KD podocytes (B) or increased in Synpo OE podocytes (D). (C) TRPC6 membrane-to-total ratios were increased in Synpo KD, SynpoKD-wtTRPC6, SynpoKD-TRPC6<sup>M131T</sup> podocytes compared their controls. (E) TRPC6 membrane-to-total ratios in Synpo OE, SynpoOE-wtTRPC6, SynpoOE-TRPC6<sup>M131T</sup> decreased compared to their controls. Quantification was based on 4 repeated wells for each condition. GraphPad’s multiple t tests were used for statistical analysis *: p<0.05; **: p<0.01; ****: p<0.0001. Graphs represent mean ± s.e.m.
Synaptopodin intensities confirmed the knockdown and overexpression (Figure 3.5 B, D). TRPC6 membrane-to-total ratios were increased in Synpo KD, SynpoKD-wtTRPC6, SynpoKD-TRPC6^{M131T} podocytes compared with SC, SC-wtTRPC6, SC-TRPC6^{M131T} podocytes (Figure 3.5 C) and decreased in Synpo OE, SynpoOE-wtTRPC6, SynpoOE-TRPC6^{M131T} compared to EV, EV-wtTRPC6, EV-TRPC6^{M131T} podocytes (Figure 3.5 E). Therefore, TRPC6 membrane-to-total ratios were elevated in synaptopodin down-regulated podocytes while decreased in synaptopodin overexpressing podocytes, consistent with the results of surface biotinylation assays.

3.5 Involvement of actin and microtubule in synaptopodin-dependent TRPC6 surface localization

Both actin and microtubules have been shown to extensively participate in membrane protein trafficking to plasma membrane (Alfonso et al., 2010). Our data show that overall expression of TRPC6 protein is not affected by synaptopodin. To explore if trafficking of TRPC6 is affected by synaptopodin downregulation, we investigated how disruption of actin and microtubules would influence TRPC6 membrane-to-total ratios in synaptopodin-depleted podocytes using Opera HCS system. We treated Synpo KD and SC podocytes with cytochalasin D (F-actin disruption agent) (Casella et al., 1981) or nocodazole (microtubule disruption agent) (Vasquez et al., 1997) overnight at concentrations indicated in Figure 3.6 A and B and measured the TRPC6 membrane-to-total ratios with Opera at the end of the treatment. Dose dependent disruption of F-actin or microtubule cytoskeleton was confirmed with phalloidin or tubulin staining by confocal microscopy (Figure 3.6 C and D). TRPC6 membrane-to-total ratios decreased as the concentrations of cytochalasin D and nocodazole increased in both SC and Synpo KD podocytes with a greater decrease
in Synpo KD (Figure 3.6 A and B) and eventually the differences diminished at 800 nM for cytochalasin D and 60 nM for nocodazole. These data suggest that synaptopodin-dependent TRPC6 membrane localization requires both functional actin and microtubule system.

Figure 3.6 Actin and microtubule cytoskeletons are involved in the regulation of TRPC6 surface expression by synaptopodin in podocytes. (A) and (B) TRPC6 membrane-to-total ratios in cytochalasin D or nocodazole treated podocytes decrease as the concentrations of cytochalasin D or nocodazole increase. GraphPad’s multiple t test was performed to compare the ratios between Synpo KD and SC podocytes at the same concentration of the same treatment. **: p<0.01; ***: p<0.001. Graphs represent mean ± s.e.m. (C)-(D) Representative immunocytochemistry images by confocal microscopy show patterns of TRPC6, F-actin and tubulin in cytochalasin D or nocodazole treated podocytes.
Chapter 4

Modification of TRPC6 activity by synaptopodin in podocytes

4.1 Methods and materials

Calcium imaging

Podocytes were grown on 35mm glass-bottomed collagen coated culture dishes (In Vitro Scientific, CA) and were transduced with lentiviruses. For calcium imaging, podocytes were incubated with 1µg of the calcium-sensitive dye Fura-2AM in HBSS (with Ca^{2+}, Mg^{2+}, Life Technologies) for 15 minutes at 37°C. Cells were rinsed and fresh HBSS was added for baseline recording. TRPC6 specific activator Hyp9, a stabilized derivative of hyperforin (Muller et al., 2008) was added 90 seconds after baseline recording at final concentration of 4µM. Fura-2AM absorbs light at 340 and 380nM depending on the binding of free calcium, and emits at 512 nm. As such, the 340/380-nm excitation ratio changes as a function of cytosolic free calcium. Ratiometric imaging was carried out on podocytes (12 to 20 cells per condition) using Attofluor Ratio Vision digital fluorescence microscopy system (Atto Instruments, Inc., MD).

Apoptosis assay

Differentiated mouse podocytes were cultured in collagen-coated 96-well CellCarrier plates and transduced with lentiviruses. Before the apoptosis assay, cells were treated with 4 µM of Hyp9 (Sigma) at 37 °C for 0, 1 or 2 hours. Apoptosis was examined with Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit (Life Technologies). Briefly, cells were gently washed with PBS in the wells then incubated with Annexin V/PI solution for 15 mins at room temperature according to manufacturer’s instructions. Stained cells were then
fixed with 4% PFA at room temperature for 15 mins and stained with CellMask Blue (Invitrogen) to outline the cytoplasm and nuclei for Opera HCS system. Cells that were Annexin V positive and PI negative were counted as early apoptotic cells and labeled as apoptotic cells in this study (Figure 4.3 A). Cells treated with 0, 0.2 mM, 0.5 mM and 1 mM of H2O2 (Sigma) for 1 hour at 37 °C were used as positive control for the assay (Liu et al., 2013) (Figure 4.3 B) and the intensity of the Annexin V and PI staining was used as references to set up cutoffs of Annexin V and PI intensities for Annexin V and PI positive cells.

4.2 TRPC6-mediated calcium influx in synaptopodin knockdown or overexpressing podocytes

We carried out calcium imaging to further investigate whether changes in synaptopodin expression can affect the calcium influx mediated by TRPC6 in podocytes. The average traces of calcium imaging are shown in figure 4.1 A-C and 4.2 A-C. For Synpo KD podocytes, average F340/F380 baselines showed no significant difference among all groups (Figure 4.1 D). The integrated intracellular calcium ([Ca^{2+}]_i) (Figure 4.1 E) as well as the normalized peaks of calcium influx (Figure 4.1 F) were greater in SynpoKD-wtTRPC6, SynpoKD-TRPC6M131T podocytes compared with their controls SC-wtTRPC6, SC-TRPC6M131T, with a greater difference between SynpoKD-TRPC6M131T and SC-TRPC6M131T than SynpoKD-wtTRPC6 and SC-wtTRPC6 (Figure 4.1 E, F). However, in wt podocytes, the normalized peak and integrated [Ca^{2+}]_i were only slightly higher in Synpo KD than SC cells but not statistically significant, possibly because the changes occurred to the very low levels of endogenous surface TRPC6 might not be enough to cause a significant difference in intracellular calcium. In contrast, the integrated intracellular
calcium significantly decreased in SynpoOE-wtTRPC6 and SynpoOE-TRPC6\textsuperscript{M131T} cells compared to their EV controls (Figure 4.2 E) except wt podocytes. The normalized peaks as well as baselines showed decreased trends in all the SynpoOE, SynpoOE-wtTRPC6, and SynpoOE-TRPC6\textsuperscript{M131T} podocytes compared with their controls, but not statistically significant (Figure 4.2 F). Under normal condition, podocytes contain adequate levels of synaptopodin for normal function and, therefore, the effect of excessive synaptopodin is limited, resulting in insignificant difference in baseline levels or normalized peaks. Note that [Ca\textsuperscript{2+}]\textsubscript{i} remained at high levels showing little trend of decreasing long after Hyp9 activation in podocytes expressing TRPC6\textsuperscript{M131T} (Figure 4.1 C and 4.2 C), in contrast to the apparent decreasing trends of [Ca\textsuperscript{2+}]\textsubscript{i} in wt podocytes and podocytes overexpressing wtTRPC6 (Figure 4.1 A, B and 4.2 A, B). This observation is consistent with the published study where TRPC6\textsuperscript{M131T} displayed increased calcium influx and delayed channel inactivation (Heeringa et al., 2009).

These calcium imaging results were consistent with the changes in synaptopodin-dependent protein expression of podocyte surface TRPC6 demonstrated previously. Together, the effects of synaptopodin levels on TRPC6 cell surface levels resulted in modification of TRPC6 activity.
Figure 4.1. TRPC6-mediated calcium influx is increased in synaptopodin down-regulated podocytes. Calcium influx is demonstrated by Fura-2AM calcium imaging and is represented by 340nm/380nm ratios in SC and Synpo KD podocytes (A), SC-wtTRPC6 and SynpoKD-wtTRPC6 podocytes (B), SC- TRPC6^{M131T} and SynpoKD-TRPC6^{M131T} podocytes (C). (D) Baselines of 340nm/380nm ratios (average of the ratios before adding Hyp9) (E) Quantification of 340nm/380nm ratio changes from 90s (adding hyp9) to 400s. \( \int (F340/F380) dt \) integrated 340nm/380nm ratio during 90s and 400s (F) peak values of 340nm/380nm ratio normalized to the according baselines. Two-way ANOVA was performed in D. GraphPad’s multiple t tests were used for E and F. *: p<0.05; ***: p<0.001; graphs represent mean ± s.e.m
Figure 4.2. TRPC6-mediated calcium influx is altered by synaptopodin in podocytes with overexpressed synaptopodin. Calcium influx is demonstrated by Fura-2AM calcium imaging and is represented by 340nm to 380nm ratios in (A) EV and Synpo OE podocytes, (B) EV- wtTRPC6 and SynpoOE-wtTRPC6 podocytes, EV-TRPC6M131T and (C) SynpoOE-TRPC6M131T podocytes. (D) Baselines of 340nm/380nm ratios (average of the ratios before adding Hyp9) showed no significant differences. (E) Quantification of 340nm/380nm ratio changes from 90s (the time point of adding hyp9) to 400s. \( \int (F340/F380) \, dt \) integrated 340nm/380nm ratio during 90s and 400s. (F) Peak values of 340nm/380nm ratio normalized to the according baselines showed no significant differences. Two-way ANOVA was used in D. GraphPad’s multiple t tests was performed in E and F. **: p<0.01 ***: p<0.001; graphs represent mean ± s.e.m
4.3 Induction of apoptosis in TRPC6 overexpressing podocytes with synaptopodin depletion

To investigate the functional consequence of the increased TRPC6 activity in synaptopodin knockdown podocytes, we performed Annexin V/PI apoptosis assay using Opera HCS system. Figure 4.3A shows the representative apoptotic cells (Annexin V positive and PI negative) in podocytes transduced with SynpoKD-TRPC6\textsuperscript{M131T} lentiviruses in contrast to non-apoptotic untreated cells (Annexin V negative and PI negative).

No significant difference was found in cells without TRPC6 activator Hyp9 treatment (Figure 4.3 C). Apoptosis was and was only induced in SynpoKD-TRPC6\textsuperscript{M131T} podocytes with 1 hour of Hyp9 treatment (1.72±0.09 % vs 5.71± 1.22%) (Figure 4.3D). When Hyp9 was used for 2 hours, SynpoKD-TRPC6\textsuperscript{M131T} podocytes had a significantly increased percentage of apoptotic cells (9.82±1.92%) compared to SC-TRPC6\textsuperscript{M131T} cells (3.89±0.76%) (p < 0.05) and all other conditions (Figure 4.3E). Increased apoptosis was also observed in SynpoKD-wtTRPC6 podocytes (3.02±0.18%) compared with SC-wtTRPC6 cells (1.32±0.44%) (p < 0.05) (Figure 4.3E).

These results suggest that the elevated intracellular calcium in Synpo KD podocytes with TRPC6 overexpression cause damage to podocytes that leads to apoptosis upon channel activation, with TRPC6\textsuperscript{M131T} causing more severe injury than wtTRPC6.
Figure 4.3. Apoptosis is induced in Synpo KD podocytes with TRPC6 overexpression. (A) Representative Opera confocal images of Annexin V/PI stained untreated podocytes showing negative Annexin V and PI staining, and Hyp9 (4 µM for 2 hours) treated SynpoKD-TRPC6M131T podocytes showing positive Annexin V and negative PI staining (cells were also stained with CellMask Blue to outline cytoplasm and nuclei). (B) Podocytes treated with H2O2 were used as positive control for Annexin V/PI staining. (C)-(E) Percentages of early apoptotic cells (Annexin V positive/PI negative) in SC, Synpo KD, SC-wtTRPC6, SynpoKD-wtTRPC6, SC-TRPC6M131T and SynpoKD-TRPC6M131T podocytes with no Hyp9 treatment (C), treated with 4 µM for 1 hour(D), treated with 4 µM Hyp9 for 2 hours(E). Quantification was based on 3 repeated wells for each condition. GraphPad’s multiple t tests *: p<0.05; graphs represent mean ± s.e.m.
Chapter 5

Regulation of TRPC6 podocyte surface expression by synaptopodin \textit{in vivo}

5.1 Methods and materials

Animal experiment

All animal studies were approved by the Rush University Medical Center Animal Institute Committee. 12-15 week-old female C57BL/6J mice from Jackson Laboratory (Bar Harbor, ME) were used as wild-type (WT) mice. TRPC6\textsuperscript{-/-} mice (Dietrich et al., 2005)(originally with 129Sv/J : C57BL/6J = 1:1 background) were backcrossed with wt C57BL/6J mice for more than 8 generations to obtain a pure C57BL/6J background. 12-14 week-old female TRPC6\textsuperscript{-/-} mice were used in this study.

\textit{In vivo} synaptopodin knockdown experiment

The siRNAs used in \textit{in vivo} synaptopodin knockdown were based on the two shRNA sequences used \textit{in vitro} studies. siSTABLE chemically modified siRNAs targeting synaptopodin mRNA and non-target control siRNA were manufactured by Dharmacon (Lafayette, CO). 120 \text{ug/mouse} of siRNAs mixed with Kidney \textit{In Vivo} Transfection reagents (Altogen Biosystems, NV) were injected into wild-type mice (4 to 5 mice per group) through tail vein. A secondary injection was performed 12 to 14 hours after the first injection. Urine and kidneys were collected 24-26 hours after the secondary injection. Amount of siRNA used in the injections were determined by a pilot experiment which involved test injections of a series of different doses of the synpo siRNAs and the analysis of synaptopodin levels in the kidneys.
LPS and cyclosporine A injection experiment

Wild-type and TRPC6−/− mice were divided into 3 groups for each genotype (7 to 9 mice per group for WT, 6-7 for TRPC6−/−). Group 1 (control group) were subcutaneously injected with olive oil (Sigma) 100uL/day for 4 consecutive days as control for cyclosporine A (CsA) and on day 3, were intraperitoneally injected with 300uL saline (G-biosciences, MO) as control for LPS. Group 2 (experimental group with LPS) were subcutaneously injected with olive oil 100uL/day for 4 consecutive days, then on day 3, were intraperitoneally injected with ultrapure LPS (Invivogen, CA) 7mg/kg in 300 uL saline. Group 3 (experimental group with LPS and CsA) were subcutaneously injected CsA dissolved in 100uL olive oil 15mg/kg/day for 4 consecutive days, then on day 3, were intraperitoneally injected one dose of ultrapure LPS 7mg/kg in 300uL saline. 16 hours after LPS injection, 500uL saline was injected to all mice to prevent hypovolemia. 36 hours after LPS injection, urine samples were collected (Figure 5.3 A). Urinary albumin levels were determined by mouse albumin ELISA kit (Bethyl Laboratories, TX). Urinary creatinine levels were measured by Creatinine Assay Kit (Cayman Chemical Company, MI).

Glomerular isolation and podocyte enrichment

The method used in this study was adapted from glomerular and podocyte preparation method described by Boerries et al.(Boerries et al., 2013). Briefly, kidneys were harvested from control and treated mice and minced into 1-mm³ pieces. Rinsed kidney pieces were incubated in digestion buffer which was made of DMEM containing collagenase 300 U/ml (Worthington, Collagenase type IV, NJ) and DNase I 50 U/mL (New England Biolabs) at 37°C for 20 mins with gentle shaking. The digested kidneys were sieved twice through two 100-µm cell strainers to block the undigested tissues. Rinsed flow through was added onto
a 40-µm cell strainer (glomeruli remained on the mesh). The mesh was washed extensively with DMEM. The glomeruli (on the mesh) were then suspended with DMEM and removed from the mesh (Figure 5.1 A). Biotinylation of the isolated glomeruli was carried out as described previously in the cell surface biotinylation section. The biotinylated glomeruli were further digested into single cells with digestion buffer for 45 mins at 37°C with vigorous shaking. Single cell suspension was rinsed with DMEM then added to antibody coated dishes. Cells were allowed to attach at 4°C for 4 hours. The unattached cells were gently washed off and the cells remaining on the dish were processed for isolation of the biotinylated proteins (Figure 5.1 B).

5.2 Alteration of In vivo podocyte surface TRPC6 expression by synaptopodin knockdown

To verify our findings in vivo, we examined the cell surface TRPC6 expression levels in podocytes in mice in response to decreased synaptopodin. In order to measure protein expression exclusively in podocytes, we developed a method to isolate podocytes from mice by enriching them from a pool of glomerular cells (Figure 5.1 A and B).

In vivo knockdown of synaptopodin was achieved by intravenously injecting synpo siRNAs in mice (Figure 5.2 A, B, C). The siRNAs were chemically modified to significantly increase the stability against nuclease activity (siSTABLE siRNAs by Dharmaco). The siRNAs targeting synaptopodin mRNA and non-targeting siRNA (negative control) were used for injections. We enriched podocytes from the glomeruli isolated from the injected mice as described above and performed surface biotinylation.
Figure 5.1. *In vivo* podocytes are enriched by immunopanning. (A) Purified glomeruli after enzyme digestion of the kidneys and purification of the digested tissue with two sizes of cell strainers as described in methods. (B) The enrichment of podocytes from single cell preparation of the isolated glomeruli was done as described in methods. Nephrin (antibody from Fitzgerald, MA was used), VE-cadherin, PDGF-β, CD13 were probed as specific markers for podocytes, endothelial cells, mesangial cells and proximal tubular cells respectively.

1. Single cells digested from glomeruli (obtained by enzyme digestion) before immunopanning
2. Cells collected after immunopanning with podocalyxin antibody
3. Cells collected after immunopanning with nephrin (extracellular) (sigma-aldrich) and podocalyxin antibodies.
4. Single cells digested from glomeruli preparation by sequential sieving.

High purity of podocytes was obtained by immunopanning with both nephrin and podocalyxin antibodies. Nephrin and podocalyxin double immunopanning was then used to enrich podocytes for *in vivo* experiments in this study.

In *synpo* siRNAs injected mice, synaptopodin mRNA levels in total cell lysates decreased by approximately 62% determined by real-time PCR and protein levels decreased by approximately 54% in podocytes determined by western blotting (Figure 5.2 A B, C). An approximately 1.6-fold increase of surface TRPC6 was detected in these cells with no significant change in total expression (Figure 5.2 A, D). No proteinuria was detected in the Synpo KD mice (Figure 5.2 E), suggesting 54% down-regulation of synaptopodin is not sufficient to cause proteinuria.
Figure 5.2. *In vivo* knockdown of synaptopodin leads to increased podocyte surface TRPC6. (A) Representative western blots showing TRPC6 and synaptopodin in total and surface fraction of enriched podocytes from non-targeting siRNA and synpo siRNAs injected mice. pan-Cadherin was used as loading control. HSP90 was used to indicate the purity of surface fraction. Synaptopodin was seen in the surface fraction possibly due to its interaction with TRPC6 and other proteins on the plasma membrane such as podocin and nephrin. (B) Synpo mRNA levels were measured by real-time PCR. (C) Synaptopodin expression was normalized to GAPDH in total cell lysates. (D) TRPC6 levels were normalized to pan-Cadherin and fold changes were calculated by comparing TRPC6 level of synpo siRNAs mice to that of non-targeting mice in the same fraction. The in vivo knockdown experiment (4-5 mice in each group) was performed three times for quantification of the protein levels. Student’s t-test and Graphpad’s multiple t-test were performed in (B) and (C). (E) *: p<0.05; **: p<0.01; Graphs represent mean ± s.e.m.
5.3 TRPC6 podocyte surface expression in wild-type mice treated with LPS or LPS/CsA

We further tested our hypothesis in LPS mouse model. In this model, synaptopodin was shown to be down-regulated upon LPS treatment and can be rescued by CsA (Faul et al., 2008). We hypothesized that cyclosporine A (CsA) protects podocytes from LPS injury partially by reducing TRPC6 membrane expression through preservation of synaptopodin in podocytes.

LPS induced albuminuria in WT mice (81.6 ± 5.0 mg/g vs 2108.7 ± 219.2 mg/g, p < 0.001, figure 5.3 C). The LPS-induced proteinuria was significantly reduced by CsA co-treatment (2108.7 ± 219.2 mg/g vs 668.5 ± 253.2 mg/g, approximately 68.3% decrease, p < 0.01, figure 5.3 C). In TRPC6−/− mice, LPS proteinuria was approximately 45% less than that in WT mice (1161.3 ± 271.1 mg/g vs 2108.7 ± 219.2 mg/g, p<0.05, figure 5.3 C) suggesting that TRPC6 contributes to LPS-induced proteinuria. CsA treatment in TRPC6−/− mice was also able to reduce LPS-induced proteinuria but to a lesser extent (654.4 ± 247.6 mg/g vs 1161.3 ± 271.1 mg/g, approximately 43.6% decrease, p > 0.05) compared to its effect in WT mice (Figure 5.3 C).

To further analyze the role of TRPC6 in CsA treatment, we enriched podocytes from control, LPS and LPS+CsA treated WT mice and performed surface biotinylation assay. Total synaptopodin protein level was decreased to approximately 54% of the control synaptopodin expression level upon LPS treatment and was restored to about 79% with CsA co-treatment (Figure 5.3 E, F). Accordingly, surface TRPC6 was elevated by approximately 1.7 fold upon LPS treatment and was lowered to 1.2-fold by CsA in WT mice (Figure 5.3 E, G). Together with the in vitro data, these observations suggested that
CsA protects podocytes from LPS treatment in mice partially by lowering podocyte surface TRPC6 levels through stabilization of synaptopodin.

**Figure 5.3** (continued) Albumin and creatinine levels were measured from urine samples taken 36 hours after LPS injection. Control: mice injected with saline and olive oil; LPS: mice injected with LPS and olive oil; LPS+CsA: mice injected with LPS and CsA. Two samples with abnormally high albumin levels (over 1000μg/mL) were disregarded. 6 to 9 mice were used in each group. Ordinary one-way ANOVA was performed to analyze proteinuria within WT or TRPC6 mice; multiple t tests were performed to compare proteinuria between WT and TRPC6−/− mice. (D) Representative urine samples (1 μl/lane) from the indicated mice were run on a NuPAGE 4-12% Bis-Tris gel and stained with GelCode Blue Stain (Thermo Scientific) (E) Representative western blots showing TRPC6 and synaptopodin in total and surface fraction of isolated podocytes from LPS or LPS+CsA treated mice. (F) Synaptopodin expression was normalized to GAPDH in total cell lysates. (G) TRPC6 levels were normalized to pan-Cadherin and fold changes were calculated by comparing TRPC6 levels of LPS and LPS+CsA to that of the control in the same fraction. The LPS/CsA treatment (3 mice in each group) for the surface biotinylation assays was performed three times for quantification of the protein levels. Ordinary one-way ANOVA was performed in (F) and (G). GraphPad’s multiple t tests were performed in (A). Two-way ANOVA was performed in (B): *: p<0.05; **: p<0.01; ***: p<0.001 graphs respresent mean± s.e.m.
Figure 5.3 Cyclosporine A reduces podocyte surface TRPC6 of LPS treated mice. (A) urinary albumin (mg) to creatinine (g) ratios in saline and LPS injected mice at 24h, 36h, 48h, 72h and 96h after injection showing highest average level at 24h with large standard errors in both saline and LPS groups. 36h was selected as the time point for urine analysis in the study for higher consistency (smaller standard errors). 4 mice were used in each group. (B) Urinary Albumin (mg) to creatinine (g) ratios in cyclosporine A treated wild-type and TRPC6<sup>−/−</sup> mice compared to olive oil controls. No significant differences were found. (C) Urinary albumin (mg) to creatinine (g) ratios in WT and TRPC6<sup>−/−</sup> mice with LPS and/or cyclosporine A (CsA) treatment. (Continued on previous page)
Chapter 6

Discussion and conclusion

Gain-of-function variants of TRPC6 have been identified as a cause of hereditary FSGS (Gigante et al., 2011; Reiser et al., 2005; Winn et al., 2005) Upregulation of TRPC6 was found in several forms of acquired proteinuric diseases (Heeringa et al., 2009; Moller et al., 2007). Synaptopodin, a key regulator of actin cytoskeleton homeostasis in podocytes, was down-regulated in various human and rodent glomerular diseases (Barisoni et al., 1999) (Srivastava et al., 2001) (Faul et al., 2008) (Tian et al., 2010) (Marko et al., 2012). Our study provides evidence that expression of TRPC6 on cell membrane and calcium entry via TRPC6 in podocytes are affected by synaptopodin. We first demonstrated that TRPC6 and synaptopodin interact and significantly associate with each other in podocyte foot processes (Figure 2.1, 2.2). In differentiated mouse podocytes, knocking down synaptopodin results in increased surface expression of TRPC6 accompanied by enhanced TRPC6-mediated calcium influx. Apoptosis is induced in synaptopodin depleted podocytes with TRPC6 overexpression. On the contrary, overexpression of synaptopodin leads to decreased podocyte surface TRPC6 and calcium influx. These findings suggest that synaptopodin limits podocyte surface TRPC6 expression and the interaction of these two proteins is likely to be involved in the mechanism.

6.1 Indirect interaction between TRPC6 and synaptopodin

We showed that TRPC6 and synaptopodin co-immunoprecipitated together in HEK293 and podocytes (Figure 2.1 A, B and C). However, our experiment also showed no direct interaction between TRPC6 and synaptopodin (Figure 2.1 E). These data suggest possible
existence of mediators that contribute to the interaction between the two proteins and the regulation of cell surface TRPC6 by synaptopodin. Recent publications have provided supportive evidence:

Compared to other members of the TRPC family channels which traffic upon cellular stimulation (Cayouette et al., 2004; Cheng et al., 2011; Odell et al., 2005), relatively less is known about protein interactions with TRPC6 and its trafficking and activity. It has been suggested that interaction of TRPC6 with nephrin near the slit diaphragm underlies regulation of gating and trafficking of the channel (lack evidence in podocytes) (Kanda et al., 2011). Nephrin has been shown to be tightly associated with tight junction protein MAGI-1 which serves as a scaffolding protein in the slit diaphragm (Hirabayashi et al., 2005). Interestingly, MAGI-1 was also shown to interact with synaptopodin as well as another important actin binding protein α-actinin4, suggesting a role in regulation of actin cytoskeleton (potential connection to the involvement of actin in TRPC6 trafficking shown in Figure 3.6) (Patrie et al., 2002). In addition, BK$_{Ca}$ channel, a potassium channel that is activated by voltage and calcium is physically and functionally coupled with TRPC6 in podocytes (Kim et al., 2010). BK$_{Ca}$ also interacts with nephrin, MAGI-1 and synaptopodin (Dryer and Reiser, 2010). As demonstrated in our study, TRPC6 and synaptopodin interact in podocyte foot processes, and synaptopodin affects TRPC6 surface expression and calcium influx. Together, these data suggest a complicated network that may play a role in regulating TRPC6 localization and function in podocytes. It requires a large and self-standing study to decipher the mechanism and functional consequences of these interactions.
6.2 Differential regulation of wild-type and mutated TRPC6 by synaptopodin

Most of the TRPC6 mutations associated with FSGS exhibit detectable increase in channel activity compared with wild-type channels (Gigante et al., 2011; Heeringa et al., 2009; Reiser et al., 2005; Riehle et al., 2016; Winn et al., 2005; Zhu et al., 2009). The mechanism that how these mutants cause glomerular abnormalities remains unclear. Schlondorff et al showed elevated NFAT activity in podocytes by overexpressing wt or FSGS-associated TRPC6 mutants, suggesting that calcineurin-NFAT pathway as a mediator of FSGS. (Schlondorff et al., 2009). Kanda et al demonstrated in HEK 293T cells that nephrin suppresses the surface expression and activity of wild-type TRPC6 but not the FSGS-associated mutants, suggesting that the lack of regulation of the disease-related channels' surface expression played a role in disease formation (Kanda et al., 2011). We included in our in vitro study the mouse mutant TRPC6^{M131T} which corresponds to the human childhood FSGS-causing mutant TRPC6^{M132T} that gives rise to significantly higher currents and delayed inactivation compared to wild-type TRPC6 channel (Heeringa et al., 2009). We observed by surface biotinylation assays that synaptopodin was able to modify surface expression of the wild-type and the mutant TRPC6 to different degrees (Figure 3.3 and 3.4). Specifically, in synaptopodin down-regulated podocytes, surface wtTRPC6 was increased by approximately 2.5 fold while TRPC6^{M131T} by approximately 1.4 fold. In synaptopodin overexpressing podocytes, surface wtTRPC6 was reduced by 41.3% whereas TRPC6^{M131T} by 23.2%. These findings indicate that the same manipulation of synaptopodin expression results in less modification of surface expression of the mutant, suggesting that the mutation may alter the ability for synaptopodin to regulate the localization and function of the channel.
However, quantification of the calcium imaging data revealed that calcium influx invoked in SynpoKD-TRPC6\textsuperscript{M131T} was significantly higher (both peak values and integrated intracellular calcium) than that in all other podocytes including SynpoKD-wtTRPC6 (Figure 4.1 E, F). In addition, our apoptosis assay showed that SynpoKD-TRPC\textsuperscript{M131T} podocytes had the highest percentage of apoptotic cells upon TRPC6 activation compared to all other conditions (Figure 4.3 E). Although surface level of TRPC6\textsuperscript{M131T} was less affected by synaptopodin than that of wtTRPC6, the changes in intracellular calcium concentrations and the damage caused to the cells were more significant with TRPC6\textsuperscript{M131T} than with wtTRPC6. These observations suggest that when synaptopodin is down-regulated (as often observed in glomerular damage), the mutant TRPC6\textsuperscript{M131T} is able to cause severe podocyte damage due to abnormally high intracellular Ca\textsuperscript{2+}. Physiologically or pathologically present TRPC6 activators such as Angiotensin II (Ilatovskaya et al., 2014) may trigger podocyte injury or induce podocyte apoptosis through this mechanism. It is noteworthy that SynpoKD-wtTRPC6 podocytes also showed significantly higher calcium influx (Figure 4.1 E, F) and more apoptosis (Figure 4.3E) than its control SC-wtTRPC6 podocytes, suggesting that synaptopodin down-regulation in wtTRPC6 overexpressing podocytes is still detrimental to the cells although it is less significant than TRPC6\textsuperscript{M131T}.

It is of great importance to study the mechanism underlying the differential regulation of wild-type and the mutant channels by synaptopodin. Most of the known FSGS-associated gain-of-function mutations are point mutations located on the intracellular N- or C- terminus including TRPC6\textsuperscript{M131T} (Dryer and Reiser, 2010), rather than the pore region that’s directly involved in ion conduction. For future studies, it would be worth
investigating whether these mutations alter the interaction between TRPC6 and other proteins that affect its trafficking. The mutations might also alter or modify the downstream signaling pathways that result in changes in TRPC6 activity and podocyte function.

6.3 Downstream effectors of TRPC6-mediated calcium influx

This study did not address what are the downstream effectors of synaptopodin regulated calcium influx mediated by TRPC6. We tested one of the candidates CaMKII which has been shown to be activated in complement treated podocytes (Kistler et al., 2013). We blotted the active form of CaMKII (pThr\textsuperscript{286}-CaMKII) in Synpo KD podocytes and their controls with or without TRPC6 activator Hyp9 at 0, 15 mins, 30 mins and 60 mins. However, we were unable to detect any pThr\textsuperscript{286}-CaMKII, indicating CaMKII was not activated under these conditions. For positive control of CaMKII activity, we used podocytes treated with 3% complement (Kistler et al., 2013).

Our experiment showed that apoptosis was induced in TRPC6 overexpressing podocyte with synaptopodin depletion (Figure 4.3). However, during this process CaMKII is not activated suggesting this process is not mediated by CaMKII.

Calcineurin-NFAT pathway was shown to be involved in podocyte dysfunction caused by Angiotensin II or Adriamycin-induced elevation of TRPC6 expression (Nijenhuis et al., 2011). Another group demonstrated that FSGS-associated TRPC6 mutants caused enhanced NFAT activity mainly using HEK-293T cells that overexpresses the M1 acetylcholine receptor (Schlondorff et al., 2009). It is worth studying whether NFAT pathway also plays a role in podocyte damage/apoptosis caused by TRPC6 overexpression in synaptopodin depleted podocytes. Chen et al demonstrated that under the condition of
albumin overload, TRPC6 is able to induce ER stress and apoptosis (Chen et al., 2011). ER stress could be considered as one of the candidate mechanisms that lead to podocyte apoptosis under disease condition (albumin overload and TRPC6 hyper-activity) for further study.

6.4 The role of actin and microtubules in synaptopodin-dependent TRPC6 surface expression.

Our study also provided evidence that regulation of podocyte surface TRPC6 levels depends on both microtubules and actin filaments since disruption of both cytoskeleton structures resulted in abortion of the elevated surface TRPC6 levels in SynpoKD podocytes (Figure 3.6). It has been well accepted that microtubules and actin filaments are major components of protein trafficking in a cell (Alfonso et al., 2010). Long range transport between organelles is generally mediated by microtubule motors. In contrast, actin motors myosin act in short range movement around organelles and especially through the cortical actin network at the plasma membrane (Alfonso et al., 2010). Cortical actin filaments have been reported to be more resistant to disruption by cytochalasin D (Lamaze et al., 1997), providing a possible explanation for why relatively high concentration of this drug was required to cancel the difference of surface TRPC6 levels between SC and SynpoKD podocytes (Figure 3.4). Considering the association shown in Figure 2.1, 2.2 and the discussion earlier, we postulate that TRPC6 and synaptopodin may form a protein complex (with possible mediators) in the cytoplasm under physiological conditions. When synaptopodin is down-regulated, TRPC6 dissociates and transports to the plasma membrane by a cytoskeleton mediated mechanism. Thus when this cytoskeleton structure is disrupted, less TRPC6 can be transported to the membrane yielding a lower membrane-
to-total ratio. As for scrambled podocytes, TRPC6 membrane-to-total ratio also generally followed a decreasing trend as the concentration of the drugs increased, but slower than in Synpo KD podocytes (Figure 3.6 A, B), possibly due to disruption of normal transport of TRPC6 to the membrane upon cytochalasin D and nocodazole treatment, causing cytoplasmic retention of the channel (Figure 3.6 C, D).

It is noteworthy that in 800 nM cytochalasin D treated podocytes TRPC6 staining presented an aster-like aggregated pattern corresponding to the pattern of actin staining. In 100 nM nocodazole treated podocytes, TRPC6 became unevenly distributed and appeared more concentrated in cytoplasm. These observations suggest that actin and microtubules differentially regulate TRPC6 trafficking.

An alternative mechanism is proposed based on the findings that synaptopodin stabilizes RhoA which is an important regulator of cytoskeleton (Asanuma et al., 2006; Zaoui et al., 2008). Synaptopodin may play a role in signaling pathways that regulates actin and microtubule dynamics and/or membrane protein trafficking that affect TRPC6 localization.

6.5 In vivo models to study TRPC6 podocyte surface expression regulated by synaptopodin

In order to verify our findings in vivo, the most suitable and straightforward experiment would be to examine the podocyte surface TRPC6 levels in synpo−/− mice compared to their littermates. However, the only synpo−/− mouse that has been published (Asanuma et al., 2005; Deller et al., 2003) might not be the ideal model for our study. In this synpo−/− mouse, an isoform of synaptopodin, SynpoT, is induced which is able to partially compensate for the loss of full-length synaptopodin (Asanuma et al., 2005; Huber et al., 2006). There has
been no report that SynpoT protein exists in humans under any physiological or pathological conditions. Using this mouse model would obscure the identification of the role of synaptopodin in regulating podocyte surface TRPC6.

In order to directly show the causal relationship between decrease of synaptopodin and increase of surface TRPC6 \textit{in vivo}, we performed \textit{in vivo} knockdown of synaptopodin by intravenously injecting \textit{synpo} siRNAs (targeting full-length \textit{synpo} and \textit{synpoT} mRNAs) in mice (Figure 5.2 A, B, C) and examined surface TRPC6 levels of the enriched podocytes. We found podocyte surface TRPC6 increased by approximately 1.6 fold (p<0.05) while the overall TRPC6 remained unchanged (Figure 5.2 D). Therefore, we provided direct evidence that down-regulation of synaptopodin in podocytes causes increased surface TRPC6.

In another mouse model, we reproduced the previously published finding (Faul et al., 2008) that rescue of synaptopodin by cyclosporine A partially protected WT mice from LPS-induced proteinuria (Figure 5.3 C). It was suggested by Faul \textit{et al} (Faul et al., 2008) that the protection was partially due to preservation of podocyte actin cytoskeleton through stabilization of synaptopodin. We further showed that preserving synaptopodin by CsA is beneficial by maintaining podocyte surface TRPC6 levels low during glomerular damage. LPS caused a lower level of proteinuria in TRPC6$^{-/-}$ mice and the protective effect of CsA was of a lesser magnitude in TRPC6$^{-/-}$ mice compared to WT mice (Figure 5.3 C). These observations indicate that TRPC6 contributes to the induction of albuminuria resulting from LPS-induced glomerular damage, and furthermore, TRPC6 is involved in the protective effect of CsA. This was supported by our surface biotinylation results of the \textit{in
vivo podocytes showing reduced TRPC6 surface expression when co-treated with CsA (Figure 5.3 E, G).

6.6 Proposed model for regulation of podocyte surface TRPC6 by synaptopodin

Based on the experimental results, we proposed a model for regulation of TRPC6 podocyte surface expression by synaptopodin (Figure 6.1). In the plasma membrane cortex region in wild-type podocytes, TRPC6 and synaptopodin, together with other participating proteins, form a putative protein complex that is associated with the cortical actin cytoskeleton through synaptopodin. When synaptopodin is knocked down, the interaction inside the protein complex no longer exists. TRPC6 that used to be bound in these protein complexes can now be trafficked to the cell membrane resulting in increased surface expression levels. Note that there is synaptopodin-TRPC6 protein complexes remained in the cytoplasm due to incomplete knockdown of synaptopodin.

![Proposed model for regulation of TRPC6 cell membrane localization by synaptopodin](image)

**Figure 6.1.** Proposed model for regulation of TRPC6 cell membrane localization by synaptopodin. TRPC6 and synaptopodin form a putative protein complex that is associated with cortical actin, preventing TRPC6 from expressing on the plasma membrane. Under the condition of synaptopodin knockdown, the number of the protein complexes decreases and TRPC6 is no longer bound in the cytoplasm. Therefore, this part of TRPC6 can be trafficked to the plasma membrane.

6.7 Conclusion
In summary, our study provides \textit{in vitro} and \textit{in vivo} evidence that podocyte surface expression levels of TRPC6 is limited by synaptopodin, demonstrating a new role for this actin-binding protein in protecting podocyte function. Loss of synaptopodin leads to elevated calcium influx mediated by TRPC6 and increased apoptosis in podocytes. Synaptopodin requires functional actin and microtubule cytoskeletons for its regulation of podocyte surface TRPC6. Cyclosporine A, a commonly used drug for FSGS and other nephrotic syndrome, protects podocytes from LPS-induced damage partially by maintaining podocyte surface TRPC6 levels. Limiting TRPC6 surface levels and thereby lowering its activity could provide a new approach for the promotion of glomerular health.
REFERENCE


