Characterization of signaling pathways regulating nephrin endocytosis in kidney podocytes: novel roles for Nck and ShcA adaptor proteins

by

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ABSTRACT

CHARACTERIZATION OF SIGNALING PATHWAYS REGULATING NEPHRIN ENDOCYTOSIS IN KIDNEY PODOCYTES: NOVEL ROLES FOR NCK AND SHCA ADAPTOR PROTEINS

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Kidney podocytes maintain blood filtration selectivity through a network of actin-based projections termed foot processes. The slit diaphragm is a molecular barrier that lies laterally between foot processes and acts as a discerning filtration pore. Nephrin is a key component of this barrier and disrupted nephrin trafficking is suggested to be a source of filtration breakdown, although evidence of this within the complexity of the body remains incomplete. Further, although nephrin’s tyrosine phosphorylation is implicated in its trafficking, the binding partner(s) that facilitate these phospho-dependent mechanisms remain unidentified.

Using several acute injury mouse models, we first demonstrate that nephrin tyrosine phosphorylation is commonly disrupted in the injured podocyte and that this disturbs connections between nephrin and Nck, a cytoskeletal adaptor. Targeted genetic disruption of nephrin/Nck interactions further exacerbates disease in mice, verifying a crucial role for nephrin/Nck signaling in withstanding insult.

We next characterized a fundamental role for Nck in mediating recruitment of actin and the endocytic scission engine dynamin in late stages of nephrin endocytosis, which required nephrin’s tyrosine phosphorylation. Disruption of nephrin/Nck binding in podocytes in vivo and in cell models led to accumulation of nephrin in endocytic pits on the cell surface and this accompanied progressive barrier demise in mice. Interestingly, aberrant activation of this mechanism could also initiate disease, highlighting a requirement for tight regulation of this apparatus for barrier maintenance.
Our final investigations revealed ShcA as a novel nephrin phosphotyrosine binding partner and modulator of nephrin trafficking. ShcA is normally expressed in low levels within podocytes and we identified a stark upregulation of ShcA in a rat model of kidney disease, concurrent with internalization of nephrin and barrier breakdown. In cell–based studies, we demonstrated that ShcA overexpression promotes phospho-mediated nephrin internalization. Upregulated ShcA gene and protein expression were also observed in several human kidney diseases, supporting the clinical relevance of this signaling pathway.

Collectively, this work has identified Nck and ShcA as two novel mediators of phospho-dependent nephrin endocytosis. Integration of these pathways into the larger framework of nephrin trafficking remains an important objective for future work.
DEDICATION

This work is dedicated to my children, Caleb, Lila and Rylan, whose patience is unparalleled, whose support is unwavering and whose zest for life is infectious

and

to my parents, Andrea and Barry, who taught me that, if you do what you love, you will never need to work a day in your life.

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**AUTHOR CONTRIBUTIONS**
CHAPTER 1


CHAPTER 3


Parts of Figure 3.4 were adapted from the following article also published in 2016: Keyvani Chahi A, Martin CE, Jones N (2016). Nephrin Suppresses Hippo Signaling through the Adaptor Proteins Nck and WTIP. J Biol Chem. 291(24):12799-808.

New LA designed and completed much of the data presented in Figures 3.1 and 3.2, along with contributions from Martin CE, Scott RP and Platt MJ. Martin CE designed and conducted the experiments presented in Figures 3.3 and 3.4.

CHAPTER 4


Martin CE designed and conducted several experiments throughout including the components presented in Figures 4.1A-D, 4.3B-C, 4.4 and 4.5A-D. Martin CE prepared graphs, figures and tables, performed statistical analyses, and wrote and edited the manuscript.

SEMs in Figure 4.1 and 4.6 were performed by New LA, as were proteinuria quantifications in Figure 4.6. Martin CE aided in the collection of urine throughout the timecourse of these experiments. Immunofluorescence experiments were performed by Blasutig IM (Figures 4.1E-F, 4.2, Supplementary Figure 3.1) and McNeilly R (Figure 4.3A). SR-SIMs (Figure 5E) were
performed by Aoudjit L and analyzed and compiled by Martin CE. Co-precipitation experiments in Figure 4.4 were performed by Mitro A and Martin CE. All biotinylation experiments and analysis (Figures 4.1A-D, Figures 4.3B-C, Figures 4.4 D-E, Figure 4.5 B-C) were performed by Martin CE, with the exception of one replicate provided kindly by Keyvani Chahi A.

CHAPTER 5


Martin CE designed and conducted most experiments throughout including Figures 5.1A, 5.1D, 5.2A, 5.2D, 5.3 and 5.4 in entirety, 5.5B-C, and all supplemental tables and figures. Martin CE prepared graphs, figures and tables, performed statistical analyses, and wrote and edited the manuscript.

Figures 5.1B-C, 5.1E and Figure 5.2C were designed, carried out and prepared by Petersen KA. Figure 5.2B was designed and carried out by Jones N. Figure 5.5A and 5.6 SR-SIMs were performed by Aoudjit L and analyzed and compiled into figures by Martin CE. Rat sample collections were also led by Aoudjit L with assistance from Martin CE. SEMs in Figure 4.1 and 4.6 were performed by New LA, as were proteinuria quantifications in Figure 4.6. Preparation of Table 5.1 was aided by Tilak M.

Jones N aided in the design of experiments and editing of the manuscripts written in the course of thesis. Significant contributions and technical assistance were also provided by Lu P throughout. Mouse husbandry and injections were largely performed by the technical staff at the University of Guelph’s Central Animal Facility.

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LIST OF ABBREVIATIONS

ACR: albumin/creatinine ratio
AE: anion exchanger
ANGII: angiotensin II
ANOVA: analysis of variance
AP-1: activator protein 1
AP-2: clathrin adaptor complex 2
aPKC: atypical protein kinase C
ARHGAP: Rho GTPase activating protein
ARHGDIA: Rho GDP dissociation inhibitor alpha
Arp: actin-related protein

BCA: bicinchoninic acid
BSA: bovine serum albumin

CA: constitutively active
Cas: p130 Crk-associated substrate
CCV: clathrin-coated vesicle
CD151: tetraspanin
CD2AP: CD2 associated protein
CH: H chain constant
CIE: clathrin-independent endocytosis
CIN85: cbl-interacting protein of 85 kDa
CKD: chronic kidney disease
cKO: conditional knockout
CME: clathrin-mediated endocytosis
CNS: congenital nephropathy syndrome
CNSF: congenital nephrotic syndrome of the Finnish type
Crk: Cdc2-related kinase

DMEM: Dulbecco’s high glucose modified eagle’s medium
DMSO: dimethyl sulfoxide
DN: diabetic nephropathy
DNA: deoxyribonucleic acid
DSS: disuccinimidyl suberate

ECL: enhanced luminol-based chemiluminescent
EDTA ethylenediaminetetraacetate
EEA: early endosome antigen
EGFR epidermal growth factor receptor
EGTA [ethylenebis(oxyethylenenitrilo)]tetraacetic acid
ELISA: enzyme-linked immunosorbent assay
ESRD: end-stage renal disease

F-actin: filamentous actin
FAT: FAT atypical cadherin
FBS: fetal bovine serum
FP: foot processes
FSGS: focal segmental glomerulosclerosis

GAPDH: glyceraldehyde-3-phosphate dehydrogenase
GBM: glomerular basement membrane
GFB: glomerular filtration barrier
GFP: green fluorescent protein
GFR: glomerular filtration rate
GLEPP: glomerular epithelial protein
Grb: growth-factor receptor binder
GST: glutathione s transferase
GTP: guanosine triphosphate

HBSS: Hank’s balanced salt solution
HEK: human embryonic kidney
Hepes: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPC: human podocyte cell
HRP: horse radish peroxidase
HS: heparin sulfate
HTS: high-throughput screen

IB: immunoblot (see WB)
IFN: inverted formin
i.p.: intraperitoneal(ly)
Ig: Immunoglobulin
IgAN: IgA nephropathy
IP: immunoprecipitate(ion)
IQGAP1: IQ motif containing GTPase activating protein 1

KD: kinase dead

LPS: Lipopolysaccharide

MAGI: membrane-associated guanylate kinase inverted
MAPK: mitogen-activated protein kinase
MCD minimal change disease
MEF: mouse embryonic fibroblast
MN: membranous nephropathy
MPC: mouse podocyte cell
Myo: myosin
Myh: myosin heavy chain

Nox: NADPH oxidase
Nck: non-catalytic region of tyrosine kinase
Neph: nephrin-like
N-WASp: Neuronal Wiskott-Aldrich Syndrome protein

OE: overexpressed

p: phospho
PACSIN: protein kinase C and casein kinase substrate in neurons
PAGE: polyacrylamide gel electrophoresis
Pak: p21 activated kinase
PAN: puromycin aminonucleoside
Par: protease activated receptor
PBS: phosphate-buffered saline
PCP: planar cell polarity
PDK: pyruvate dehydrogenase kinase
PHN: Passive Heymann Nephritis
PIP2: phosphatidylinositol-4,5-bisphosphate 2
PIP3: phosphatidylinositol-3,4,5-triphosphate 3
P13K: phosphatidylinositol 3-kinase
PKB/Akt: protein kinase B
PKC: protein kinase C
PLC: phospholipase C
PLC+: PLC+ lysis buffer
PMSF: phenylmethylsulfonyl fluoride
PP2: 4-amino-5-(4-chlorophenyl)-7-(t-buty1)pyrazolo[3,4-d]pyrimidine PPi inorganic pyrophosphate
PPR: proline rich region
PS: protamine sulfate
PTB: phosphotyrosine-binding domain
PTP: protein tyrosine phosphatase
PVDF: poly(vinylidene difluoride)

Rab: Ras-related protein
RhoA: Ras homolog family member A
RNAi: ribo-nucleic acid interference
Robo: roundabout, axon guidance receptor
RPMI: Roswell Park Memorial Institute
rtTA: reverse tetracycline Trans-activator

SD: slit diaphragm
SDS: sodium dodecyl sulfate
S.E.M.: standard error of mean
SEM: scanning electron microscopy
SFK: src family kinase
SH2: src homology domain
Shc: src homology 2 domain-containing
Ship: SH2-containing 5'-inositol phosphatase
SHP-1/2: SH2 domain-containing protein tyrosine phosphatase-1/2
siRNA: short interfering ribonucleic acid
SIRP: signal-regulatory protein alpha
SRN: steroid resistant nephropathy
SR-SIM: Super Resolution Structured Illumination Microscopy

TBS(T): tris-buffered saline (with tween)
TEM: transmission electron microscopy
Tris: tris(hydroxymethyl)aminomethane
TRPC6: transient receptor potential cation channel, subfamily C, member 6

VAMP: vesicle-associated membrane protein
Vangl: Van Gogh-like
VEGFR: vascular endothelial growth factor receptor
Vps: vacuolar protein sorting

WASp: Wiskott-Aldrich Syndrome protein
WB: Western immunoblot (IB)
WT: wildtype
WT-1: Wilms tumour 1

α: alpha
β: beta
γ: gamma
ε: epsilon
λ: lambda
ζ: zeta
CHAPTER 1: INTRODUCTION(1)
1.1 Chronic kidney disease and its impact on Canadian’s health

The kidneys are responsible for filtering wastes and excess fluids from the blood, which are then excreted from the body as urine. When the kidneys fail, dangerous levels of fluid, electrolytes and wastes can build up, wreaking havoc on the body. Patients suffering from chronic kidney disease (CKD) display progressive renal dysfunction and irreversible kidney damage. CKD is one of the top causes of death for Canadians (2) and its prevalence continues to grow in Canada and worldwide. Between 2007-2009, 12.5% of Canadians were suffering with chronic kidney disease (2) and the number of people being treated for kidney failure grew a striking 36% from 2006-2015 (kidney.ca). It is estimated that 30% of Canadians will experience acute or chronic kidney failure within their lifetime (kidney.ca).

Kidney disease can be a result of inherited susceptibility, but is more commonly a consequence of acquired injury (3). The elderly are particularly susceptible to disease and over 50% of newly diagnosed cases are individuals over 65 years of age (kidney.ca). The United States Renal Data System estimates diabetes to be the cause of approximately 44% of kidney failure cases within the United States, making diabetic nephropathy (DN) the most common form of disease (4).

In the early stages of CKD, patients often display few signs or symptoms and many patients remain unaware until their kidney function is significantly impaired. In 2015, 27% of patients referred to a nephrologist in Canada unknowingly were already suffering with end-stage renal disease (ESRD) in which the kidneys hold less than 15% of their blood filtering function (kidney.ca). There is no cure for ESRD and these individuals must undergo the only currently available therapies: dialysis or transplantation. These both induce a major burden on the quality of life of patients and their families as well as the healthcare field. Currently, over 24,000 patients in Canada are undergoing dialysis, representing an approximate cost of $2.4 billion per year (kidney.ca). Dialysis only delays the need for a kidney transplant and 76% of those on the transplant waiting list in Canada are awaiting a kidney (kidney.ca).

Current treatments for CKD focus on the prevention of disease progression as well as supportive therapies to treat consequences of suboptimal kidney function rather than the underlying cause of disease. Unfortunately, it is difficult to predict those who will respond well
to treatment, or those who will ultimately progress to ESRD. Clearly, it is of the utmost importance to better understand the underlying mechanisms of kidney damage, to identify individuals who are likely to progress to ESRD and to develop novel means to treat all stages of disease.

1.2 The nephron is the filtration subunit of the kidney

Within the kidney’s cortex lies millions of blood filtering subunits known as nephrons. Each nephron is made up of a glomerulus, the site of primary filtration, as well as a network of tubules where this filtrate is concentrated and further refined before it passes to the bladder to await excretion as urine. Glomerular dysfunction is a fundamental feature of kidney disease. It is for this reason that the glomerulus remains the focus of much of the investigation into the pathogenesis and treatment of kidney disease (1).

1.2.1 The glomerulus and its filtration barrier

The glomerulus resembles a ball of tiny capillaries twisted around a core of mesangial cells (Figure 1.1). Each capillary is lined with a fenestrated endothelium and is surrounded by highly specialized epithelial cells called podocytes that envelope them. The glomerular basement membrane (GBM), a compilation of proteins made by podocytes and endothelial cells (5), lies at the interface of the two cell types, also connecting with the mesangium in some areas. The glomerular filtration barrier (GFB) is collectively comprised of the fenestrated endothelium, the GBM and podocytes and it acts as a size and charge-selective barrier (6). The GFB is freely permeable to water and small and midsized solutes within the plasma, yet maintains considerable size and charge selectivity for proteins and larger molecules, allowing these vital components to be retained in the blood (7). Central to the GFB’s filtration selectivity is the unique three-dimensional architecture of the podocyte.

1.2.2 The specialized structure of the podocyte and the filtration slits

Podocytes extend a series of microtubule and intermediate filament-based primary and secondary processes from their cell body, which branch into an elaborate network of actin-based tertiary processes known as ‘foot processes’ (8). Foot processes (FP) interdigitate with each other to surround the glomerular capillaries and ultimately act as the main barrier to loss of proteins
and macromolecules into the urine. Extensive intercellular connections between adjacent podocytes, which are referred to as filtration slits or slit diaphragms (SD), account for much of the size-dependency of the barrier (9).

The SD is a unique cell-cell adhesion module that acts a molecular barrier and filtration sieve. It contains components that resemble both adherens (10) and tight junctions (11) as well as those not found elsewhere within the body such as nephrin (12, 13), the nephrin-like (neph) family of proteins (neph1/2/3) (14) and podocin (15). Early experiments showed that proteins like horseradish peroxidase, with a molecular weight of 40 kDa, penetrate through the endothelial layer, the GBM, and the slit pores to enter the subsequent chamber known as ‘Bowman’s space’. Larger proteins such as myeloperoxidase, about 160 kDa, cross the endothelium and the GBM but pile up at the SD, identifying a principal role for the SD in size-based sorting. Much of the charge-dependent selectivity, on the other hand, is dictated by the anionic glycocalyx of the podocyte, constituted mainly by podocalyxin (16). This allows for the negatively charged podocyte FPs to exert repulsive effects against negatively charged proteins, providing an additional charged-based barrier. Collectively, the podocytes contribute to both the size and charge basis for selective filtration, positioning them as a key component of the barrier.

1.2.3 Podocyte disruption in disease

Podocyte dysfunction is a hallmark of nearly all forms of renal disease. FP disruption, characterized by deregulation of the podocyte’s actin cytoskeleton, is identified by characteristic retraction of their processes into broader, more simplified structures in a process known as effacement. Loss of FP ultrastructure or podocyte surface charge results in increased filtration of macromolecules (typified by albumin) across this barrier, ultimately resulting in loss of protein into the urine, or proteinuria. Not only does this result in loss of important substances from the body, but also often overwhelms the kidney’s salvaging mechanisms in the nephron tubules, leading to persistent damage and fibrosis.
1.3 Nephrin: the core of the slit diaphragm

1.3.1 Structure and expression profile of nephrin

While we have understood the link between the SD and proteinuria for nearly fifty years, it was only recently that molecular components of the SD were first identified, starting with the gene \textit{NPHS1} and its protein product nephrin (17). This initiated a new phase of intense research into SD-associated proteins and their essential roles in filtration. The \textit{NPHS1} gene (OMIM *602716) is located on chromosome 19q13.1 and has a size of 26 kb. Translation of nephrin’s 29 exons produces a 1241-residue protein (and \textasciitilde90 kDa) and, once glycosylated, this transmembrane protein doubles in molecular weight to \textasciitilde180 kDa (12). Nephrin contains a large extracellular domain, a single transmembrane region and a short \textit{c}-terminal cytoplasmic tail. Its expression is most prominent in kidney podocytes (18-20), but has also been reported in the brain (18, 19), heart (21), pancreas (18, 19), and testes (22). Mutations in nephrin, including those that cause deletion of nearly the entire protein, do not appear to lead to appreciable defects in any organ but the kidney, highlighting its predominant importance in podocytes. This also makes nephrin a tempting target for drug development (23). Key interactions both extracellularly and intracellularly position nephrin as the core protein of the SD.

1.3.2 Nephrin and neph molecules create a porous molecular sieve

Nephrin is a member of the Immunoglobulin (Ig) protein superfamily. Its large extracellular domain is made up of 8 IgG-like motifs and a single fibronectin type 3 repeat (17). Neph1, first identified as a nephrin-like protein, contains 5 IgG-like motifs in its extracellular portion (14). The IgG domains on nephrin and neph1 interact in trans with adjacent nephrin and neph molecules on neighbouring FPs, creating the zipper-like organization of the SD (24).

Nephrin/neph-like complex formation is an evolutionarily conserved adhesion module in which heterodimeric trans neph1-nephrin interactions occur between two distinct cell types, with one expressing nephrin and the other expressing neph1 (25, 26). In the glomerulus however, interactions occur between adjacent podocyte cells which each express both nephrin and neph1 proteins, allowing for the possibility of nephrin-nephrin, neph-neph and nephrin-neph interactions. The likely architecture of the SD and nephrin and neph1 components was recently
illuminated using high-resolution ultrastructural imaging (24). Unlike in other modules, nephrin and neph1 appear to minimally interact in podocytes. It instead appears that nephrin-nephrin and neph1-neph1 segments largely comprise the SD r, in an approximate 2:5 ratio. In addition, the neph1 paralogs neph2 and neph3 do not appear to be present at the SD at all as compared to their presence in other modules involving nephrin-neph complex formation (25). Neph1 molecules appear to form the lower part of the junction, closer to the GBM, with a width of 23 nm, while single nephrin molecules form an adjacent junction more apically with a width of 45 nm. In both cases, the molecules are quasiperiodically spaced 7 nm apart. It is likely that this unique nephrin and neph1 complex formation holds much of the responsibility for the unique barrier function of the podocyte SD, setting it apart from other nephrin-neph-like cell-cell adhesion modules.

1.3.3 Nephrin is required for podocyte function

The NPHS1 gene was first discovered nearly 20 years ago when its mutation was identified as causative of disease in patients suffering with severe congenital nephropathy syndromes (CNS) (17). Mutations in NPHS1 are the most common causes of congenital nephrotic syndrome of the Finnish type (CNFS), which causes massive proteinuria at the perinatal stage (17). The most common nephrin mutation, Finmaj, is a frameshift that results in the production of a truncated (90 amino acids out of 1241), non-functional protein. Further characterization of these patients (12, 27) or of mice harbouring deletion of NPHS1 (19), has demonstrated that the SD neglects to form in the absence of nephrin, providing a major breakthrough in how the SD is formed and its importance in barrier function.

Since the identification of this first mutation, at least 250 additional nephrin mutations have been reported (28). Although most result in disease that presents with massive proteinuria in utero or soon after birth, some recent reports have identified mutations that appear to cause disease in later adolescence (29) through to early adulthood (30). Downregulation of nephrin has likewise been documented in many forms of acquired glomerular diseases, such as DN (31-35), minimal-change disease (MCD) (36-38), focal segmental glomerulosclerosis (FSGS) (39), membranous nephropathy (MN) (36, 39, 40), and others (40, 41) (description and classification of diseases in Table 1.1). This led to the suggestion that, in adulthood, a reduction in nephrin can likewise cause podocyte injury in vivo. A recent study tested this theory and demonstrated that
prolonged knockdown of nephrin expression over a long period of time can induce mild disease in adulthood (42). In addition, disease was exacerbated by knockdown of nephrin in experimental disease models, even after only short-term disruption of nephrin expression. It was clear from this work that nephrin is an essential protein within the podocyte and that the expression of nephrin is important in development, adulthood and in order to cope with damage.

1.3.4 Nephrin recruits cytosolic effectors via its short cytoplasmic domain

Nephrin not only serves as the physical barrier for the podocyte, but also as a signaling platform due to its extensive network of cytoplasmic binding partners (1) (Figure 1.2). The importance of nephrin’s cytoplasmic domain was first recognized by the identification of the Fin\textsuperscript{minor} NPHSI frameshift mutation which results in the truncation of nephrin’s cytoplasmic region (loss of 132/155 amino acids) (17). Although this mutation caused a much smaller truncation of nephrin as compared to the Fin\textsuperscript{major} mutation, it displayed an identical clinical manifestation including the absence of the SD, identifying nephrin’s cytoplasmic tail as key to its function in forming and maintaining the SD barrier as well as FP structure (27). Many nephrin binding partners have been identified including CD2-associated protein (CD2AP) (43), podocin (15, 44), phosphoinositide 3-kinase (PI3K) (45, 46), Fyn (47, 48), IQ motif containing GTPase activating protein (IQGAP) 1 (48, 49), Transient receptor potential cation channel, subfamily C, member 6 (TRPC6) (50), membrane-associated guanylate kinase inverted (MAGI)-1 (51) and MAGI-2 (49), beta (β)-arrestin2 (52), non-catalytic region of tyrosine kinase (Nck)1/2 (53, 54), vesicle-associated membrane protein (VAMP) 2 (55), protease activated receptor (Par) 3/ atypical protein kinase C (aPKC) zeta (ζ) (56, 57), aPKC lambda (λ)/t (58, 59), BKCa channel Slo1VEDEC (60), phospholipase C (PLC)- gamma (γ) 1 (61), cbl-interacting protein of 85 kDa (CIN85) (62), anion exchanger (AE) 1 (63), vascular endothelial growth factor receptor (VEGFR) 2 (64, 65), protein-tyrosine phosphatase (PTP) 1B (66), Van Gogh-like (Vangl) 2 (67), septin-7 (68), signal-regulatory protein (SIRP) alpha (α) (69), p130 Crk-associated substrate (Cas)/ Cdc2-related kinase (Crk) 1/2/ like (L) (70, 71), plexinA1 (72), src homology (SH) region 2 domain-containing phosphatase (SHP)-1 (73) and SHP-2 (74). These proteins represent key components of diverse signaling cascades that affect podocyte cell polarity, survival, calcium mechano-signaling, focal adhesion turnover, endocytosis and actin organization (Figure 1.2). Nephrin binds many of these proteins via phosphorylation of various conserved threonine-serine
and tyrosine residues found in binding motifs on its cytoplasmic tail (Figure 1.3). Of note, Y1114, Y1138, Y1193 and Y1217 are conserved between human, mouse and rat nephrin, while Y1176 is absent in rats, but present in humans and mice (Table 1.2).

1.3.5 Engagement of nephrin results in its tyrosine phosphorylation, which influences a variety of cellular pathways

The complex interplay between SD structure and FP morphology appears to be accomplished in large part through cell signaling events centered at nephrin phosphotyrosines. Phosphorylation of tyrosines within the cytoplasmic tail of nephrin by Fyn (Figure 1.4) allows for the recruitment of the SD protein podocin as well as several SH2/SH3 adaptor proteins including Crk1/2 (70), the Crk paralog CrkL (71), p85/PI3K (45, 46), PLC-γ1 (61) and Nck1/2 (3, 53, 54, 75). Much of our current understanding of intracellular signaling downstream of nephrin has been resolved using in vitro signaling investigation. Application of anti-nephrin antibodies to cells in culture results in nephrin clustering and tyrosine phosphorylation of the cytoplasmic tail of nephrin (76, 77). Likewise, in a model fashioned by Rivera et al. (78), a series of fusion protein constructs were generated in which the CD16 extracellular domain and the CD7 transmembrane domain were coupled to the cytoplasmic domain of nephrin (53). Clustering of the extracellular domains of the fusion protein using anti-CD16 antibody stimulates tyrosine phosphorylation on nephrin’s cytoplasmic tail and recruitment of downstream effectors. Use of these artificial systems has allowed for the investigation of events downstream of inducible nephrin signaling that are otherwise hampered by difficulties in modeling the SD’s unique 3-dimensional structure and thereby trans nephrin-nephrin signaling ex vivo.

1.3.5.1 Podocin and lipid raft dynamics

Podocin is a member of the stomatin family and localizes to lipid rafts at the SD (15). Mutation in its gene, NPHS2 are the second most common mutations found in CNS (79), although the severity of disease in these patients is less than those possessing mutations in NPHS1 (80). The overall importance of nephrin-podocin communication in normal podocyte function is highlighted in instances of CNS in which single mutations in either nephrin or podocin are benign in respective parents, but their dual presence in a child leads to congenital disease of a digenic kind (81), which notably is a rare phenomenon (82). Mutations affecting the
NPHS2 gene have been shown to disrupt nephrin localization to lipid rafts and podocin is thereby believed to be essential for nephrin’s recruitment to the membrane at the SD (15, 45).

Although nephrin-podocin interactions are predicted to occur at nephrin arginine 1160, phosphorylation of Y1193 has been shown to promote nephrin-podocin interactions (44, 45). Studies have demonstrated that this may, at least in part, be due to competition for nephrin between podocin and the endocytic effector β-arrestin 2, which binds nephrin in the absence of its phosphorylation at Y1193 (52).

1.3.5.2 Crk1/2/L and focal adhesion turnover

Crk1 and Crk2 (collectively Crk), and CrkL are recruited to tyrosine phosphorylated nephrin indirectly via p130Cas (70, 71), an important mediator of focal adhesion turnover that works downstream of nephrin phosphorylation. The redundant role of Crk and CrkL was revealed in a study in which their simultaneous deletion in podocytes led to disruption of FP structure and proteinuria, while deletion of either protein individually caused no alterations into adulthood (70, 71). Unlike the congenital nephropathy caused by deletion of nephrin, Crk/CrkL knockout mice display delayed pathogenesis starting at 6 weeks after birth, inferring a role for Crk/CrkL in maintenance of physiological steady state (71). In this regard, both single Crk and CrkL conditional knockout (cKO) mice are protected from damage induced by acute kidney injury models (Table 1.3) including from protamine sulfate (PS)-induced FP effacement (characterization of animal disease models in Table 1.3), which is predicted to involve rapid focal adhesion turnover characterized by lamellipodia protrusion (70, 71). This highlights a unique role for these adaptors in preservation of actin morphology through modulation of focal adhesion dynamics.

1.3.5.3 PI3K and Akt survival signaling

The protein kinase B (PKB)/Akt family of proteins (Akt1, Akt2 and Akt3) are serine/threonine kinases that are involved in multiple cellular pathways including cell migration, actin remodeling and cell survival (83). Akt2 alone is expressed in glomeruli and its expression in mouse podocytes is essential for maintenance of the filtration barrier as animals age and in response to injury (84). It is well documented that Akt activity can be influenced by PI3K, which is composed of a catalytic subunit p110 and a regulatory subunit p85 (83). Tyrosine
phosphorylation of nephrin on Y1114 and Y1138 results in the recruitment and binding of the p85 subunit to nephrin (46, 85). P85/P13K subsequently phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to form phosphatidylinositol-3,4,5-triphosphate (PIP3), which, along with pyruvate dehydrogenase kinase (PDK) 1, stimulates the translocation of Akt to the membrane where it becomes serine phosphorylated (83). Podocyte-specific knockout of Akt2 in mice ultimately results in reduced cell survival signaling, priming podocytes for apoptosis (84). Strikingly, a correlation between decreased Akt2 phosphorylation and development of albuminuria was recently observed in a subset of kidney transplant recipients treated with the immunosuppressive drug sirolimus (84), a finding which has immediate clinical implications for graft survival.

1.3.5.4 TRPC6, PLC-γ1 and calcium signaling

The importance of calcium regulation in podocytes was fully realized when gain-of-function mutation of TRPC6 was identified in FSGS patients (50, 86). Aberrant calcium signaling in podocytes is linked to the pathogenic remodeling of the actin cytoskeleton that results in FP effacement (87). PLC is a class of membrane-associated enzymes that cleave phospholipids at phosphate groups, and it is made up of six structurally unique isotypes (β, γ, δ, ε, ζ, η). Previous studies have demonstrated that nephrin Y1193 phosphorylation promotes its interaction with PLC-γ1, stimulating its activation and triggering calcium mobilization at TRPC6 channels (61, 88) in a PLC-γ1-dependent manner (88). PLC-γ1 levels are increased in rats perfused with PS (61) and TRPC6 is commonly upregulated in proteinuric diseases (89) making this axis an exciting area for future investigations. Interestingly, nephrin may also impede calcium signaling through PLC-γ1 and TRPC6 by directly binding and inhibiting TRPC6 activity in a non-tyrosine-dependent mechanism (88). In support of this, several disease-causing TRPC6 mutations leave the calcium channel unresponsive to nephrin inhibition in cell culture experiments (88), identifying an important role for nephrin-mediated inhibition of TRPC6 in podocytes.

More in-depth analysis of the PLC-γ1 pathway has also demonstrated an essential role for PLC-γ1 in TRPC6 trafficking (61), which may dictate its requirement in TRPC6-mediated calcium signaling. PLC-γ1 may also play a role in controlling nephrin’s own trafficking by
enhancing nephrin’s phosphorylation at T1120/T1125 (90, 91), which promotes nephrin’s interaction with β-arrestin and ultimately its endocytosis.

1.3.5.5 Nck and actin polymerization

The Nck family of cytoskeletal adaptor proteins (made up of Nck1 and Nck2) are essential for formation of podocyte FPs as demonstrated in podocyte-specific Nck1/2 cKO mice (53). Nck adaptors are multivalent proteins, owing to the presence of a single SH2 domain, which targets phosphorylated tyrosine (Y) residues embedded in YDxV motifs, and three SH3 domains, which target a variety of effector proteins containing proline-rich residues (PRR) in the PxxP conformation (where x is any amino acid) (92). Upon nephrin tyrosine phosphorylation, Nck can be recruited to any of 3 distinct YDxV residues (3, 53, 54, 75) in an independent fashion (93, 94), thereby creating a cluster of multivalent interactions (93, 94). Similarly to nephrin, knockout of Nck in podocytes appears to not only be essential in development but also throughout life as demonstrated by the development of nephrosis in mice after its inducible deletion in adulthood (95). This appears to be intimately linked to Nck’s role in the recruitment of actin to nephrin (3, 53), which may facilitate stabilization of the actin-based FP.

1.3.6 Actin reorganization downstream of nephrin tyrosine phosphorylation

Polymerization of actin in podocytes allows for the unique structure of the FPs and nephrin tyrosine phosphorylation is an important regulator of podocyte actin dynamics. Phosphorylation of nephrin results in modulation of two forms of actin reorganization in vitro: lamellipodia formation, which is associated with pathogenic FP effacement, and the growth of actin polymers (also referred to as ‘tails’ or ‘comets’) at nephrin, which are believed to stabilize FP ultrastructure and the SD laterally. Phosphonephrin-dependent lamellipodia formation appears to be modulated by p85/PI3K/ Akt/Cas/Crk through phosphorylation of Y1114/1132 (46, 70, 71, 96). Conversely, the production of actin tails at nephrin punctae is dependent on phosphorylation of Y1176, Y1193 and Y1217 and via recruitment of Nck (53, 97). This is clearly depicted by characterization of mutants in which all three of these tyrosines (Y) are mutated to phenylalanine (F) residues, which, although structural similar, cannot undergo phosphorylation. This triple Y-to-F conversion (Y3F) leads to a complete absence of actin tail
formation and demonstrates the importance of recruitment at these phosphorylated tyrosines residues in nephrin-actin interactions (53, 97).

1.3.7 Multivalent and redundant connections between nephrin, Nck and actin

Clustering experiments have demonstrated that Nck is engaged at nephrin concomitantly with actin tails (97) and that phosphorylation at any single one of these tyrosine residues is sufficient for recruitment of Nck and generation of actin tails at nephrin. This demonstrates a potential redundancy in nephrin-Nck interactions, at least in vitro (97). The amount of actin recruited to nephrin appears to be somewhat additive in that the most actin polymerization is observed when all three residues remain intact and successive loss of residues reduces the intensity of actin tail formation (97).

Clustering of CD16/7-Nck SH3 domains at the plasma membrane is also able to independently induce actin polymerization (78), while introduction of mutant Nck that can no longer bind downstream effectors via the SH3 domains (SH3*x3) results in severe disruption. Likewise, actin polymerization at nephrin-Nck clusters in the presence of the Nck-SH3* mutant are disrupted in a dominant negative manner (97). This points to a key role for Nck in actin recruitment downstream of nephrin tyrosine phosphorylation.

Nck facilitates the recruitment of actin nucleator actin-related protein (Arp) 2/3 at nephrin via binding of Neuronal Wiskott-Aldrich Syndrome protein (N-WASp) to its second or third SH3 domain (98-100). Similar to Nck, podocyte-specific knockout of N-WASp also results in disrupted FP formation in development and into adulthood (101). However, disease onset is marginally delayed in N-WASp versus Nck cKO mice, perhaps denoting additional roles for Nck beyond maintenance of actin organization or functional overlap between N-WASp and other actin nucleation factors such as WASp (98) and p21 activated kinase (Pak) (102), which can also be recruited by Nck SH3 domains. Nck was also recently characterized to play a role in stabilization of the Guanosine triphosphate (GTP)ase Ras homolog family member A (RhoA) in an N-WASp-independent manner in podocytes (103). However, unlike the requirement for Nck or N-WASp, podocytes lack a strict requirement for RhoA in development and adulthood (104), thereby suggesting that this pathway may be involved in the fine-tuning of Nck signaling in established podocytes.
Nck remains the only known binding partner that can bind all three tyrosine residues responsible for actin tail formation at nephrin, although the reasoning for this redundancy has not yet been illuminated. Nck does appear to show a preference for binding the Y1217 residue (95), possibly because of a preference for the YDEV motif found here versus YDQV at Y1176 and Y1193 (Table 1.2). Redundancy in Nck itself is also observed in the kidney, with total body deletion of Nck1 or Nck2 alone leading to no obvious phenotype in the kidney, while Nck1/2 podocyte cKO leads to congenital disease (53). It was recently demonstrated that the multivalency of Nephrin-Nck-N-WASP is essential to actin polymerization at nephrin in vitro (93, 94, 105) and Nck’s ability to bind nephrin on multiple sites plays a significant role in this multivalency.

1.3.8 Regulation of nephrin phosphorylation by kinases and phosphatases

1.3.8.1 Kinase-mediated regulation of nephrin signaling

Nephrin tyrosine residues can be phosphorylated by several Src family kinases (SFK) including Src (77), Fyn (44, 47, 77), Lyn (77) and Yes (47, 77). Of the six most highly conserved tyrosine residues, the majority can be phosphorylated by Fyn in vitro, though each residue may not be phosphorylated to the same extent (44, 48, 53, 61). Podocyte-specific Fyn cKO mice demonstrate reduced nephrin tyrosine phosphorylation and display FP effacement and proteinuria within the first few weeks of life, whereas knockout of Yes does not appear to lead to major defects (47), identifying Fyn as the likely central SFK within podocytes.

Recently, a positive feedback loop wherein Nck binding to Fyn promotes phosphorylation of nephrin tyrosine residues was identified (106). Fyn can directly bind to nephrin, reportedly via either its SH2 and SH3 domain (47, 48). Nck/Fyn/nephrin complex formation increases Fyn’s activity, leading to hyperphosphorylation of nephrin, a phenotype that is amplified by Nck2 versus Nck1 (106). Likewise to knockout of Fyn (47), knockout of Nck appears to reduce nephrin phosphorylation in mice (106), indicating that this pathway is likely also important for basal levels of nephrin phosphorylation. Of note, Fyn can also interact with the p85 subunit of PI3K (107), which may also influence nephrin signaling at Y1114 and Y1138, although this has not been directly investigated.
1.3.8.2 Phosphatase-mediated regulation of nephrin signaling

Fyn activity is modulated by several protein tyrosine phosphatases (PTP) known to be expressed within the podocyte. Phosphatases catalyze the removal of phosphate groups from target proteins, which often affects their activity status. De-phosphorylation of Fyn by the SHP-2 phosphatase (108), which also binds nephrin (74), was recently found to release intramolecular inhibition of Fyn, ultimately enhancing its kinase activity on nephrin (74). Conversely, the active site of Fyn (Y418) was found to be a substrate for the phosphatase PTP-PEST, and de-phosphorylation of this site leads to reduced Fyn activity, which indirectly leads to reduce nephrin phosphorylation (66).

PTPs have also been demonstrated to act directly on nephrin. PTP-1B, which is upregulated in the puromycin aminonucleoside (PAN) model of MN, can directly dephosphorylate rat tyrosines residues that correlate to the human sites 1193 and 1217 (66). Likewise, the SHP-1 phosphatase has been shown to dephosphorylate Y1176, 1193 and 1217 (73) and its upregulation has been observed in instances of hyperglycemia and diabetes by several groups (73, 109-113).

In podocytes, hyperglycemia induces a persistent increase of SHP-1 expression, due to epigenetic modification in the SHP-1 promoter (112) leading to insulin signaling resistance, podocyte dysfunction and cell death. Interestingly, PTP-1B and SHP-1 both are unable to dephosphorylated nephrin’s Y1138 site (66, 73), indicating that these phosphatases likely do not exert their influence by modulating PI3K-Akt signaling, but rather through tyrosine residues that bind Nck and PLC-γ1.

1.3.9 Disruption of nephrin tyrosine phosphorylation in podocyte-based kidney diseases

Previous studies have established the presence of nephrin tyrosine phosphorylation in human, mouse and rat glomeruli (46, 47, 53, 75, 114, 115) (Table 1.4). Multiple groups have developed phospho-specific antibodies recognizing individual tyrosine residues and used them to characterize tyrosine phosphorylation throughout disease (95, 116). Phosphorylation of Y1217 (or the equivalent) has been observed in normal healthy glomeruli of all three species (95, 116),
while phosphorylation of Y1193 has been reported in both mice (95, 114, 115) and in rats (95, 114, 116).

The role of nephrin tyrosine phosphorylation during disease has become a recent area of interest since the identification of reduced nephrin tyrosine phosphorylation in instances of human disease and in various disease models (Table 1.4). Alterations in nephrin tyrosine phosphorylation at various sites have been described in MCD (116) and MN (117) as well as in PAN (95), PS (54, 118), nephrotoxic serum (NTS) (74, 91, 119), lipopolysaccharide (LPS) (118) rodent injury models and type I diabetic Akita mice (73, 113). Unfortunately, there is often a lack of consensus about whether nephrin tyrosine phosphorylation is up or downregulated and whether this is associated with promoting podocyte damage or protecting from it. The role of nephrin tyrosine phosphorylation in disease thereby remains an intense area of research within the podocyte field.

1.4 Nephrin endocytosis: an emerging focus in podocyte biology

Investigation of nephrin trafficking mechanisms has become a keen area of interest in recent years owing to the recognition of nephrin mislocalization in a broad range of human disease including MN (117), CNSF (120), steroid resistant nephropathy (SRN) (121), MCD (38), DN (62) and hypertensive nephropathy (91). However, relatively little is known about the specific mechanisms that dictate nephrin trafficking (122).

1.4.1 Endocytosis in mammalian cells

Endocytosis is the process by which cells internalize membrane-bound components including embedded surface receptors and their ligands. In the podocyte, two endocytic pathways have been identified: clathrin-mediated endocytosis (CME) and clathrin-independent endocytosis (CIE) (123). CME is most commonly recognized as mediating the regular turnover of receptors (124). Clathrin is recruited in the initiation stage of pit invagination, supported by various proteins including α-adaptin and synaptojanin. Here, it coats the cytosolic portion of the membrane and acts as a scaffold for the recruitment of endophilin-1, which aids in membrane curvature, and actin, which facilitates continued elongation of the pit. Subsequently, the GTPase dynamin is recruited to the vesicle where it wraps and constricts its neck until the small
membrane-encapsulated vesicles is cut free, completing its internalization. The clathrin-coated vesicle (CCV) will lose its coat before fusing with the early endosome. It is here that the vesicle’s components are sorted and either recycled back to the cell membrane or transported to the late endosome and ultimately the lysosome to be degraded. The entire plasma membrane, and its embedded components, is turned over from 1-5 times each hour (122). The majority of cargo endocytosed in this manner, about 95%, is believed to be recycled back to the plasma membrane and this pathway thereby defines a constitutive turnover mechanism in cells. The other 5% that is targeted for degradation may be damaged, requiring replacement by synthesizing mechanisms, or a means to finesse receptor activity.

CIE involves largely the same mechanisms observed in CME except it occurs independent of clathrin recruitment. Caveolae, a common site of CIE, are invaginations of the plasma membrane with a characteristic flask-shaped morphology. Importantly, unlike CME, CIE is a stimulation-dependent, non-constitutive means of internalization. Several studies have demonstrated a dependence for SFK, local actin polymerization and dynamin in CIE (125-127). However, the precise mechanisms of CIE are believed to be as diverse as the receptors that use them to regulate cell functions.

1.4.2 Roles for actin throughout endocytosis

Actin polymerization has been shown to play many roles throughout endocytosis: it can help create protrusions to encompass extracellular materials, support invagination of a membrane segment into the cytoplasm, elongate invaginations and aid in scission of the vesicle from the membrane (128, 129). To date, Arp2/3 is the only actin filament nucleator that has been found at endocytic sites and its mutation can result in the disruption of endocytosis (130). N-WASp, a potent activator of Arp2/3, has been shown to arrive early to endocytic sites, even before Arp2/3 or actin (131). This collectively identifies the N-WASp and Arp2/3 as key early recruits upstream of actin polymerization during endocytosis.

Much of our understanding about the mechanisms of endocytosis has been elucidated using yeast. Interestingly, unlike yeast, endocytosis in mammalian cells does not hold a strict requirement for actin polymerization (132). This may be attributed to the unique physical restraints placed on the endocytic machinery of yeast and mammalian cells. More specifically,
the requirement for actin during endocytosis in double-walled yeast may be as a direct result of the increased surface turgor that must be counteracted in order to induce membrane invagination. Interestingly, endocytic invaginations in yeast and mammalian cells are visually distinct: mammalian endocytic pits are roughly spherical in shape while those in yeast are tubular. These differences in shape have been proposed to be a result of differences in surface tension dynamics and thereby their distinct requirements for actin (133).

In polarized mammalian cells, actin polymerization does appear to be required for endocytosis at the apical, but not basal or basolateral membranes. It has been demonstrated that membrane tension is greater on the apical surface of polarized cells, which results in increased membrane tension and presumably dictates this requirement for actin to aid in constriction (134, 135). However, subjecting cells to hypotonic swelling (135) or mechanical stretch (135) can create a requirement for actin in CME on the basolateral surface of mammalian cells. Collectively this demonstrates a potential role for surface tension in dictating the requirement of actin in endocytosis of the SD.

1.4.3 Essential role for endocytosis in podocytes

Reports characterizing the effects of podocyte-specific deletion of dynamin1/2 (136, 137), synaptojanin (136) and endophilin (136) have clearly established the functional importance of endocytic machinery within podocytes. Dynamin1/2 is required to establish and maintain podocyte FP structure as demonstrated in podocyte-specific cKO mice (136). Dynamin’s role in endocytic scission has been well characterized (138, 139). As a vesicle invaginates, it polymerizes into a helical tube and twists around the neck of the vesicle. The polymer tightens upon guanosine triphosphate (GTP) binding and hydrolysis. This constricts the underlying membrane and causes its fission, ultimately resulting in the vesicle’s release from the parent membrane. Indeed, arrested endocytic pits can be observed in both dynamin-deficient podocytes and mouse FPs (136). It has been further demonstrated that dynamin complexes with and supports nephrin’s endocytosis through a phosphotyrosine-dependent mechanism, although it performs this function indirectly through a yet-to-be-identified intermediary (136, 140).

Evidence indicates that dynamin also holds a key endocytosis-independent role in pathogenic actin polymerization in podocytes (141, 142). Pharmacological inhibition of
dynamin’s influence on actin can rescue proteinuria and podocyte damage induced by disease-causing mutations of various distinct proteins that disrupt actin plasticity (142). This may hold major clinical implications for individuals suffering from podocytopathies of diverse origins.

1.4.4 Nephrin endocytosis via β-arrestin2 and friends

β-arrestin was the first nephrin binding partner identified to influence nephrin trafficking (52). β-arrestin binds phosphorylated T1120/T1125 on nephrin’s cytosplasmic tail, which facilitates nephrin endocytosis in a CME fashion (91). β-arrestin mediated nephrin endocytosis is relatively well-characterized within podocytes and several pathways seem to converge on this mechanism. Protein kinase C (PKC)α mediates nephrin phosphorylation of T1120/1125, leading to β-arrestin recruitment and nephrin internalization and this axis has been shown to be relevant in several diseases including in human diabetes (143) and in response to the vascular protein angiotensin II (ANGII) (90, 91).

During glomerular development, activation of the planar cell polarity (PCP) pathway also appears to stimulate nephrin endocytosis via a clathrin/β-arrestin-dependent mechanism (67, 144). Disruption of Vangl2 activity, which is involved in the PCP pathway, results in increased surface expression of nephrin in podocytes, and this leads to disruption of glomerular maturation in a mouse model (145). Notch activation likewise has been shown to induce nephrin internalization via a β-arrestin/ dynamin-dependent, raft-independent route (146) and animals overexpressing activated Notch display proteinuria and damage that is associated with enhanced nephrin endocytosis and loss of SDs.

1.4.5 Role of nephrin tyrosine phosphorylation in its endocytosis

Although much of the characterization of nephrin trafficking in podocytes has focused on the role of β-arrestin, tyrosine phosphorylation of nephrin has also been implicated in the regulation of nephrin endocytosis (123). However, there are conflicting reports regarding the potential role for site-specific nephrin phosphorylation events in the process. Reduced phosphorylation of Y1193 has been shown to induce binding of β-arrestin and promote rapid removal of nephrin from the cell surface by CME (52), while phosphorylation of this same tyrosine promotes podocin binding to nephrin, which is proposed to localize nephrin to lipid raft
microdomains where it is turned over at a slower rate by CIE (45, 123). Others have reported that mutation of mutation of Y1217 or compound mutation of Y1193/Y1176 decreases nephrin internalization (123, 140), and that enhanced dynamin-mediated phosphorylation of nephrin promotes its endocytosis (140). Interestingly, although nephrin tyrosine phosphorylation has been reported to induce its endocytosis in some instances of disease, a nephrin phosphotyrosine binding partner directly involved in its endocytosis has yet to be identified.

1.4.6 Abnormal nephrin trafficking - a new hallmark of disease?

Nephrin mislocalization has been observed in nearly all forms of human kidney disease including those of genetic and acquired origins (38, 62, 91, 117, 120, 121). Likewise, the NTS nephritis model of podocyte injury (91) and the PS model of rapid FP effacement (123) have also recently been reported to stimulate endocytosis within podocytes. Characterization of both of these models indicates they may involve alterations in nephrin tyrosine phosphorylation (54, 74, 77, 91), although there is little consensus about whether phosphorylation is increased or decreased and whether this represents a protective or deleterious effect within the podocytes.

Relatively few disease-causing NPHS1 mutations have been characterized for precisely how they affect nephrin function from a cell biology or cell signaling perspective. From the studies available, it appears that most of the NPHS1 mutations lead to abnormal retention of nephrin in the endoplasmic reticulum, and therefore it fails to traffic to the cell surface (147, 148). This suggests then that the majority of NPHS1 mutations result in a loss of function of nephrin due to the inability for it to localize to the SD, which leads to the early-onset and severe disease often associated with NPHS1 mutations (147). Assumedly, this also affects the ability of nephrin to be phosphorylated and there is some evidence that disruption of nephrin localization to the membrane results in its sub-maximal phosphorylation (45). Although less commonly reported, some mutations, such as the V822M NPHS1 mutation, do not appear to affect nephrin trafficking to the cell membrane, but rather they affect normal nephrin trafficking from the plasma membrane (149). This likewise causes congenital nephrotic disease (150), indicating that trafficking from the membrane is equally important to normal nephrin function. Interestingly, although nephrin-V822M is able to traffic normally to the cell membrane, it also displays sub-maximal nephrin phosphorylation and is unable to reorganize actin filaments (149).
1.5 Genetic disruption of nephrin tyrosine phosphorylation causes kidney disease in mice: the nephrin-Y3F mouse model

1.5.1 Generation of the nephrin-Y3F mouse model

Our ability to study the role of nephrin tyrosine phosphorylation in podocyte function has been hampered by opposing reports and disagreements in how results should be interpreted (Table 1.4). Discrepancies between patients with the same diagnosis, between human disease and the animal models used to study them (Table 1.1), and even within the same animal models used by separate groups are common. These differences may stem from many sources including but not limited to difficulties in obtaining consistent diagnosis/grading of disease by different pathologist, diversity even within the disease subtype (especially in the severity and progression of disease), the impact of age, race and sex on human disease, innate differences between human diseases and the models employed to study them, the use of different antibodies, processing techniques and staining protocols, differences in age, genetic backgrounds or sex of animals used, animal or reagent sourcing, difference in animal housing and/or diets, urine collection methods (spot urines versus 24 hour collections), and, finally, differences in chosen timepoints for timecourse analyses. These complications have led to frustration within the field regarding our ability to unravel the pathomechanisms of kidney damage and come to a consensus to continue moving treatments forward for patients and their families.

Until very recently, it was not possible to determine whether nephrin tyrosine phosphorylation was even involved in the normal development or maintenance of podocyte FPs. To directly investigate the necessity of tyrosine phosphorylation in podocyte biology, our group generated knockin mice harbouring the nephrin-Y3F mutation. These mice were backcrossed on to two different genetic backgrounds (CD-1 and C57BL/6) for further analysis and were born in normal Mendelian ratios (118).

1.5.2 Barrier maintenance, but not establishment, is disrupted in nephrin-Y3F mice

Unlike in individuals in which total nephrin, or its cytoplasmic domain, have been deleted, nephrin-Y3F mice do not display congenital abnormalities on either the CD-1 or C57BL/6 backgrounds. However, these mice develop progressive proteinuria accompanied by
structural changes in the filtration barrier, including podocyte FP effacement, irregular thickening of the GBM, and dilated capillary loops during aging. Examination of glomeruli from nephrin-WT and nephrin-Y3F mice on outbred CD-1 background was performed at four time points: at the earliest sign of proteinuria (1 month), at the onset of moderate proteinuria (2 months), after the progression to severe proteinuria (4 months), and finally, after a period of chronic proteinuria (6 months). At 1 month of age, nephrin-Y3F glomeruli contained enlarged capillary loops, which were accompanied by haphazardly organized FPs that appeared short, thick and wavy in scanning electron micrographs. These observations became more extensive by 2 months of age and were accompanied by an irregular thickening and bulging of the GBM by 4 months of age. By 6 months, glomeruli from nephrin-Y3F were hypocellular, with numerous dilated capillary loops and animals displayed widespread podocyte FP effacement. It is noteworthy to report that effects were even observed in the heterozygous state, and this may be relevant in patients with nephrotic syndrome who display dominant forms of disease as well as those who present outside the neonatal period (151).

Structural changes were accompanied by several biochemical changes within nephrin-Y3F glomeruli. Immunoprecipitation (IP) followed by Western immunoblotting (WB) demonstrated that the interaction between nephrin and Nck was lost in nephrin-Y3F animals, as was predicted by prior in vitro results. In addition, total phosphorylation of nephrin was significantly decreased, as was activation of Akt, which importantly acts downstream of Y1114 and Y1138, highlighting the more global effects of loss of signaling at these 3 residues. Compensatory means of tethering nephrin to the actin cytoskeleton via mechanisms beyond through Nck could explain why congenital nephrosis does not manifest in these mice (as it does in nephrin or Nck-deficient animals (53)). Although this tenuous integration may be sufficient for early podocyte morphogenesis, the weakened linkage between the SD and actin cytoskeleton in the absence of phospho-nephrin signaling rendered the podocyte more vulnerable to breakdown. This may likewise leave podocytes more susceptible to damage in disease models.
1.5.3 Heterogenic disease is observed in animals with different genetic backgrounds- role for blood pressure?

Although the biological and biochemical dysfunction of nephrin-Y3F was similar in both CD-1 and C57BL/6 animals, disease onset and severity were significantly delayed in C57BL/6 mice. Most CD-1 nephrin-Y3F mice showed significant albuminuria by 1 month, which progressed markedly with age, while disease onset was not observed until at least 6 months in C57BL/6 animals. Regardless, the spectrum of glomerular pathologies associated with the nephrin-Y3F mutation portrayed on both backgrounds was very similar. Intriguingly, C57BL/6 mice have lower blood pressures than age–matched CD-1 mice (152), and it is possible that reduced glomerular strain plays a role in the delayed disease observed in C57BL/6 mice. Nonetheless, nephrin-Y3F animals provide a unique genetic resource to further pursue the role of nephrin tyrosine phosphorylation in podocyte health and disease.

1.6 Rationale and thesis objectives

The role of nephrin in the formation and maintenance of the podocyte’s SD has been a major focus over the last 20 years. Evidence from several groups suggests that loss of nephrin tyrosine phosphorylation is associated with breakdown of the GFB, while other studies suggest a pathogenic role for heightened nephrin tyrosine phosphorylation in promoting FP effacement. Similarly, the role(s) of nephrin turnover in barrier maintenance and breakdown is poorly understood. Nephrin tyrosine phosphorylation has been proposed to influence nephrin endocytosis, although whether it promotes or inhibits internalization is unclear, and a nephrin phospho-tyrosine-binding partner that facilitates endocytosis has yet to be identified.

The objective of this thesis is to define the role of nephrin tyrosine phosphorylation in promotion of and/or protection from kidney damage. These investigations are built on the framework of previous reports that have identified a role for nephrin tyrosine phosphorylation in podocyte actin cytoskeleton plasticity and in nephrin endocytosis. We attempt to unravel the signaling mechanisms that dictate these outcomes in the context of cell and animal models as well as in instances of human disease.
The findings from these investigations have been organized in the following three chapters:

1. Nephrin tyrosine phosphorylation is disrupted in various kidney injury models and is required for recovery from disease
2. Multivalent nephrin-Nck-actin interactions differentially modulate nephrin endocytosis
3. The ShcA adaptor protein promotes nephrin endocytosis and is upregulated in proteinuric nephropathies
Table 1.1 Description of common kidney diseases, their etiologies and the models used to study them.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Characteristics</th>
<th>Associated Genes</th>
<th>Animal Models</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Congenital Nephrotic Syndrome (CNS)</td>
<td>• Infantile-onset&lt;br&gt;• Glomerular alterations and proteinuria almost always observed in utero or shortly after birth</td>
<td>• NPHS1 (nephrin genes – over 250 identified&lt;br&gt;• NPHS2 (podocin – over 100 identified&lt;br&gt;• ARHGDI A (Rho GDP dissociation inhibitor 2)&lt;br&gt;• WT-1 (Wilms tumor 1)&lt;br&gt;• ACTN4 (α-actinin-4)&lt;br&gt;• LAMB2 (laminin β2)&lt;br&gt;• ITGA3 (integrin α3)&lt;br&gt;• PLCE1 (Phospholipase C ε1)</td>
<td>• nephrin and podocin knockout mice display strikingly similar phenotypes to patients harboring NPHS1 or NPHS2 mutations, whereas mice harboring deletion of Phospholipase C ε1 do not display an overt phenotype</td>
<td>• Encompasses all diseases with congenital onset&lt;br&gt;• NPHS1 mutations account for 98% of CNS disease in Finnish populations (also called CNSF); and 40-50% of disease in non-CNSF</td>
</tr>
<tr>
<td>Minimal Change Disease (MCD)</td>
<td>• Usually childhood onset&lt;br&gt;• Characterized by extensive foot process effacement in the absence of other glomerular alterations</td>
<td>• Largely idiopathic, but some genes identified&lt;br&gt;• NPHS1&lt;br&gt;• NPHS2&lt;br&gt;• WT-1&lt;br&gt;• MAGI-2</td>
<td>• Puromycin aminonucleoside nephrosis (PAN) (mice or rats)&lt;br&gt;• Lipopolysaccharide (LPS) model (mice)&lt;br&gt;• Nephrotic serum model (mice or rats)</td>
<td>• Classified broadly into steroid-sensitive and steroid-resistant subtypes; resistant types are largely the heritable forms</td>
</tr>
<tr>
<td>Focal Segmental Glomerulosclerosis (FSGS)</td>
<td>• Often adult-onset&lt;br&gt;• Affects only a portion of glomeruli and is characterized by glomerular fibrosis, leading to sclerosis and destruction of capillary loops</td>
<td>• Largely idiopathic, but some genes identified&lt;br&gt;• IFN2 (inverted formin-2)&lt;br&gt;• CD2AP (CD2-associated protein)&lt;br&gt;• NPHS2&lt;br&gt;• PLCE1</td>
<td>• Adriamycin/doxurubicin (mice or rats)&lt;br&gt;• Several genetic models (in which knockout or mutation of a specific gene leads to FSGS-like fibrosis); some of these models exploit known mutations in human instance of FSGS</td>
<td></td>
</tr>
<tr>
<td>Membranous Nephropathy (MN)</td>
<td>• Often adult-onset&lt;br&gt;• Auto-immune-mediated&lt;br&gt;• Characterized by the deposition of immune complexes in the GBM followed by complement activation</td>
<td>• Podocyte-expressed M-type phospholipase A2 receptor (PLA2R) appears to be responsible for over 80% of cases of primary disease (153)&lt;br&gt;• Polymorphisms at HLA-DQA1 (affects antigen-presenting cells) influences severity (154)</td>
<td>• Passive Heymann nephritis model (PHN) (although target antigen is the glycoprotein megalin 330 versus PLA2R) (rat) (155)</td>
<td>• Primary and secondary forms are characterized by whether MN is initiated on its own or secondarily, as a consequence of various diseases that affect the immune system</td>
</tr>
<tr>
<td>Immunoglobulin A Nephropathy (IgAN)</td>
<td>• Characterized by deposition of IgA in glomeruli, causing inflammation that, over time, disrupts filtration&lt;br&gt;• Usually affects young-aging adults versus children</td>
<td>• More than 90% of cases are sporadic&lt;br&gt;• Familial disease has been reported, but the genetic basis is not fully understood</td>
<td></td>
<td>• Also called Berger’s disease&lt;br&gt;• Individuals may also be sub-classified into broader disease categories, such as MCD</td>
</tr>
<tr>
<td>Diabetic Nephropathy (DN)</td>
<td>• Induced by chronic hyperglycemia, leading to progressive and chronic loss of filtration function&lt;br&gt;• The incidence in disease is greater in type I diabetes than type II, likely as a factor of the extent of hyperglycemic exposure</td>
<td>• Incidence is greater with a familial past of DN, but the genetic basis is not fully understood</td>
<td>• Type I diabetes: Akita spontaneous mouse model (harbor insulin 2 gene mutation)&lt;br&gt;• Type II diabetes: Streptozocin (STZ) injection of mice (destroys insulin-producing pancreatic beta-cells)</td>
<td>• Neither the Akita or STZ models display all defining characteristics of human DN (such as Kimmelstiel-Wilson nodules) (156)&lt;br&gt;• Progression of disease in these models is muted in comparison to human DN</td>
</tr>
</tbody>
</table>
Table 1.2 Conserved tyrosine (Y) residues shared between human, mouse and rat nephrin. Bold font represents YDxV motifs.

<table>
<thead>
<tr>
<th></th>
<th>Human</th>
<th>Mouse</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>1114</td>
<td>YEES</td>
<td>1128-</td>
<td>1127-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>YEES</td>
<td>YEES</td>
</tr>
<tr>
<td>1138</td>
<td>YYRS</td>
<td>1153-</td>
<td>1152-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>YYSM</td>
<td>YYSM</td>
</tr>
<tr>
<td>1158</td>
<td>YSRG</td>
<td>1172-</td>
<td>1171-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>YRQA</td>
<td>YHQG</td>
</tr>
<tr>
<td><strong>1176</strong></td>
<td><strong>YDEV</strong></td>
<td><strong>1191</strong></td>
<td><strong>YDEV</strong></td>
</tr>
<tr>
<td>1183</td>
<td>YPPS</td>
<td>1198-</td>
<td>1194-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>YGPP</td>
<td>YGPP</td>
</tr>
<tr>
<td><strong>1193</strong></td>
<td><strong>YDEV</strong></td>
<td><strong>1208</strong></td>
<td><strong>YDEV</strong></td>
</tr>
<tr>
<td>1210</td>
<td>YQDP</td>
<td>1225-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>YEDP</td>
<td></td>
</tr>
<tr>
<td><strong>1217</strong></td>
<td><strong>YDQV</strong></td>
<td><strong>1232</strong></td>
<td><strong>YDQV</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Disease Model</td>
<td>Human Disease</td>
<td>Mechanism of action</td>
<td>Patho genesis</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>-------------------------</td>
<td>------------------------------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Puromycin Aminonucleoside Nephrosis (PAN) (rat)</td>
<td>Minimal Change Disease (MCD)</td>
<td>• Podocyte toxin that induces reactive oxygen species (ROS), ribosomal dysfunction and oxidant-dependent DNA damage</td>
<td>• Proteinuria becomes evident starting around 3-5 days post injection, peaking at 12-14 and returning to baseline by 28 days. • Significant effacement observed</td>
</tr>
<tr>
<td>Lipopolysaccharide (LPS) (mouse)</td>
<td>MCD</td>
<td>• A component of the outer membrane of Gram-negative bacteria</td>
<td>• Proteinuria becomes evident at 24 hours and returns to baseline by 48 hours. • Significant effacement observed • Commonly used in various experimental models of inflammation; has many non-podocyte targets</td>
</tr>
<tr>
<td>Nephrototoxic Serum (NTS) (mouse)</td>
<td>MCD</td>
<td>• Sheep are inoculated with rat glomeruli and serum is collected and injected into mice • Characterized by infiltration of glomerular tufts by leukocytes and linear anti-GBM antibody deposits along the glomerular capillaries are observed within 2 hours of injections, leading to further infiltration of immune cells</td>
<td>• During the acute phase of disease, proteinuria becomes evident starting around 6-12 hours, peaking at 24 hours and returning to baseline around 54 hours. Significant effacement observed. • Disease is recurrent, leading to glomerulosclerosis and chronic damage approximately 1 month after initiation</td>
</tr>
<tr>
<td>Protamine Sulfate (PS) /Heparin Sulfate (HS) (mouse and rat)</td>
<td>Rapid reversible foot process effacement</td>
<td>• Reversion of the podocyte glycocalyx’s negative charge with positively charged PS; reversion to baseline with neutralizing HS</td>
<td>• PS causes foot process effacement within 15 minutes • HS reverses effacement within 15 minutes • Proteinuria cannot be monitored</td>
</tr>
<tr>
<td>Adriamycin/ Doxurubicin (rat)</td>
<td>Focal segmental glomerulosclerosis (FSGS)</td>
<td>• Doxorubicin causes damage through two mechanisms: a) by intercalating with DNA and inhibiting transcription and DNA repair, and b) by generating free radicals, leading to damage of cellular membranes, DNA and proteins</td>
<td>• Produces FSGS-like lesions • Animals develop proteinuria after 10 days with foot process effacement and eventual development of glomerulosclerosis</td>
</tr>
<tr>
<td>Passive Heymann Nephritis (PHN) (rat)</td>
<td>Membranous Nephropathy (MN)</td>
<td>• Auto-antibodies against intrinsic podocyte antigens, generated after injection with crude kidney extracts in Freund's adjuvant • Immune complex accumulation occurs within the sub-epithelial region of the GBM below podocyte foot</td>
<td>• Proteinuria becomes evident within 5 days in the “heterologous phase” • This is followed by an autologous phase during which rat IgG antibodies with specificity for the heterologous glomerular-bound IgG are deposited,</td>
</tr>
</tbody>
</table>
processes, followed by complement activation and podocyte injury

| Streptozocin (STZ) (mouse) | Type 1 Diabetes (induced) | • STZ induces cell death by causing alkylation of DNA  
• It is preferentially cytotoxic to pancreatic beta-cells because of the high expression of GLUT2 glucose transporters here (the mechanism STZ uses to enter cells), ultimately leading to type 1 diabetes  
| Akita mice | Spontaneous Type 1 Diabetes | • Mice harbor a spontaneous mutation in the insulin 2 gene, which ultimately leads to reduced insulin secretion by pancreatic beta-cells  
| Zucker Diabetic Fatty (ZDF) rats | Obesity/ Type 2 Diabetes | • Harbor a spontaneous mutation resulting in the development of obesity, leading to the development of type 2 diabetes-like characteristics.  
• Hyperglycemia, proteinuria and reduced kidney function  
• Hypoinsulinemia  
• Progression of disease leads to some characteristics of glomerulosclerosis, but not as severe as human counterparts  
| | | • Hyperglycemia, proteinuria and reduced kidney function  
• Hypoinsulinemia  
• Progression of disease leads to some characteristics of glomerulosclerosis, but not as severe as human counterparts  
• Hyperlipidemia, glucose intolerance, obesity, hyperinsulinemia |
Table 1.4 Summary of published antibodies raised against tyrosine phosphorylated nephrin residues. Grey font represents findings from this thesis work. H= human; M= mouse; Ra= rat; * = antibodies not commercially available; U= unpublished; D= development; A= adult; NTS\textsuperscript{A} and NTS\textsuperscript{C} = acute and chronic forms of NTS; PS= Protamine Sulfate; LPS= Lipopolysaccharide; NTS= Nephrotoxic Serum; PAN= Puromycin Aminonucleoside; MCD= Minimal Change Disease; MN= Membranous Nephopathy; ANGII= Angiotensin II.

<table>
<thead>
<tr>
<th>Target</th>
<th>Source</th>
<th>Actual Specificity</th>
<th>HEALTH</th>
<th>DISEASE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1176</td>
<td>Jones (95)</td>
<td>Y1176 and Y1193</td>
<td>H M Ra</td>
<td>-Increased in PS (118) -Decreased LPS (6, 24h) (118) -Increased NTS\textsuperscript{A} (2h\textsuperscript{U}, 4h\textsuperscript{U}) -Decreased -NTS\textsuperscript{A} (6, 24h) (119) -Decreased NTS\textsuperscript{A} (48h) (74)</td>
</tr>
<tr>
<td></td>
<td>Holzmann (54)</td>
<td>Y1176 and Y1193*</td>
<td>D&gt;A (54)</td>
<td>-Decreased in LPS (24h) (158)</td>
</tr>
<tr>
<td>1193</td>
<td>Uchida (116)</td>
<td>?*</td>
<td>A (116)</td>
<td>-Increased in PAN (d 7,14) (157)</td>
</tr>
<tr>
<td></td>
<td>Jones (95)</td>
<td>Y1193*</td>
<td>A (95)</td>
<td>-Decreased in PAN (d 3,7,14) (116)</td>
</tr>
<tr>
<td></td>
<td>Uchida (116)</td>
<td>?*</td>
<td>A (116)</td>
<td>-Decreased in PAN (d 3,7,14) (116)</td>
</tr>
<tr>
<td>1217</td>
<td>Jones (95)</td>
<td>Y1217</td>
<td>A (95)</td>
<td>-Decreased in PS (118) -Decreased LPS (6, 24h) (118) -Decreased NTS\textsuperscript{A} (6, 24h) (119) -Decreased NTS\textsuperscript{C} (d 18) (159) -Increased NTS\textsuperscript{A} (48h) (74) -Decreased ANGII (91)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>A (95)</td>
<td>-Decreased in PAN (d 4&lt; 7,14) (95)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>A (95)</td>
<td>-Decreased in PAN (d 4&gt; 7,14) (157)</td>
</tr>
</tbody>
</table>
Figure 1.1 Components of the kidney’s glomerular filtration barrier. (A) The kidney is supplied by blood from the afferent artery (AA) and filtered blood exits through the efferent artery (EA), while urine travels to the bladder through the ureter. Within the kidney cortex lies the glomerulus (B), which is the site of blood filtration. (C) Glomerular capillaries are surrounded by a web of podocyte processes that extend from their cell bodies, ultimately culminating in interdigitating foot processes (FP). (D) Filtration selectivity is determined by the glomerular filtration barrier (GFB), which is made up of the fenestrated endothelium of the capillary wall, the glomerular basement membrane (GBM), and podocyte FPs. The slit diaphragm (SD) lies between adjacent FPs and acts as a barrier and macromolecular filter. During disease, the ultra-fine structure of the FPs and GBM is often disrupted, leading to a loss of SDs. (E) Transmission electron micrographs display the GFB illustrated within the cartoon drawings under healthy and diseased conditions. Scale: 2 µm.
Figure 1.2 Representation of the nephrin signaling network. Nephrin signaling pathways converge on several diverse cellular processes including cytoskeletal organization, cell survival, calcium signaling, blood pressure regulation, lipid raft assembly, cell polarity, nephrin tyrosine phosphorylation and nephrin turnover. For completeness, this figure summarizes data up to the date of thesis submission. Grey font represents findings from this thesis work.
Figure 1.3 Conserved nephrin binding motifs and their key binding partners. Conserved tyrosine, serine/threonine, arginine and lysine residues on nephrin’s cytoplasmic tail have been labeled according to the amino acid position in the human sequence. Binding partners interacting directly with these motifs are shown, and those requiring phosphorylation are indicated with a ‘P’. Some complementary and competitive interactions have been denoted.
Figure 1.4 Molecular architecture of the podocyte slit diaphragm. Nephrin is the primary component of the slit diaphragm (SD). Cross-linking of nephrin molecules on adjacent foot processes create a porous molecular sieve that allows small blood solutes to pass while blocking larger molecules and blood proteins. Several phosphorylated tyrosines on nephrin’s cytoplasmic tail link the SD to actin nucleators through binding partners including the Nck family of adaptors. This maintains healthy foot (FP) process structure. Disruption of nephrin tyrosine (Y) phosphorylation (P) by mutation of tyrosine residues to phenylalanines (F; nephrin-Y3F) disrupts interactions between nephrin and the actin cytoskeleton, resulting in FP effacement and loss of filtration selectivity.
CHAPTER 2:
MATERIALS AND METHODS
2.1 *In vivo* investigations

2.1.1 Subjects

2.1.1.1 Human Patients

Formalin-fixed paraffin embedded samples from freshly curated human biopsy material were obtained from the McGill University Health Centre Kidney Disease Biorepository. Information on human patients has been summarized in Supplemental Table 5.1.

2.1.1.2 Mice

Nephrin-Y3F knockin mice were generated by homologous recombination targeting mouse Y1191, Y1208 and Y1232 (corresponding to human Y1176, Y1193 and Y1217) to phenylalanine (F) as described previously (118). Animals on the CD-1 background were used for studies analyzing spontaneous disease in the nephrin-Y3F model and indicated throughout. Animals on the C57BL/6N background were used for all injury model studies, at 2-6 months of age, which is prior to the onset of significant renal injury. Animal age and sex is indicated in the description of each model.

2.1.1.3 Rats

Male Sprague-Dawley rats (250 to 300 g) were obtained from Charles River (St. Constant, Quebec).

2.1.1.4 Study approval

Informed consent was obtained to use kidney biopsy samples left unused after clinically-indicated biopsies. This study was approved by the Research Ethics Board of the McGill University Health Centre. Animal studies were carried out in accordance with Canadian Council on Animal Care protocols and approved by the University of Guelph Animal Care Committee.
2.1.2 Acute injury models

2.1.2.1 Lipopolysaccharide (LPS) model

Non-proteinuric female C57BL/6 nephrin-WT and nephrin-Y3F mice between the ages of 3 and 6 months were used for this experiment. Mice were injected intraperitoneally (i.p.) with 200 mg LPS (1 mg/ml in phosphate-buffered saline (PBS); L2630; Sigma-Aldrich, St. Louis, MO) or an equal volume of PBS. Spot urine samples were collected before injection, at 24 hours after injection, and for recovery analysis, every 12 hours until 72 hours after injection. For biochemical analyses, mice were euthanized with CO₂ at 6 and 24 hours after injection; kidneys were removed and immediately processed for glomerular isolation.

2.1.2.2 Protamine sulfate (PS) and heparin sulfate (HS) model

Non-proteinuric male and female C57BL/6 nephrin-WT and nephrin-Y3F mice aged 8–12-weeks old were used in this model. All perfusion solutions were maintained at 37°C in a water bath. Mice were anesthetized with a 2%: 98% mix of isoflurane: oxygen and kept warm with a lamp.

The abdomen was opened, and a 27-gauge tube was inserted into the abdominal aorta just below the renal arteries. The inferior vena cava was then nicked below the renal veins, and 10 ml warmed HBSS was infused at a rate of 5 ml/min by hand. The kidneys were then perfused at a rate of 4.5 ml/min using a Pump 11 Elite Infusion Syringe Pump (Harvard Apparatus, Holliston, MA) via the abdominal aorta serially with Hank’s Balanced Salt Solution (HBSS) for 2 minutes, protamine sulfate (2 mg/ml; Sigma-Aldrich) for 15 minutes, HBSS for 2 minutes, and heparin sulfate (800 mg/ml) for 15 minutes. Kidneys were immediately processed for glomerular isolation or placed in appropriate fixative for further processing.

2.1.2.3 Nephrotoxic serum (NTS) model

Male C57BL/6N mice (475, Charles River Laboratories Canada) aged 8–10 weeks were injected with 0.04 mg/g of IgG2 subclass sheep anti-rat nephrotoxic serum (a gift from Dr. David Salant, Boston University) via tail vein injection. Prior to injection, mice were warmed in an empty cage under a heat lamp for 15 min to induce tail vein dilation. Spot urine samples were
collected prior to and at various times after injection. At 0, 6, or 24 h post-injection, mice were euthanized with CO₂, and both kidneys were removed and immediately processed for glomerular isolation.

2.1.2.4 Puromycin aminonucleoside (PAN) model

Male Sprague-Dawley rats (250 to 300 g) were injected with a single dose of PAN (50 mg/kg body weight) to induce podocyte injury, as described previously (46).

2.1.3 Evaluation of proteinuria

Mice were placed into a metabolic cage until they urinated freely, and spot urines were collected. Rats were placed in metabolic cages for 24 hours and allowed to urinate freely. For Coomassie urine gels, urine samples (2 or 5 ml) were diluted in sodium dodecyl sulfate (SDS) sample buffer, separated by 10% SDS- polyacrylamide gel electrophoresis (PAGE), and stained with Coomassie brilliant blue R. The urinary albumin/creatinine ratio (ACR) was determined using the Albuwell M enzyme-linked immunosorbent assay (ELISA) (Exocell) and Creatinine Companion (Exocell) kits according to the manufacturer’s instructions. For time course recovery studies, the total urinary protein-to-creatinine ratio (in micrograms per milligram) was determined using standard protocols on the basis of a modified Bradford assay(160) and the Jaffe reaction.

2.1.4 Immunofluorescence of kidney tissue sections

2.1.4.1 Indirect immunofluorescence

Kidneys were either snap frozen in cryomatrix (Fisher Scientific) or fixed and embedded in paraffin prior to sectioning and immunostaining. Cryosections (6 µm) were dried at room temperature for 10 minutes, then fixed and permeabilized in acetone at -20°C for 10 minutes prior to immunostaining. For paraffin embedding, kidneys were first fixed in fresh 4% paraformaldehyde (PFA) (Thermo Scientific #28908) for 30 minutes at room temperature and washed 3x 30 minutes in PBS, then stored in 70% ethanol until embedding. Paraffin sections (4 µm) were de-paraffinized and re-hydrated using standard protocols. After antigen retrieval with heated 10 mM trisodium citrate dihydrate buffer, immunostaining was performed as with frozen
kidneys. All subsequent steps were carried out at room temperature. When using the phospho-
nephrin antibodies, phosSTOP tablets (Roche) were added to all solutions. Slides were blocked
for 1 hour in 10% goat serum, then incubated with primary antibodies for 1 hour (See Table 2.1
for summary). After 3 washes in PBS, slides were incubated with secondary antibodies (1:400)
for 1 hour. Slides were washed, then mounted using Prolong Gold anti-fade mounting medium
(Invitrogen). Epifluorescence images were obtained from cryosections using Volocity software
version 5.3.2 (Improvision) on a DMIRE2 microscope (Leica) using a 40x objective. Stacks were
captured at 0.2 µm z- intervals, then deconvolved using an iterative restoration function (95%
confidence with 15 iterations) in Volocity.

2.1.4.2 Super Resolution Structured Illumination Microscopy

For Super Resolution Structured Illumination Microscopy (SR-SIM) imaging, kidneys
were fixed and embedded in paraffin prior to sectioning. Prior to staining, slides were blocked
for 1 hour in 10% goat serum, then incubated with respective antibodies for 1 hour. After 3
washes in PBS, slides were incubated with anti-rabbit secondary antibodies (1:400) for 1 hour.
Slides were washed, then mounted using Prolong Gold anti-fade mounting medium (Invitrogen).
SR-SIM images were gathered using an Elyra LSM 880 microscope (Zeiss) and processed with
ZEN software (Zeiss) to obtain the SIM.

2.1.5 Ultrastructural analyses

For electron micrographs, sagittal slices (scanning electron micrographs (SEM)) and
small pieces (transmission electron micrograph (TEM)) of kidney tissue were fixed in 0.1 M
sodium cacodylate buffer containing 4% paraformaldehyde and 2% gluteraldehyde (Electron
Microscopy Sciences), postfixed in 1% OsO₄, and dehydrated through graded ethanols. TEM
samples were embedded in Quetol–Spurr resin. Ultrathin sections were cut and stained with
uranyl acetate and lead citrate and viewed using an FEI CM100 TEM. For SEM, samples were
critical point dried and sputter coated with gold. Samples were viewed using an FEI XL30 SEM
or Hitachi S-540 SEM (Hitachi, Yokohama, Japan).
2.1.6 Glomerular isolation

Mouse kidneys were dissected and cortices separated from medulla, minced and digested by incubating with 1 mg/mL type 4 collagenase (Worthington) in PBS at 37°C with 140 rpm agitation for 30 minutes. Digested cortices were passed through 100-mm sterile nylon cell strainer (431752, Corning,) with chilled PBS to obtain glomeruli. Red blood cells were lysed in sterile Ack lysis buffer (150 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM ethylenediaminetetraacetate (EDTA)) and glomeruli were pelleted by spinning at 3503g for 1 minute. Rat glomerular isolations were similarly performed through differential sieving as described previously (44).

2.2 Cell culture investigations

2.2.1 Cell culture

Human embryonic kidney (HEK)293T cells and Nck1/2 null mouse embryonic fibroblasts (MEF) (53) were grown in Dulbecco’s high glucose modified eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (both HyClone), 200 units/mL penicillin and 200 µg/mL streptomycin (Invitrogen) and maintained at 37°C with 5% CO₂. Mouse podocyte cells (MPCs) and human podocyte cells (HPCs) were grown in Roswell Park Memorial Institute (RPMI) 1640 with 10% FBS at 33°C and 5% CO₂ and thermoswitched to 37°C with 2% FBS for 14 days to induce differentiation.

2.2.2 Plasmids

Constructs encoding human FLAG-tagged Nck1/2, human Myc-tagged full-length and green fluorescent protein (GFP)-CD16/7-nephrin and fusions and mutants thereof, wildtype and constitutively active Fyn, podocin and Activator Protein (AP)-1 luciferase were generated previously (44, 53, 97, 119). Plasmids for Flag-tagged SH2 domain-containing (Shc)A variants in pcDNA3 were also generated previously (161), and used as templates to construct glutathione s transferase (GST)-fused ShcA SH2 and phosphotyrosine-binding domain (PTB) domains in pGEX-4T-1. Plasmid for Flag-tagged β-arrestin was provided by Dr. Stephen Ferguson (Western University, ON) and the plasmid for renilla luciferase was provided by Dr. Ray Lu (University of Guelph, ON). GFP-Dynamlin-WT and GFP-Dynamlin-K44A were provided by Dr. Costin
Antonescu (Ryerson University) and GFP-N-WASP was provided by Dr. Samantha Gruenheid (McGill University).

2.2.3 Transient transfections

Transient transfection of HEK293Ts was performed using polyethyleneimine (PEI) for 48 hours. Transient transfection of MEFs was performed using Lipofectamine 2000 (Invitrogen) as per manufacturer’s specifications.

2.2.4 Cell treatments

For CD16 clustering experiments, transfected cells were serum-starved for 12 hours before stimulation with 1 µg/mL anti-CD16 antibody for 15 or 30 minutes at 37°C. For inhibition of SFKs, cells were treated with 10 µM 4-amino-5-(4-chlorophenyl)-7-(t-buty1)pyrazolo[3,4-d]pyrimidine PP1 inorganic pyrophosphate (PP2) (Sigma-Aldrich) for 4 hours prior to CD16 stimulation. For PAN treatments, 10mg/mL of PAN (in PBS) was incubated with cells for 24 hours.

2.2.5 AP-1 luciferase assay

HEK293T cells were transfected with 1 µg AP-1 firefly luciferase, 80 ng renilla luciferase and additional vectors as indicated, to a total DNA amount of 2 µg/ 35mm dish. Serum starved cells were harvested on ice in 1 mL PBS, spun at 4,000 rpm at 4°C for 2 minutes, lysed in 100 µL of 1x passive lysis buffer (Promega) and spun again at 13,000 rpm at 4°C for 2 minutes to remove insoluble material. Luciferase activity was determined using a commercial dual luciferase assay system (Promega) on a POLARstar Omega microplate reader (BMG Labtech) and normalized to renilla activity to correct for transfection efficiency. Protein expression was confirmed by immunoblot.

2.2.6 Indirect immunofluorescence of cells

Prior to fixing, cells were gently washed 3 times in PBS to remove residual CD16 antibody. Slides were then fixed for 10 minutes in 4% PFA before either undergoing permeabilized in 1% TritonX-100 for 10 minutes to stain surface and cytosolic proteins or being left non-permeabilized to label surface proteins. Samples were then blocked for 1 hour in 10%
goat serum prior to further incubations. Select slides were then incubated with Texas-red phalloidin (30 minutes) or the indicated primary antibody for 1 hour. After 3 washes in PBS, slides were incubated with appropriate secondary antibodies (1:400) for 1 hour. Slides were washed, then mounted using Prolong Gold mounting medium (Invitrogen). Epifluorescence images were obtained using Volocity software version 5.3.2 (Improvision) on a DMIRE2 microscope (Leica) using a 40x objective. Stacks were captured at 0.2 μm z-intervals, then deconvoluted using iterative restoration (95% confidence with 15 iterations) in Volocity.

To view surface nephrin, non-permeabilized slides transfected with various GFP-CD16/7-nephrin constructs (visible in the 488 wavelength) were stimulated with mouse anti-CD16 antibody and stained with anti-mouse Alexa Fluor 594 (to label surface nephrin in the 594 wavelength). In the merged immunofluorescent image, surface nephrin, which appears in both the 488 (total, green) and 594 (surface alone, red) channels, appears yellow, while endogenous nephrin, protected from anti-mouse Alexa Fluor 594 labelling, remains green.

2.3 Biochemical analysis of glomerular and cellular samples

2.3.1 Biotinylation experiments

Isolated glomeruli or adherent cells were incubated with 5mL of 1 mg/mL EZ-Link Biotin-X-NHS dissolved in borate buffer (10 mM boric acid, 150 mM NaCl, pH 8.0) for 45 minutes under gentle agitation at 4°C. Coupling was terminated by washing 2 times (2 minutes each) with 15 mM glycine in PBS at 4°C. After a final wash in PBS, cells were lysed, and sonicated for 10 seconds. Lysates were then subjected to streptavidin precipitation overnight at 4°C or re-suspended in 2 x SDS loading buffer to act as the input loading control. After overnight precipitation, beads were washed 3 times successively in PLC+ and spun at 3,000 x g for 1 minute between washes before finally being re-suspended and boiled in 50 μL 2 x SDS loading buffer. Quantification of surface nephrin in each sample was determined by dividing the density of band observed in immunoblotting of the streptavidin precipitated sample by the density of band observed in the input sample. The ratio of this value in test samples to the value obtained in control samples was calculated and represents the amount of surface nephrin in indicated test groups relative to control samples.
2.3.2 Glomerular and cellular lysis

Glomeruli or cells were lysed in cold in PLC+ lysis buffer (PLC+) (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 15 mM MgCl₂, 1 mM [Ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA), 10 mM NaPPi, 100 mM NaF) supplemented with fresh protease and phosphatase inhibitors (1 mM Phenylmethylsulfonyl Fluoride (PMSF), 1 mM sodium orthovanadate, 10 µg/mL aprotinin, and 10 µg/mL leupeptin) by sonicating on ice for 10 seconds, incubating on ice for 5 minutes, and then, centrifuging at 14,000g for 10 minutes at 4°C. Protein concentrations in supernatants were determined using a bicinchoninic acid (BCA) Protein Assay (Pierce, Rockford, IL). Supernatants were mixed with appropriate amounts of SDS sample buffer and incubated at 100°C for 5 minutes.

2.3.3 Antibodies

Primary antibodies were used for IP (Table 2.2), WB (Table 2.3) and IF (Table 2.1). For IB detection, secondary horseradish peroxidase (HRP)-conjugated goat anti-mouse (170-6516, Biorad), goat anti-rabbit (170-6515, Biorad) and donkey anti-goat (sc-2020, Santa Cruz Biotechnology) antibodies were used at a 1:10,000 dilution. Secondary antibodies for IF (all Invitrogen) were goat anti-rabbit Alexa Fluor 488 (A11008) and 594 (A11037), goat anti-mouse Alexa Fluor 350 (A31553), Alexa Fluor 488(A11001) and 594 (A11005), and goat anti-guinea pig Alexa Fluor 594 (A11076) and were each used at a 1:400 dilution.

2.3.4 Immunoprecipitation

For IP of Nck or ShcA, protein A beads (Invitrogen) were first conjugated to respective antibodies to avoid interference from IgG heavy chain during IB as described elsewhere (162) with the following alterations: 0.5 ml antibody was incubated per 100 ml beads in 500 ml PBS for 1 hour at room temperature. Excess unbound antibody was removed by washing three times in PBS followed by spinning at 3000xg for 3 minutes; 100 ml 1.3 mg/ml disuccinimidyl suberate (DSS) (Thermo Fisher Scientific) in dimethyl sulfoxide (DMSO) was added to crosslink the antibody and beads in 500 ml PBS and left for an additional 1 hour at room temperature. Conjugated beads were successively washed 4x in tris-buffered saline (TBS) (0.02 M Tris base
and 0.15 M NaCl) and an additional 4x in 100 mM glycine to remove unconjugated antibody. Conjugated beads were washed four times in TBS before use; 100 ml conjugated anti-Nck and anti-ShcA beads were incubated with 2 mg glomerular lysate for 5 hours at 4°C with rotation.

IP of flag or ShcA from cells was performed overnight at 4°C with rotation. IP of GFP was performed for 1 hour at 4°C with rotation. IP of Nck or ShcA from glomeruli was performed for 5 hours at 4°C with rotation. IP incubations were followed by washing three times with PLC+ and protein complexes were eluted from the beads in 2 x SDS loading buffer by boiling at 100°C for 2 minutes prior to proceeding to IB.

2.3.5 Immunoblotting and spot peptide array

Protein samples were resolved on 10% SDS-PAGE gels with 10-100 ug protein loaded as total lysate and transferred to poly(vinylidene difluoride) (PVDF) membranes (EMD Millipore, Billerica, MA). Spot peptide arrays were constructed as described previously (53) and incubated with 1 µM eluted GST fusion proteins prior to Far Western analysis. Membranes were blocked in 5% skim milk or 5% bovine serum albumin (BSA) in TBS with tween (T). Membranes were incubated with primary antibodies overnight at 4°C. After washing, membranes were incubated with horse radish peroxidase (HRP)–conjugated secondary antibody for 1 hour at room temperature. Signals were detected using an enhanced luminol-based chemiluminescent (ECL) substrate (Pierce) or Luminata Crescendo (EMD Millipore). Blots were imaged using a ChemiDoc XRS+ (Bio-Rad) or exposed to film (Pierce). Densitometry was performed using ImageLab version 2.0 analysis software (Bio-Rad).

2.4 Nephroseq Analysis

Human SHCA gene expression data was downloaded from Ju et al. (163, 164) and Hodgin et al. (165) datasets using the Nephroseq data mining platform (www.nephroseq.org, 2017, University of Michigan, Ann Arbor, MI).

2.5 Statistical Analyses

Values are presented as mean standard error of means (S.E.M.). Differences between two groups were analyzed by t-test, and differences between more than two groups were analyzed by
analysis of variance (ANOVA) using SAS version 9.4 (SAS Institute Inc., Cary, NC). Graphs were prepared using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA). P* < 0.05 or P** < 0.05 were considered statistically significant.
**Table 2.1 Summary of primary antibodies and fluorescent conjugates used for immunofluorescence and SR-SIM experiments.** M= mouse; R= rabbit; GP= guinea pig; Ra= Rat; PBS= Phosphate Buffered Saline; IF= Immunofluorescence; SR-SIM= Super Resolution Structured Illumination Microscopy.

<table>
<thead>
<tr>
<th>Target Protein</th>
<th>Antibody Species (M/R/GP)</th>
<th>Source</th>
<th>Concentration Used (in PBS)</th>
<th>Species Reactivity (M/H/Ra)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEA1</td>
<td>M</td>
<td>610456; BD Biosciences</td>
<td>1:100</td>
<td>Ra</td>
<td>IF</td>
</tr>
<tr>
<td><strong>F-actin</strong></td>
<td></td>
<td>Texas-Red Phalloidin conjugate</td>
<td>T7471; Thermo Fischer Scientific</td>
<td>1:40</td>
<td>M/H/Ra</td>
</tr>
<tr>
<td><strong>FLAG M2</strong></td>
<td>M</td>
<td>F3165; Sigma-Aldrich</td>
<td>1:100</td>
<td>-</td>
<td>IF</td>
</tr>
<tr>
<td>Nephrin</td>
<td>R</td>
<td>Tomoko Takano (44)</td>
<td>1:50</td>
<td>M, H, Ra</td>
<td>SR-SIM</td>
</tr>
<tr>
<td>Nephrin</td>
<td>GP</td>
<td>20R-NP002; Fitzgerald</td>
<td>1:100</td>
<td>M, Ra</td>
<td>IF</td>
</tr>
<tr>
<td><strong>Phospho Nephrin (Y1217)</strong></td>
<td>R</td>
<td>Ab-80298; Abcam</td>
<td>1:50</td>
<td>M, H, Ra</td>
<td>IF</td>
</tr>
<tr>
<td>Podocin</td>
<td>R</td>
<td>P0372; Sigma-Aldrich</td>
<td>1:100</td>
<td>M, Ra</td>
<td>IF</td>
</tr>
<tr>
<td>ShcA</td>
<td>M</td>
<td>1:50</td>
<td>M, H, (Ra background high)</td>
<td>IF and SR-SIM;</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.2 Summary of primary antibodies used for immunoprecipitations. H= human; M= mouse; Ra= rat; R= rabbit; Y= yes; N=no; Pr-A= proteinA. Conjugation of antibodies (Ab) allows for the elimination of heavy and light chains in the immunoprecipitation (IP) eluant allowing for unencumbered visualization of the protein in the subsequent Western immunoblot.

<table>
<thead>
<tr>
<th>Target Protein</th>
<th>Source</th>
<th>Ab Species (H/R)/beads used</th>
<th>Volume/IP</th>
<th>Conjugation (Y/N)</th>
<th>Molecular Weight (kDa)</th>
<th>Species Reactivity (H/M/Ra)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLAG M2</td>
<td>F3165; Sigma-Aldrich</td>
<td>M/10% mouse beads</td>
<td>1 µl Ab/100uL beads</td>
<td>N</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>GFP</td>
<td>Abcam 290</td>
<td>R/20% Pr-A beads</td>
<td>.25 µl Ab/25 uL beads</td>
<td>N</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Myc 9E10</td>
<td>05-419; Millipore</td>
<td>M/10% mouse beads</td>
<td>1 µl Ab/100uL beads</td>
<td>N</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Nck 1794</td>
<td>Louise Larose</td>
<td>R/20% Pr-A beads</td>
<td>1 µl Ab/100uL beads</td>
<td>Y</td>
<td>50</td>
<td>M,H,Ra</td>
<td>Nck1&gt;&gt;Nck2</td>
</tr>
<tr>
<td>ShcA</td>
<td>Ab-24787; Abcam</td>
<td>R/20% Pr-A beads</td>
<td>1 µl Ab/100uL beads</td>
<td>Y</td>
<td>46,52,66</td>
<td>M, H</td>
<td></td>
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</tbody>
</table>
Table 2.3: Summary of primary antibodies used for Western immunoblotting. P= phospho; Ab= antibody; TBST=; BSA= Bovine Serum Albumin; H= human; M= mouse; Ra= rat; R= rabbit; G= goat.

<table>
<thead>
<tr>
<th>Target Protein</th>
<th>Ab Species</th>
<th>Source</th>
<th>Block (5% in TBST)</th>
<th>Concentration Used (in TBST)</th>
<th>Molecular Weight (kDa)</th>
<th>Species Reactivity (H/M/Ra)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akt (C67E7)</td>
<td>R #4691; Cell Signaling Technology</td>
<td>BSA</td>
<td>1:1000</td>
<td>63</td>
<td>H</td>
<td>Recognizes Akt1/2/3</td>
<td></td>
</tr>
<tr>
<td>Dynamin2</td>
<td>G sc-6400; Santa Cruz Biotechnology</td>
<td>BSA</td>
<td>1:500</td>
<td>100</td>
<td>M</td>
<td>Endogenous dirty</td>
<td></td>
</tr>
<tr>
<td>FLAG M2</td>
<td>M F3165; Sigma-Aldrich</td>
<td>Milk</td>
<td>1:1000</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fyn (Fyn3)</td>
<td>R sc-16; Santa Cruz Biotechnology</td>
<td>Milk</td>
<td>1:1000</td>
<td>60</td>
<td>H</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH 1D4</td>
<td>M G041; Applied Biologic Materials Inc.</td>
<td>Milk</td>
<td>1:2000</td>
<td>35</td>
<td>M, H</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GFP</td>
<td>R Abcam 290</td>
<td>Milk</td>
<td>1:2000</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GFP B2</td>
<td>M sc-9996; Santa Cruz Biotechnology</td>
<td>Milk</td>
<td>1:1000</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GST (B-14)</td>
<td>M sc-138; Santa Cruz Biotechnology</td>
<td>Milk</td>
<td>1:1000</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myc 9E10</td>
<td>M 05-419; Millipore</td>
<td>Milk</td>
<td>1:1000</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nck 108</td>
<td>M 610100; BD Transduction Laboratories</td>
<td>Milk</td>
<td>1:500</td>
<td>47</td>
<td>M, H</td>
<td>Nck1&gt;Nck2</td>
<td></td>
</tr>
<tr>
<td>Nck 1794</td>
<td>R Louise Larose</td>
<td>BSA</td>
<td>1:2000</td>
<td>47</td>
<td>M, H, Ra</td>
<td>Nck1&gt;&gt;Nck2</td>
<td></td>
</tr>
<tr>
<td>Nphrin</td>
<td>R Tomoko Takano</td>
<td>BSA</td>
<td>1:1000</td>
<td>180/ 110</td>
<td>M, H, Ra</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-Nphrin (Y1176/1193)</td>
<td>R Ab-80299-Abcam</td>
<td>BSA</td>
<td>1:1000-1:2500</td>
<td>180</td>
<td>M, H, Ra</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-Nphrin (Y1217)</td>
<td>R Ab-80298;Abcam</td>
<td>BSA</td>
<td>1:1000-1:2500</td>
<td>180</td>
<td>M, H, Ra</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-Shc (Y317)</td>
<td>R #2431; Cell Signaling Technology</td>
<td>BSA</td>
<td>1:1000</td>
<td>50,55,70</td>
<td>H</td>
<td>Endogenous dirty</td>
<td></td>
</tr>
<tr>
<td>P-Src (Y416; active)</td>
<td>R #2101; Cell Signaling Technology</td>
<td>BSA</td>
<td>1:1000</td>
<td>60</td>
<td>H</td>
<td></td>
<td></td>
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<tr>
<td>P-Akt (Ser473) D9E</td>
<td>R #4060; Cell Signaling Technology</td>
<td>BSA</td>
<td>1:1000</td>
<td>63</td>
<td>H</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Podocin</td>
<td>R P0372; Sigma-Aldrich</td>
<td>BSA</td>
<td>1:1000</td>
<td>46</td>
<td>M, Ra</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospho Y (4G10)</td>
<td>M 16-101; Upstate Biotechnology</td>
<td>BSA</td>
<td>1:500-1:1000</td>
<td>-</td>
<td>M, H</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ShcA</td>
<td>R Ab-24787; Abcam</td>
<td>BSA</td>
<td>1:2000</td>
<td>46,52,66</td>
<td>M, H</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubulin (B-5-1-2)</td>
<td>M T5168; Sigma-Aldrich</td>
<td>Milk</td>
<td>1:2000</td>
<td>60</td>
<td>Ra</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin AC15</td>
<td>M A1978; Sigma-Aldrich</td>
<td>Milk</td>
<td>1:2000</td>
<td>46</td>
<td>M, H, Ra</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 3:
NEPHRIN TYROSINE PHOSPHORYLATION IS DISRUPTED IN VARIOUS KIDNEY INJURY MODELS AND IS REQUIRED FOR RECOVERY FROM DISEASE (118, 119)
3.1 Abstract

Podocytes are specialized epithelial cells of the kidney blood filtration barrier that contribute to permselectivity via a series of interdigitating actin–rich foot processes. Positioned between adjacent projections is a unique cell junction known as the slit diaphragm, which is physically connected to the actin cytoskeleton via the transmembrane protein nephrin. Evidence indicates that tyrosine phosphorylation of the intracellular tail of nephrin initiates signaling events, including recruitment of proteins that regulate actin cytoskeletal dynamics. Nephrin-Y3F mice, which possess tyrosine-to-phenylalanine replacements at three of these key tyrosine residues, develop spontaneous disease characterized by foot process effacement. Likewise, most forms of acquired human and experimental renal disease are characterized by pathologic foot process remodeling. Conflicting reports remain on whether nephrin tyrosine phosphorylation is altered in disease and how this might affect foot process remodeling. We now demonstrate that nephrin tyrosine phosphorylation is decreased in the commonly utilized PS, LPS and NTS experimental kidney disease models. Furthermore, compared with wild-type mice, nephrin-Y3F mice display heightened disease and delayed recovery in podocyte injury models. Profiling in wild-type mice subjected to podocyte injury indicated site-specific changes in nephrin tyrosine phosphorylation at baseline, injury, and recovery, and nephrin’s ability to interact with the Nck cytoskeletal adaptor protein was transiently lost in the PS and LPS models during injury. Our results define an essential requirement for nephrin tyrosine phosphorylation in stabilizing podocyte morphology during disease, and conferring plasticity to the podocyte actin cytoskeleton.

3.2 Introduction

Blood filtration in the kidney is critically dependent on the size-selective barrier wall of the glomerulus, which is composed of an inner layer of fenestrated endothelium, a GBM and an outer layer of podocytes. Differentiated podocytes adopt a unique and complex three-dimensional architecture that is fundamental to their function (166, 167). Their cell bodies extend numerous microtubule-based major processes, which branch into an interdigitating network of smaller actin-rich FP bridged by SDs that serve as the final filtration element to limit loss of urinary protein. Podocyte injury has emerged as a significant contributor to many forms
of renal disease and is characterized by remodeling of the actin cytoskeleton, loss of SDs, and FP effacement, leading to proteinuria (168).

The SD is a specialized cell-cell junction that is anchored to the actin cytoskeleton via a series of transmembrane proteins, including the central scaffolding protein nephrin (169). Mutations in nephrin in humans and rodents lead to FP effacement and proteinuria. The Fin<sup>minor</sup> mutation, which results in near-complete truncation of the short cytoplasmic tail of nephrin, causes a similar disease phenotype as complete loss of nephrin (17), suggesting that this segment is essential for nephrin function. Within this segment, there exists a number of highly conserved tyrosine residues that, once phosphorylation by Fyn kinase (53, 54, 61), serve as docking sites for intracellular signaling proteins (1). Through recruitment of actin adaptors, such as p85/PI3K (46, 85), the Cas/Crk complex (70), and Nck1/2 (53, 54, 75), nephrin phosphorylation is postulated to facilitate direct and dynamic connection to the podocyte cytoskeleton. Moreover, Nck enhances nephrin phosphorylation via activation of Fyn (106), and loss of Nck within podocytes leads to reduced nephrin tyrosine phosphorylation and widespread FP effacement (95, 106), inferring a reciprocal relationship between Nck and nephrin in the maintenance of podocyte structure.

Nephrin is tyrosine phosphorylated during glomerulogenesis and throughout life (44, 54, 95), and decreases in nephrin phosphorylation on Y1217 are seen coincident with FP effacement in human kidney diseases, including MCD (116) and MN (117). Similarly, reduced phosphorylation of the downstream survival factor Akt on serine (S) 473 can be observed in MCD (158), and inactivation of the actin binding protein cofilin is detected in patients with MCD, MN, and FSGS (170). These observations raise the intriguing possibility that uncoupling of phospho-nephrin signaling from actin leads to pathologic disruptions in FP morphology in human disease.

We recently developed the nephrin-Y3F mouse model to directly test the role of phosphorylated nephrin in the complex in vivo environment (118). Nephrin-Y3F mice possess tyrosine-to-phenylalanine replacements at Y1176, Y1193 and Y1217, abrogating the ability of these residues to become phosphorylated upon nephrin engagement. The collective loss of nephrin phosphorylation at these sites results in spontaneous disease in mouse models.
characterized by FP effacement, GBM abnormalities and proteinuria. These data support a role for nephrin tyrosine phosphorylation in long-term maintenance of the barrier. We reasoned that this might reflect a similar requirement for nephrin phosphorylation in controlling dynamic actin regulation and podocyte remodeling in response to various insults. To directly test this, we will now employ several established podocyte injury models in nephrin-WT and nephrin-Y3F mice and investigate alterations in nephrin’s tyrosine phosphorylation as well as downstream effects. We demonstrate that nephrin tyrosine phosphorylation is decreased at key timepoints in the PS, LPS and NTS acute injury models. Recovery from disease induction is delayed in nephrin-Y3F mice in the PS and LPS models and biochemical investigations revealed that this was associated with nephrin-Nck uncoupling. This is in accordance with a key requirement for nephrin tyrosine phosphorylation and Nck recruitment in protection from damage and promotion of disease recovery.

3.3 Results

3.3.1 C57BL/6 nephrin-Y3F mice show impaired recovery from protamine sulfate and lipopolysaccharide podocyte injury

To test whether nephrin tyrosine phosphorylation is required to preserve glomerular function in response to disease, we initially utilized the PS and LPS podocyte injury models, which each stimulate acute, reversible injury associated with FP effacement. We used these two models in young C57BL/6 nephrin-WT or nephrin-Y3F mice, prior to the development of spontaneous disease, which allowed us to directly investigate whether phosphorylation of nephrin tyrosine residues is required for disease initiation and/or recovery.

We first used the PS model, where perfusion of PS causes rapid FP effacement, which can be reversed through subsequent perfusion with HS (54, 171). Kidneys within nephrin-WT and nephrin-Y3F mice were perfused with HBSS control buffer, PS, or PS followed by HS, fixed, and analyzed by TEM. Compared with buffer-treated animals, mice from both genotypes perfused with protamine sulfate showed extensive FP spreading (Figure 1, A and B). Interestingly, however, although podocyte FP architecture was restored to baseline after HS treatment in nephrin-WT animals, this recovery was impaired in the majority of nephrin-Y3F glomeruli analyzed.
We next examined the LPS model of acute injury, whereby LPS induces transient mild albuminuria from 24-48 hours after injection, which correlates with FP effacement (171). Nephrin-WT and nephrin-Y3F mice were injected with PBS or LPS, and spot urine samples were collected at regular intervals up to 72 hours after injection. Urinary protein was increased at 24 hours post-LPS injection in nephrin-Y3F mice compared with control mice (Figure 3.2A), and although proteinuria started to decline at 24 hours in both genotypes, nephrin-Y3F mice still showed elevated levels at 36 hours post-injection (Figure 3.2B). Quantitation of the urinary ACRs in mice before (0 hours) and 24 hours after injection showed that LPS treatment induced a significant increase in albuminuria in nephrin-Y3F mice compared with nephrin-WT mice and that injection of PBS alone did not alter urinary protein levels in either genotype (Figure 3.2C). Ultrastructural analysis using SEM demonstrate significant alterations in both nephrin-WT and nephrin-Y3F animals as compared to PBS control mice (Figure 3.2D). Collectively these findings are consistent with a defect in FP restoration in nephrin-Y3F mice.

3.3.2 Nephrin tyrosine phosphorylation and binding to Nck are altered during podocyte effacement

The recovery defect in nephrin-Y3F mice prompted us to explore the dynamics of nephrin signaling that occur coincident with FP actin remodeling in these injury models. First, glomeruli were isolated from nephrin-WT mice exposed to PS or LPS challenge and IB with phosphospecific nephrin antibodies recognizing Y1176/Y1193 and Y1217 (95). Within the PS model, we observed a significant increase in nephrin phosphorylation on Y1176/Y1193 after PS perfusion, which decreased after HS recovery (Figure 3A and B). Relative to PS–treated mice, Y1176/Y1193 was weakly phosphorylated in glomeruli perfused with buffer alone. By contrast, Y1217 was highly phosphorylated with buffer alone and after HS treatment, and a modest decrease in phosphorylation on this tyrosine was observed after PS perfusion (Figure 3A and C). Strikingly, this transient decrease in phosphorylation of Y1217 after PS was paralleled by reduced interaction between nephrin and Nck, which was likewise restored after HS treatment (Figure 3A). Similar findings were obtained within the LPS model. At 6 hours post-LPS injection, which precedes marked proteinuria, we detected a significant decrease in nephrin phosphorylation on Y1217 and reduced association of nephrin with Nck, both of which were restored by 24 hours when proteinuria peaks and subsequently resolves (Figure 3D and F). No
changes in Y1176/Y1193 were detected at either time point analyzed (Figure 3D and E). These results further support a role for Nck in nephrin phosphotyrosine-based signaling pathways in preservation and restoration of FP architecture and filtration barrier function in the face of podocyte injury.

3.3.3 Nephrin tyrosine phosphorylation is altered during nephrotoxic serum-induced disease

The acute NTS-mediated disease has recently become a popular model within the field because of its ability to directly target the glomerulus during disease induction (70, 74, 172). We thus decided to investigate whether NTS injections results in nephrin tyrosine phosphorylation alterations. To first investigate the timecourse of disease induction in this model, 8-week old C57BL/6 mice were injected with sheep anti-rat glomerular NTS and spot urines were collected over 96 hours. Coomassie urinalysis determined that NTS injection induces nephrotic range proteinuria within 24 hours, which resolves by 72-96 hours post-injection (Figure 3.4A).

We next investigated whether alterations in nephrin tyrosine phosphorylation were apparent in this model. Based on the urine timecourse, we chose to investigate the 6 hour and 24 hour timepoints, which represented timepoints during the initiation period, before significant albuminuria was present, and the peak in albuminuria respectively. We determined that nephrin tyrosine phosphorylation on Y1176/93 was decreased at both 6 and 24 hours post injection with NTS (Figure 3.4B and C). A similar decrease in phosphorylation at Y1217 was observed at 24 hours (Figure 3.4D). Future investigation of whether disease is affected by the nephrin-Y3F mutation will provide a better understanding of nephrin phosphorylation’s role in the initiation and recovery from NTS-induced disease.

3.4 Discussion

The potential for the cytoplasmic tail of nephrin to be regulated by tyrosine phosphorylation has been recognized since the initial characterization of nephrin (17, 77). Given the clinical relevance of alterations in nephrin expression and function, numerous efforts have been made to determine the role of this phosphorylation. The nephrin-Y3F knockin mouse model now provides unequivocal evidence that phospho-nephrin signaling is crucial for restoration of
podocyte cytoarchitecture after injury and that this may be due to a critical requirement for interactions between nephrin and Nck.

The delayed phenotype of nephrin-Y3F mice on the C57BL/6 background (118) allowed us to use these animals to explore the dynamics and overall necessity of nephrin tyrosine phosphorylation in experimental models of effacement, proteinuria, and recovery, which mimic the podocyte injury associated with acquired glomerulopathies. Nephrin-Y3F mice undergo FP spreading on PS perfusion and enhanced LPS-induced proteinuria, clearly showing that tyrosine phosphorylation of nephrin on these sites is not required to actively promote effacement in these models (173). In concordance with our findings, recent work has established nephrin-independent Rac activation as an important pathomechanism that drives effacement (70, 174, 175). Instead, we find that FP recovery is impaired in nephrin-Y3F animals in both injury models, similar to that observed in mice lacking the actin regulators synaptopodin and cofilin (96, 176). Taken together, our data support a model wherein nephrin tyrosine phosphorylation is required to counteract normal podocyte stress and loss of this phosphorylation, as seen in nephrin-Y3F mice and some human diseases, leads to irreversible injury and chronic disease.

The injury phases in the disease models studied herein are coincident with de-phosphorylation of nephrin Y1217 and uncoupling from Nck as well as decreased pS473Akt in the LPS model (158). Reductions in nephrin phosphorylation, Nck/p85 binding, and Akt activation have also been detected in the PAN model of reversible podocyte injury (46, 75, 95, 116) in parallel with increased expression of the phosphatase PTP1B, which can dephosphorylate nephrin Y1217 (66). By contrast, phosphorylation of nephrin on Y1176/Y1193 is increased with protamine sulfate as noted previously (54, 61). However, this change does not occur in the LPS model, and it does not seem to be essential for effacement; thus, the significance of this site remains to be elucidated. Further, phosphorylation at all 3 YDxV sites appears to be reduced during the peak of injury in the NTS model, which was recently shown to involve nephrin endocytosis and barrier breakdown (159). We posit that differential phosphorylation on particular tyrosines may facilitate preferential recruitment of specific signaling proteins to nephrin, such as Nck, as well as p85/PI3K, β-arrestin, and Cas/Crk, which in turn, control distinct processes that regulate FP remodeling. In the context of Nck, YDxV phosphopeptides show similar binding affinities for the isolated SH2 domains (97); however, Y1217 is found
within a YDQV motif in contrast to the YDEV motif of both Y1176 and Y1193, and Nck seems
to have the highest affinity for Y1217 in vitro (53).

In summary, since induced loss of Nck in podocytes results in rapid demise of the
filtration barrier (95) and the inability to induce phosphorylation on Y1217 and subsequent Nck
binding in nephrin-Y3F mice impairs the rapid actin remodeling required for reformation of FPs,
we refine the existing model to include Nck as a critical signaling axis between nephrin Y1217
and the actin cytoskeleton to maintain podocyte plasticity. Interactions between Nck and Y1176
and Y1193 may hold additional refined roles in actin reorganization or in other yet to be
investigated pathways and the site-specific role of nephrin tyrosines remain of interest to
investigate in future studies. Establishment of this causal relationship may support the
development of novel therapeutic approaches for human proteinuric kidney diseases that
selectively preserve nephrin phosphorylation and thereby the podocyte actin cytoskeleton.
Figure 3.1 C57BL/6 nephrin-Y3F mice display delayed recovery in the protamine sulfate and heparin sulfate reversible podocyte injury model. (A) Transmission Electron Micrographs of foot processes in nephrin-WT (WT) and nephrin-Y3F (Y3F) mice perfused with HBSS, protamine sulfate (PS), or protamine sulfate followed by heparin sulfate (PS/HS). PS induces foot process spreading in both genotypes. A proportion of foot processes is restored with HS in Y3F mice (region 1), although the majority remains spread (region 2, arrows). Images are representative of two mice of each genotype per condition. Scale bar, 2 mm. (B) Box and whisker (5%-95%) plots of individual foot process widths measured via transmission EM (n=2 kidneys analyzed per treatment with a minimum of 60 measurements per mouse). Comparisons were made between treatments as indicated. \*P<0.05 by ANOVA.
Figure 3.2 C57BL/6 nephrin-Y3F mice show enhanced lipopolysaccharide-induced podocyte injury. (A) Coomassie stained gels of consecutive urine samples from nephrin-WT (WT) and nephrin-Y3F (Y3F) animals injected with equal volumes of PBS (control) or lipopolysaccharide (LPS). (B) Protein-creatinine ratios demonstrate 24 h (hours) as the peak of proteinuria in WT and Y3F mice. Quantification of total urinary protein (ug) normalized to urine creatinine (mg) shows that Y3F mice have an enhanced response to LPS–induced podocyte injury at 24 and 36 h compared with control (Y3F: n=8–13 per time point; WT: n=4–6 per time point except at 60 h, where n=3). (C) Quantification using albumin/creatinine ratio (ACR; ug/mg) (n=4-5/genotype). *P<0.05 by ANOVA. (D) Scanning Electron Micrographs of podocyte foot processes in WT and Y3F animals 24 h after injection with PBS or LPS. Foot processes in WT mice show mild retraction while those in Y3F mice are regionally effaced (asterisk). Scale bar: 2 µm.
Figure 3.3 Analysis of nephrin tyrosine phosphorylation and Nck binding in glomeruli isolated from wildtype mice exposed to podocyte injury. (A) Nephrin tyrosine phosphorylation and nephrin/Nck co-immunoprecipitation (IP) after injection with HBSS, protamine sulfate (PS), or protamine sulfate followed by heparin sulfate (PS/HS). Interaction of nephrin with Nck is decreased on PS perfusion. (B and C) Densitometric quantitation of results in A shows a significant increase in nephrin tyrosine phosphorylation on Y1176/Y1193 but not Y1217 in PS-treated samples. (D) Nephrin tyrosine phosphorylation and nephrin/Nck IP 6 and 24 h (hours) after injection with PBS or lipopolysaccharide (LPS). Interaction of nephrin with Nck is decreased at 6 h after LPS injection. (E and F) Densitometric quantitation of results in D shows a significant decrease in nephrin tyrosine phosphorylation on Y1217 but not Y1176/Y1193 at 6 h after LPS injection. The fold change in nephrin phosphorylation was normalized to control samples (i.e., HBSS or PBS). Mice were analyzed individually, and results are representative of 3-5 mice per treatment. *P<0.05; **P<0.01.
Figure 3.4 Nephrotoxic serum induces loss of nephrin tyrosine phosphorylation throughout disease. (A) Coomassie-stained SDS-PAGE gel of urine collected from mice injected with nephrotoxic serum (NTS) at the indicated time points. Proteinuria peaked at 24 h (hours) post-injection, and animals recovered by 72–96 h post-NTS injection. (B) Representative immunoblots (IB) of glomerular lysates from mice injected with NTS at 0, 6, or 24 h for nephrin tyrosine phosphorylation at Y1176/Y1193 and total nephrin as well as GAPDH as a loading control. (C) Densitometry of B, showing statistically significant decreases in phosphorylation at Y1176/Y1193 in glomeruli of mice injected with NTS (n=3). *P<0.05; **P<0.01. (D) Representative IB of glomerular lysates from mice injected with NTS or control sheep immunoglobulin (IgG) serum at 0 or 24 h for nephrin tyrosine phosphorylation and total nephrin as well as Nck and GAPDH as controls. Similar to results with the Y1176/Y1193 antibody, phosphorylation using antibodies specific for Y1193 and Y1217 demonstrate a significant reduction in phosphorylation at 3 tyrosine residues at the 24 h peak timepoint in NTS animals.
CHAPTER 4:
MULTIVALENT NEPHRIN-NCK-ACTIN INTERACTIONS MODULATE NEPHRIN ENDOCYTOSIS (manuscript in prep)
4.1 Abstract

Nephrin constitutes the core of the slit diaphragm and its anchorage to the podocyte actin cytoskeleton is essential for barrier function. The slit diaphragm is susceptible to hemodynamic stress and regular turnover of its components facilitates barrier remodeling. Decreased nephrin tyrosine phosphorylation and nephrin mislocalization are commonly observed in glomerular disease and, although roles for nephrin tyrosine phosphorylation in actin plasticity and nephrin trafficking have been separately characterized, the interplay between these signaling events has not yet been considered. Here we characterize a requirement for the Nck adaptor protein in the recruitment of actin and the endocytic GTPase dynamin to tyrosine phosphorylated nephrin during its endocytosis. Heightened surface nephrin and arrested endocytic pits are observed in the effaced foot processes of animals harbouring a mutated form of nephrin in which these three tyrosine residues have been converted to phenylalanines (nephrin-Y3F), which abolishes nephrin/Nck signaling. Cells expressing nephrin-Y3F also accumulate nephrin on the surface in stalled endocytic pits and we further characterize a striking absence of actin recruitment to these tubulated invaginations. These phenotypes are recapitulated with mutation of Nck’s SH3 domains, while restoration of nephrin-Nck signaling through the nephrin-Y3F-Nck-SH3X3 fusion protein rescues arrested endocytosis and normalizes surface nephrin. Finally, we establish that filtration barrier demise in the nephrotoxic serum model of podocyte injury is driven by phospho-dependent removal of nephrin from the cell surface, and that nephrin-Y3F mice, which are sheltered from phospho-dependent endocytosis, are protected from injury. We conclude that regulation of phospho-nephrin and Nck signaling plays a pivotal role in nephrin’s internalization by facilitating ample recruitment of actin and dynamin to the site of endocytosis, and that alterations in this signaling mechanism disrupts the delicate balance of slit diaphragm turnover, leading to barrier breakdown.

4.2 Introduction

Podocytes are an essential component of the GFB. Positioned within the outer layer of the glomerulus, their ability to withstand the continuous mechanical forces associated with hemodynamic flow is attributed to their specialized actin-rich FPs (177). Podocyte FPs contribute to filtration selectivity via a unique intercellular junction known as the SD. Mounting evidence indicates that the SD is physically anchored to actin through the scaffold protein
nephrin (53, 95, 104, 105, 118), and that nephrin is mislocalized in numerous hereditary and acquired forms of kidney disease associated with FP effacement and proteinuria (38, 91, 117, 120, 121, 157). However, the molecular mechanisms that facilitate maintenance and replacement of integral SD components are poorly understood (122).

In addition to its structural role, nephrin also serves as a signaling platform to connect the SD to various pathways that dictate podocyte cell shape and adhesion, polarity and survival. Nephrin signaling is mediated via several conserved tyrosine residues on its intracellular tail, which, once phosphorylated by Src family kinases such as Fyn (47), bind key cytosolic SH2 domain-containing proteins including p85/p110 (85), PLC-γ1 (61), and Nck1/2 (53, 54). Recruitment of Nck to tyrosine phosphorylated nephrin via its SH2 domain induces actin nucleation via SH3 domain-dependent N-WASp (94, 100, 105) recruitment and subsequent activation of Arp2/3. Genetic evidence supports the fundamental importance of Nck (53, 54, 95) and N-WASp (101) signaling in shaping actin architecture within the podocyte, as demonstrated by congenital defects in FP formation in knockout animals. Intriguingly, the phenotype of animals harbouring mutations in the three Nck binding sites on nephrin (nephrin-Y3F) is less severe than cKO of Nck or N-WASp (118). Nephrin-Y3F animals show a progressive background-dependent phenotype that is characterized by FP disorganization and flattening, GBM thickening and proteinuria. Together these findings suggest a role for nephrin phospho-signaling in maintaining the integrity of the GFB, though the nature of this adult-onset phenotype warrants further investigation.

Tyrosine phosphorylation of nephrin has been further linked to its turnover at the cell surface, with its phosphorylation signature inducing differential recruitment of endocytic regulators such as podocin (123), CIN85 (62, 178), aPKC (90, 91, 143), β-arrestin (52, 90, 91), Vangl2 (144), PACSIN2 (179) and the GTPase dynamin (140). Of note, the N-WASp/ Arp2/3 complex is a well-established driver of endocytosis (130, 131), along with actin binding proteins cofilin (96) and α-actinin (180), which also play vital roles within the podocyte. Actin assembly can contribute at all stages of endocytosis and is a particularly important participant in endocytosis at membranes under strain (134, 135), helping to drive membrane invagination, narrowing/ elongation of invaginating pits and vesicle scission, which further requires dynamin
Despite these advances, the interplay between actin signaling and endocytosis in podocytes remains to be explored.

Here, we uncover a pivotal role for Nck in connecting the actin machinery to phosphorylated nephrin within the SD to facilitate nephrin turnover. Using our nephrin-Y3F mouse model and complementary cell-based approaches, we show that unphosphorylated nephrin accumulates on the cell surface, and that it resides within endocytic pits that are continuous with the membrane. We show a similar effect in the presence of Nck2 lacking SH3 domain functionality, and that fusion of the Nck2 SH3 domains to nephrin-Y3F can rescue the phenotype, owing to their ability to create a multivalent node for actin and dynamin recruitment. Lastly, we demonstrate that filtration barrier demise in the NTS model of podocyte injury is driven by a rise in nephrin phosphorylation which correlates with its removal from the cell surface, and that nephrin-Y3F mice are protected from injury in this model, as they cannot undergo phospho-mediated endocytosis. Altogether, these findings highlight a fundamental role for nephrin phosphorylation and Nck/actin signaling in regulating the dynamics of nephrin turnover and barrier integrity in the healthy and injured podocyte.

4.3 Results

4.3.1 Genetic disruption of nephrin phosphotyrosine signaling perturbs nephrin endocytosis

Changes in nephrin tyrosine phosphorylation have been correlated with its altered endocytosis, thus we were interested to determine whether nephrin-Y3F mice might exhibit defects in nephrin turnover. We first performed biotinylation assays on intact glomeruli isolated from 4-week old nephrin-Y3F mice and WT littermate controls on the CD-1 background, which shows an early proteinuric phenotype (118). Nephrin IB of avidin-enriched and lysate input fractions shows a significant increase in surface nephrin levels in mutant animals (Figure 4.1A and B). We confirmed this effect of the nephrin-Y3F protein in vitro, using biotinylation assays on HEK293T cells expressing full-length WT or Y3F nephrin (Figure 4.1C and D). We next sought to investigate the subcellular distribution of the nephrin-Y3F protein. Nephrin immunostaining of kidney sections from 4-week old CD-1 WT and Y3F mice revealed abnormal congregation of nephrin throughout the glomeruli of nephrin-Y3F mice, compared to its thin,
continuous linear organization in control animals (Figure 4.1E). In TEMs, we noted the appearance of unusual elongated endocytic structures within the FP of podocytes of nephrin-Y3F mice (Figure 4.1F). To further explore the nature of these cellular changes, we used CD16 fusion inducible nephrin clustering system in MEFs (53, 97). CD16/7 nephrin-WT or CD16/7 nephrin-Y3F (both GFP-tagged) were transfected into MEFs, and live cells were subject to CD16 antibody-induced clustering for 30 minutes. After clustering, cells were fixed and stained with fluorescent secondary antibody (red) in the absence of membrane permeabilization, to visualize surface nephrin. In the merged image, nephrin on the cell membrane appears yellow, while internalized nephrin, which was protected from secondary antibody, appears green. As we and others have reported (47, 53, 97), clustered CD16/7-nephrin-WT forms diffuse, small punctae throughout the cell that are mostly internalized (Figure 4.1G, top row). By contrast, we observed the formation of long, cylindrical nephrin aggregates or ‘tubules’ in many cells expressing CD16/7 nephrin-Y3F, and these structures are continuous with the cell surface (Figure 4.1G, bottom row). Similar to nephrin-Y3F, additional investigation of nephrin dual tyrosine point mutants (Y1176/1193F, Y1176/1217F and Y1193/1217F) revealed varying degrees of tubulation (Supplemental Figure 4.1). Tubules are most prominent when Y1193 is disrupted and least when Y1217 is disrupted, identifying phosphorylation at the Y1193 site as a key regulator of nephrin patterning. Collectively, these results confirm that defects in nephrin trafficking occur in vivo in the absence of nephrin tyrosine phosphorylation, and that this process can be reliably modelled in in vitro systems.

4.3.2 Disruption of Nck SH3 domain signaling promotes formation of tubulated nephrin and impairs actin assembly

Nephrin tyrosine phosphorylation is regulated in part by recruitment of the Nck actin adaptor proteins, and this effect is more pronounced with Nck2 than Nck1 (106). Thus we next sought to investigate whether disruption of nephrin signaling through Nck might induce a similar defect in endocytosis. MEFs lacking Nck1/2 expression were co-transfected with CD16/7 nephrin-WT-GFP and various FLAG-tagged Nck mutants. Following addition of CD16, samples were fixed and permeabilized to allow immunostaining for nephrin (GFP) and Nck (FLAG), as well as contrast-stained with Texas-red phalloidin to visualize F-actin. Expression of wildtype Nck2 (Figure 4.2, top panel) promoted the formation of discrete nephrin/Nck punctae which
were associated with polymerized actin, consistent with our previous findings (53). Nephrin clusters were similarly observed upon co-expression of the Nck2-SH2* mutant which cannot bind nephrin (Figure 4.2, bottom panel), though this mutant did not relocalize into punctae or induce actin polymerization at nephrin, as we have reported previously for the Nck1-SH2* mutant (97). Intriguingly, the Nck2-SH3*x3 mutant induced the formation of tubulated nephrin (Figure 4.2, middle panel), and Nck was colocalized into these elongated structures. As we showed earlier for the Nck1-SH3*x3 mutant (97), actin does not reorganize with the triple SH3 domain mutant of Nck2 (Figure 4.2, middle panel). Together these findings imply that binding of Nck to nephrin promotes actin-dependent internalization of nephrin, and that the Nck SH3 domains are critical for this response.

4.3.3 Nck2 SH3 domains rescue the tubulation phenotype induced by nephrin-Y3F mutation

Having identified a potential requirement for the Nck2 SH3 domains in localizing endocytic machinery to phosphorylated nephrin, we reasoned that fusion of this region to the defective nephrin-Y3F protein should restore complex formation and rescue the tubulation phenotype. Therefore, we generated a construct in which the 3 SH3 domains of Nck2 were cloned in-frame downstream of the intracellular tail of CD16/7 nephrin-Y3F, creating the CD16/7 nephrin-Y3F-Nck2-SH3x3-GFP fusion protein (119). CD16/7 nephrin-WT-GFP, CD16/7 nephrin-Y3F-GFP or CD16/7 nephrin-Y3F-Nck2-SH3x3-GFP were independently transfected into Nck1/2-null MEFs, followed by CD16 clustering and immunostaining. Cells expressing the fusion protein show diffuse nephrin punctae on the cell surface, reminiscent of the patterning of wildtype nephrin, and in contrast to the cylindrical surface aggregates in nephrin-Y3F-expressing cells (Figure 4.3A). Quantification indicates a total of 78% of nephrin-Y3F cells displaying this defective organization, whereas cells expressing nephrin-WT or the fusion protein displayed tubulated nephrin within only 4% and 8% of the transfected population, respectively (Figure 4.3B). Biotinylation experiments further confirm that surface nephrin levels are normalized by fusion of the Nck2 SH3 domains to mutant nephrin-Y3F (Figure 4.3C and D). These data further support the notion that recruitment of an Nck2 SH3 domain binding partner is required for appropriate nephrin endocytosis.
4.3.4 Dynamin2 binds Nck2 and mediates nephrin internalization

Core components of the endocytic machinery including dynamin2 have been shown to regulate nephrin internalization (136), and previous studies have reported an interaction between dynamin and Nck SH3 domains (181), thus we next examined the significance of this complex in nephrin trafficking. We first explored whether dynamin2 could interact with both Nck proteins. Full-length FLAG-tagged Nck1 or Nck2 were transiently co-expressed with GFP-dynamin2, GFP-N-WASp (positive control) or GFP alone (negative control) in HEK293T cells, and cell lysates were subject to GFP IP. IB for FLAG (Nck) revealed a more prominent interaction between dynamin2 and Nck2 vs. Nck1 (top panel, lanes 1 and 2), while interaction with N-WASp was comparable between Nck1 and Nck2, and notably more robust than with dynamin2 (Figure 4.4A). Endogenous binding of Nck with dynamin2 was confirmed in mouse glomerular extracts (Figure 4.4B). To next confirm that the interaction between Nck2 and dynamin2 is mediated by the Nck2 SH3 domains, we employed the Nck2 SH2 and SH3 domain mutants used in Figure 4.2. Disruption of Nck SH2 domain function (Nck2-SH2*) did not inhibit binding between FLAG-Nck and GFP-dynamin2, while mutation of the 3 tandem SH3 domains (Nck2-SH3X3*) abolished the interaction (Figure 4.4C). Lastly, we investigated the effect of impaired dynamin signaling on nephrin internalization in our HEK293T biotinylation assay. Expression of a dominant negative dynamin2 protein (K44A, defective in GTP binding) increased nephrin surface expression compared to wildtype dynamin2 (Figure 4.4D and E), similar to our observations with the nephrin-Y3F protein (Figure 4.1C and D). Collectively, these findings position Nck2 at the interface between nephrin and dynamin2 to facilitate endocytosis of phosphorylated nephrin.

4.3.5 Nephrin tyrosine phosphorylation initiates endocytosis during nephrotoxic serum-induced disease

Changes in nephrin endocytosis and nephrin phosphorylation have been reported within several human and rodent proteinuric kidney diseases, including the NTS nephritis model of reversible podocyte injury (74, 119, 159). However, the cause/effect relationship between these processes has not yet been investigated, and there is no consensus on the role of nephrin phosphorylation in disease progression. In the NTS model, injection with sheep anti-rat
glomerular antiserum induces nephrotic range proteinuria within 24 hours, which resolves by 72-96 hours post-injection (Figure 4.5A). We have recently shown that nephrin tyrosine phosphorylation decreases throughout the disease timecourse coincident with the onset of proteinuria (119), and we herein set out to characterize the kinetics and interplay between nephrin tyrosine phosphorylation and endocytosis prior to filtration barrier breakdown. Wildtype C57BL/6N mice were injected with NTS, and glomeruli were then isolated at 0, 2, 4, 6 or 12 hours post-injection for use in biotinylation assays, as described in Figure 4.1A. Nephrin immunoblotting of avidin-precipitated samples demonstrates a significant loss of nephrin from the cell surface at 2 and 4 hours post-injection, with a return to baseline at 6 and 12 hours post-injection, corresponding to the onset of proteinuria (Figure 4.5B and C). Remarkably, an inverse relationship is observed with phosphorylated nephrin on Y1176/Y1193, with an increase at 2 and 4 hours post-injection, and a decrease at 6 and 12 hours post-injection to below baseline (Figure 4.5 B and D), which is consistent with our previous report at 6 and 24 hours post-NTS injection (119). Using SR-SIM on kidney sections immunostained for nephrin, we verified that alterations in nephrin localization could be seen in glomeruli as early as 2 hours post-NTS injection (Figure 4.5E). Altogether, these data demonstrate that hyperphosphorylation of nephrin is an early event in disease progression leading to rapid loss of nephrin from the cell surface and subsequent demise of the GFB.

4.3.6 Nephrin-Y3F animals are protected from NTS-induced disease

The preceding findings indicate that a rise in nephrin tyrosine phosphorylation represents a rapid signaling event triggering nephrin endocytosis and disease in the NTS model. We thereby hypothesized that the inability to initiate phosphorylation on the Nck binding sites in our nephrin-Y3F animals would mediate protection from NTS-induced podocyte injury. To test this, we used nephrin-Y3F mice and WT littermate controls on the C57BL/6N background, as they show a delayed onset phenotype (10 months versus 1 month on the CD-1 background) (118). Mice at 8-10 weeks of age were injected with a single dose of NTS or sheep IgG as a control, and urine samples were collected at various timepoints. Unlike WT mice, Y3F mice showed significant attenuation of proteinuria during the injury timecourse (Figure 4.6A). Quantification of the ACR in the urine at 0 and 24 hours post-injection indicated an approximate 5-fold decrease in average proteinuria in Y3F mice compared to WT at 24 hours post-NTS injection,
and control sheep IgG injection had no effect on either genotype (Figure 4.6B and C). Consistent with these findings, ultrastructural analysis revealed widespread FP rearrangement in WT mice injected with NTS, while Y3F mice exposed to NTS resembled IgG-injected controls (Figure 4.6D). Collectively these findings underscore the role of nephrin tyrosine phosphorylation in induction of podocyte injury in the NTS model.

4.4 Discussion

Nephrin mislocalization commonly accompanies kidney dysfunction and mounting evidence indicates that disordered endocytosis is a driving force behind breakdown of the filtration barrier (91, 157, 178). We now demonstrate that loss of nephrin tyrosine phosphorylation on its 3 Nck-binding residues disrupts nephrin internalization. We further characterize an essential role for Nck SH3 domains in mediating the recruitment of dynamin and actin (through N-WASp) to sites of nephrin internalization, which seemingly is required during the later stages of endocytosis. Significantly, podocyte FP and cultured cells expressing nephrin-Y3F or Nck SH3 domain mutation display elongated endocytic pits and these long tubules are connected to the plasma membrane and contain surface nephrin. Such membrane invaginations are similarly seen in podocytes and other cell types lacking expression of the endocytic regulator dynamin (136, 182), and they are proposed to arise as a consequence of arrested endocytosis. Dynamin-mediated scission of endosomal vesicles from the plasma membrane is dependent on its GTPase activity (183) as well as its C-terminal PRR (182). Further, dynamin co-localizes with the N-WASp/Arp2/3 actin nucleation complex at late-stage endocytic pits (136, 184), indicating dynamin and actin work in concert during endocytosis in podocytes.

Although dynamin colocalizes with F-actin during endocytosis in podocytes (136) and is itself a potent modulator of podocyte actin dynamics (142), it is seemingly incapable of directly connecting to Arp2/3 endocytic actin machinery during endocytosis (136). This was demonstrated in a landmark study by Soda et al. (136) in which knockout of dynamin1/2 led to accumulation of Arp2/3 at the ensuing arrested pits. Actin’s recruitment to endocytic pits must thereby be independent of dynamin in podocytes, implicating a requirement for a linker protein in the connection between dynamin and actin. A intermediary was likewise proposed to facilitate indirect interactions between tyrosine phosphorylated nephrin and dynamin in pancreatic beta-
cells, although it’s identity remains unknown (140). We now demonstrate that dynamin and N-WASp bind to Nck’s SH3 domains, and that fusion of the Nck SH3 domains to nephrin-Y3F can rescue the tubulation phenotype. These findings position Nck as a central facilitator of simultaneous interactions with phosphorylated nephrin, dynamin and N-WASp, thus connecting nephrin to endocytic machinery at the SD to enable nephrin internalization and vesicle scission.

We have previously reported that the presence of a single Nck binding site on nephrin is sufficient to induce actin polymerization comparable to wildtype nephrin (97), however, arrested membrane invaginations remain prominent with these same mutants (Supplemental Figure 4.1). Tubulation of nephrin is particularly apparent when the Y1193 residue is disrupted, suggesting that this site may recruit additional regulators of endocytosis beyond Nck. PLC-γ1 can also be recruited to this site (61), and although not yet characterized in podocytes, PLC-γ1 and dynamin interactions may enhance dynamin-dependent endocytosis (185, 186). The clathrin adaptor β-arrestin2 can also associate with nephrin and promote its removal from the cell surface, although this interaction still occurs with the Y1193F mutation in the presence of threonine phosphorylation (52, 90). Other nephrin interaction partners may also play roles in nephrin internalization, such as CIN85 (62, 178) and CD2AP (136), and CD2AP accumulates at pits during the late stages of endocytosis in podocytes, identifying it as a possible additional source for actin recruitment during endocytic scission (136). Taken together, these findings implicate a diverse set of effectors and nephrin’s own phosphoregulation in the mechanism dictating its internalization.

Phosphoregulation of nephrin tyrosine residues has already been indentified as a means to modulate the role of clathrin in its endocytosis. In culture, nephrin tyrosine phosphorylation promotes its CIE, while nephrin dephosphorylation stimulates CME (123). CME mechanism are largely considered a constitutive means of internalization, essential for regular turnover of membrane components and in order to facilitates reorganization of SD-like adhesions (187, 188). Podocyte-specific cKO of dynamin1/2, which is required for scission of endosomes induced by CME and CIE alike, results in accumulation of predominantly clathrin-coated pits in FPs of 4-week old mice, possibly highlighting a preference for CME in the podocyte during development and/or maintenance, as might be expected. Alternatively, CIE mechanisms are receptor and stimulation-dependent and, hypothetically, they may be required for more reactive responses,
such as to hemodynamic changes or injury remodeling. Importantly, several these processes have already been demonstrated to be influenced significantly by nephrin tyrosine phosphorylation and to involve nephrin internalization (1, 136, 157, 159, 189).

Regardless of the mechanisms initiating nephrin’s endocytosis, this work demonstrates a clear requirement for nephrin tyrosine phosphorylation in facilitating actin and dynamin recruitment through Nck in the later stages of endocytosis. Such a significant requirement for actin recruitment is uncommon in endocytosis at the basolateral membrane of mammalian cells. The elongated endocytic pits observed in mice and cells expressing nephrin-Y3F are reminiscent of those observed in yeast rather than those generally observed in mammalian cells, including in podocytes, where they are roughly spherical in shape (136). Tubulation of endocytic pits suggests a heightened requirement for actin in membrane invagination and vesicle neck constriction, as occurs in turgid yeast (133, 134, 140, 211). Similar requirements can be induced artificially in mammalian cells by heightening the cells’s surface tension, such as through mechanical stretch (134). As a utility of its barrier function, it is well documented that the SD is subject to significant mechanical stretch and significant shear strain (177, 190), both which induce heightened membrane tension. We thereby propose that actin recruitment to sites of nephrin endocytosis is uniquely required for appropriate invagination of the membrane and scission of the vesicle as a means to overcome the strain present at the SD, although future work is required to validate this theory.

Nck is specially positioned in its ability to facilitate abundant actin recruitment to nephrin during endocytosis because of the multivalency of Nck/N-WASp SH3/PRR interactions (93, 94, 105). Spontaneous complex formation between SH3 and PRR segments occurs in the presence of a string of 5 SH3 and 5 PRR motifs, identifying a hypothetical minimum requirement for SH3 and PRR signaling node formation (93). Nck and N-WASP are prototypical SH3/PRR modules, defined by Nck’s 3 SH3 modules and N-WASP’s lengthy PRR and multivalent Nck and N-WASP interactions are sufficient to induce Arp2/3 actin polymerization at nephrin (93, 94). Importantly, clustering-induced phosphorylation of 2 of nephrin’s tyrosines reduces the amount of Nck-N-WASp required to induce Arp2/3 recruitment to nephrin by half by enhancing the valency of the complex (93). This is further augmented with phosphorylation of all 3 nephrin tyrosines, underscoring the unique ability of the phospho-nephrin/Nck/N-WASp multivalent
module to facilitate pronounced interactions between nephrin and actin (93). The importance for this theoretical relationship in FP function has now been solidified in the nephrin-Y3F mouse model and we have further identified its central importance in facilitating the force generation required to induce of nephrin’s endocytosis at the unique SD barrier.
Figure 4.1. Genetic disruption of nephrin phosphotyrosine signaling perturbs nephrin endocytosis. (A) Glomeruli from 4-week old CD-1 wildtype (WT) or Y3F mice (n=6) were isolated and subjected to surface biotinylation, followed by lysis, streptavidin agarose precipitation (Ppt) and nephrin immunoblotting (IB). A portion of the initial lysate was saved to represent the total input p-nephrin Y1176/Y1193 and nephrin. (B) Densitometric quantitation of (A). All values were made relative to surface nephrin levels in WT samples. (C) HEK293Ts transiently expressing WT or Y3F Myc-nephrin (n=4) were also subjected biotinylation analysis of surface nephrin, which was quantified in (D). (E) Nephrin and podocin immunostaining of kidney sections from 4-week old CD-1 WT and Y3F mice reveal abnormal congregation of nephrin-Y3F in mouse glomeruli. Scale bar: 10 µm. (F) Transmission electron micrographs reveal abnormal endocytic pits with elongated necks in distorted foot process of CD-1 nephrin-Y3F mice. Scale bar 100 nm. (G) Immunofluorescent imaging of MEFs transfected with WT or Y3F CD16/7 nephrin-GFP reveal long, cylindrical nephrin aggregates of nephrin-Y3F but not WT. Concurrent surface labelling of nephrin in non-permeabilized samples (red) indicates that this ‘tubulated’ nephrin-Y3F is restricted to the pool of nephrin continuous with the surface (yellow in merge). Scale bar: 20 µm. *P<0.05 by two-tailed Student’s t-test.
Figure 4.2. Disruption of Nck SH3 domain signaling promotes formation of tubulated nephrin and disrupts the recruitment of F-actin to nephrin. Nck1/2 knockout MEFs were co-transfected with wildtype (WT) CD16/7 nephrin-GFP and various FLAG-Nck2 mutants and immunofluorescence microscopy was performed. Expression of FLAG-Nck2-WT (blue) did not disrupt the formation of normal nephrin punctae (green). Normal localization of F-actin (red) comet formation was observed at nephrin (merge). Expression of Nck2-SH3*X3 (blue), which does not disrupt nephrin-Nck2 interaction, but rather the recruitment of downstream Nck2 binding partners to nephrin, resulted in the formation of tubulated nephrin (green) accompanied by abolishment of F-actin tail formation at nephrin (red, merge with phalloidin). Although F-actin tail formation at nephrin was also significantly disrupted in Nck2-SH2* expressing cells (red, merge with phalloidin), nephrin tubulation was not observed. Scale bar: 20 µm.
Figure 4.3. Nck2 SH3 domains rescue the tubulation phenotype induced by nephrin-Y3F mutation. (A) Wildtype (WT) CD16/7 nephrin-GFP, CD16/7 nephrin-Y3F-GFP or CD16/7 nephrin-Y3F-Nck2-SH3x3-GFP was transfected in Nck1/2 knockout MEFs. Immunofluorescence imaging demonstrates evidence that this fusion protein normalizes surface nephrin (red) patterning from the cylindrical aggregates in nephrin-Y3F cells to diffuse punctae in fusion cells. Scale bar: 20 µm. (B) Quantification indicates a total of 78% of nephrin-Y3F cells displaying this defective organization, whereas cells expressing nephrin-WT or the fusion protein displayed tubulated nephrin only 4% and 8% of the time respectively (at least 100 cells per replicate, n=3). (C,D) Immunoblots (IB) of biotinylation assays on HEK293T cells transfected with CD16/7 nephrin-WT-GFP, CD16/7 nephrin-Y3F-GFP or CD16/7 nephrin-Y3F-Nck2-SH3x3-GFP demonstrates enhanced surface (avidin precipitate (ppt)) relative to total (input) nephrin in CD16/7 nephrin-Y3F-GFP expressing cells, but not CD16/7 nephrin-Y3F-Nck2-SH3x3-GFP expressing cells relative to CD16/7 nephrin-WT-GFP expressing cells (n=5). These data indicate that it is the recruitment of an Nck2-SH3 binding partner that is required for appropriate nephrin endocytosis. *P<0.05 by two-tailed Student’s t-test.
**Figure 4.4. Dynamin2 preferentially binds Nck2 vs. Nck1, and mediates nephrin internalization.** (A) To investigate if full-length (FL) Nck and dynamin2 interact, FLAG-Nck1 or FLAG-Nck2 were transiently co-expressed with GFP-dynamin2, GFP-N-WASp (as a positive control for Nck1 and Nck2 co-immunoprecipitation (IP)) or GFP alone (as a negative control) in HEK293T cells. Cell lysates were harvested for IP of GFP and representative immunoblots (IB) for GFP and FLAG in IP and whole cell lysates reveals a specific interaction between GFP-dynamin2 and FLAG-Nck1 and FLAG-Nck2. The Co-IP between dynamin and Nck2 is greater than that between dynamin and Nck1. Co-IPs with N-WASp confirm that both FLAG-Nck1 and FLAG-Nck2 bind the actin nucleation activator to similar degrees. No binding was observed in the GFP alone negative control (n=4). (B) Co-IPs between nephrin, dynamin2 and Nck were confirmed in glomerular lysates IP’d for total Nck. (C) Co-IP between dynamin2 and Nck were disrupted using a Nck2-SH3X3* mutant, but not a Nck2-SH2* mutant protein. (D) HEK293T cells transiently co-expressing WT or K44A GFP-dynamin2 and WT Myc-nephrin were subject to surface biotinylation, followed by lysis, streptavidin agarose precipitation (Ppt) and Myc (nephrin) or GFP (dynamin) IBs. (E) Densitometric comparison of streptavidin-precipitated biotinylated nephrin (surface) to total nephrin (input) indicates an increase in surface nephrin in the presence of K44A dynamin 2 relative to cell co-expressing WT dynamin 2 (n=3). *P<0.05 by two-tailed Student’s t-test.
Figure 4.5 Nephrin tyrosine phosphorylation initiates endocytosis during nephrotoxic serum-induced disease. 8-week old C57BL/6 wildtype mice were injected with nephrotoxic serum (NTS) and analyzed at indicated timepoints. (A) Urine samples were analyzed for the peak in albuminuria by running 2 uL of spot urines at timepoints between 0 and 96 hours after injection. A representative Coomassie urine gel indicates albuminuria peaks at 24 hours. Positive is 2.5 ug bovine serum albumin. (B) Glomeruli from 8-week old C57BL/6 mice were subjected to surface biotinylation, followed by lysis, streptavidin agarose precipitation (Ppt). A portion of the initial lysate was saved to represent the total input and Ppt and input samples were immunoblotted (IB) for p-nephrin Y1176/93 and nephrin at 0 (n=4), 2 (n=10), 4 (n=4), 6 (n=6) and 12 (n=3) hours post-injection to characterize the signaling events in damage initiation. (C) Densitometric quantitation of streptavidin-precipitated biotinylated nephrin (surface) compared to total nephrin (input) from (B) demonstrates an increase in surface nephrin levels at 2 and 4 h post-injection. All values were made relative to surface nephrin levels in WT samples. (D) IB of total tyrosine Y1176/Y1193 phosphorylated nephrin revealed a trend for increased nephrin tyrosine phosphorylation from 2-4 h post-injection and decreased phosphorylation thereafter. Nephrin Y1176/Y1193 phosphorylation was significantly increased at 2 h post-injection, which was accompanied by a significant decrease in surface nephrin. (E) High resolution microscopy of animals injected with control immunoglobulin (IgG) serum or NTS reveals significant alterations in nephrin localization in glomeruli at 2 h post-injection with NTS but not IgG. Scale bar: 10 µm. *P<0.05 by two-tailed Student’s t-test
Figure 4.6 Nephrin-Y3F animals are protected from nephrotoxic serum-induced disease. Nephrin wildtype (WT) and Y3F animals were injected with control Immunoglobulin (IgG) serum or NTS and monitored over 96 hours (n=3-5 mice per genotype). (A) Protein/creatinine ratios ((µg/mg)^10^-1) of spot urines collected over the timecourse reveals significant proteinuria in nephrin-WT between 12-48 hours, peaking at 24 hours and declining back to baseline between 54-96 hours post-injection. Nephrin-Y3F mice injected with NTS do not display significant proteinuria at any timepoint monitored. *P<0.05 by two-tailed Student’s t-test. (B) A representative Coomassie urine gel demonstrates the presence of albuminuria in spot urines collected from WT mice injected with NTS but not control IgG serum at the peak 24 hour timepoint. Albuminuria was not observed in Y3F mice injected with IgG or NTS at this peak timepoint. (C) Albumin/creatinine ratios (µg/mg) were quantified in WT and Y3F animals injected with IgG or NTS at 24 hours and confirm a significant increase in albuminuria in WT-NTS as compared to WT-IgG urine samples. Significant albuminuria was not observed in Y3F-NTS urine samples and, accordingly, WT-NTS albuminuria was significantly greater than that quantified in Y3F-NTS. *P<0.05 by two-tailed ANOVA. (D) Scanning electron micrographs the induction of significant foot process disorganization and broadening in WT mice exposed to NTS as compared to IgG. Conversely, foot process organization appears analogous in Y3F-IgG and Y3F-NTS samples. Scale bar: 2 µm.
Supplemental Figure 4.1 Dual disruption of nephrin phosphotyrosine signaling is sufficient to induce nephrin tubulation. (A) MEF cells transfected with various CD16/7 nephrin-GFP dual point mutants were evaluated for nephrin punctae formation. Similar to nephrin-Y3F, nephrin Y1176/1193F, nephrin Y1176/1217F and nephrin Y1193/1217F all form long, cylindrical nephrin aggregates (green) post-clustering with CD16 antibody, although to differing degrees. Scale bar 20 µm.
CHAPTER 5:
THE SHCA ADAPTOR PROTEIN PROMOTES NEPHRIN ENDOCYTOSIS AND IS UPREGULATED IN PROTEINURIC NEPHROPATHIES (157)
5.1 Abstract

Nephrin is a key structural component of the podocyte slit diaphragm, and proper expression of nephrin on the cell surface is critical to ensure integrity of the blood filtration barrier. Maintenance of nephrin within this unique cell junction is proposed to require dynamic phosphorylation events and endocytic recycling, although the molecular mechanisms that control this interplay are poorly understood. Here we have identified the phosphotyrosine adaptor protein ShcA as a novel signaling molecule regulating nephrin turnover. ShcA is expressed in podocytes, and it associates with several phosphorylated tyrosine residues on nephrin via its SH2 domain. We demonstrate that ShcA directly promotes nephrin tyrosine phosphorylation, in addition to reduced nephrin signaling and surface expression. Using a rat model of reversible podocyte injury and proteinuria, we further show that phosphorylated nephrin is temporally colocalized within endocytic structures coincident with upregulation of ShcA, and we use in vivo biotinylation to confirm that nephrin is removed from the cell surface during the injury timecourse. Finally, we demonstrate overexpression of ShcA in several human proteinuric kidney diseases. Our results suggest that increases in ShcA perturb nephrin phospho-signaling dynamics, leading to aberrant nephrin turnover and SD disassembly.

5.2 Introduction

Podocytes play a critical role in maintaining the integrity of the kidney’s GFB. These specialized epithelial cells extend a network of interdigitating actin-based FPs which are bridged by a unique cell junction known as the SD (167). A fundamental component of the SD is the transmembrane protein nephrin. Through cis and trans interactions of its extracellular domain, nephrin contributes to the filtration pore that is largely responsible for permselectivity (3). Aberrant expression of nephrin and other SD molecules such as podocin leads to FP effacement and loss of essential plasma proteins into the urine (proteinuria) (167). Accumulating evidence suggests that stable expression of nephrin on the cell surface is regulated by endocytosis, and that defects in nephrin turnover might lead to proteinuria (191).

Nephrin can be internalized via both CME and CIE mechanisms (123). Nephrin endocytosis is reduced in podocytes lacking the endocytic effector dynamin (136), and enhanced in the presence of CIN85, which further induces its ubiquitination (62). Additionally, nephrin
endocytosis appears to be triggered by its tyrosine phosphorylation (62, 123, 136, 140), with the phosphorylation signature instructing differential binding of endocytic regulators β-arrestin and podocin (52). Nephrin contains several tyrosine residues on its intracellular tail which undergo SFK-mediated phosphorylation and serve as docking sites for SH2 domain-containing proteins (1). We have recently demonstrated that sustained phosphorylation on several of these sites is essential to stabilize FP morphology and maintain barrier function (118). Despite these observations, the molecular mechanisms that facilitate nephrin turnover are poorly understood, and only a single nephrin phosphotyrosine binding partner involved in endocytosis has been identified (Nck, Chapter 4).

Herein, we reveal the adaptor protein ShcA as a novel signaling molecule within the SD. Shc family adaptors (ShcA-D) contain both a phosphotyrosine binding PTB domain and a C-terminal SH2 domain, each of which can bind phosphotyrosine residues (Figure 5.1A) (192). The central H chain constant (CH1) region contains three phosphorylatable tyrosine residues, in addition to an α-adaptin binding motif, which facilitates binding of the μ2 subunit of the clathrin assembly complex AP-2 (adaptor complex 2). Splicing and alternative translation start sites produce the 46, 52 and 66 kDa isoforms of ShcA (193). The p46 and p52 isoforms are well established activators of the Ras/mitogen-activated protein kinase (MAPK) pathway via their recruitment of growth-factor receptor binder (Grb)2 to the central tyrosines, and they also promote CME of endothelial growth factor receptor (EGFR) through the α-adaptin binding motif (194). The p66 isoform uniquely controls the oxidative stress response through its extended CH2 region (195). Global knockout of all ShcA isoforms in mice causes major cardiovascular abnormalities resulting in embryonic lethality, and ShcA-deficient fibroblasts exhibit defects in actin organization (196). In the kidney, deletion of p66 renders proximal tubule cells more resistant to oxidative stress (197), and it also suppresses hyperglycemia-induced glomerular injury (198); however, the in vivo role of ShcA in podocytes has not yet been explored.

We now report that ShcA interacts via its SH2 domain with phosphorylated nephrin to regulate nephrin tyrosine phosphorylation and surface expression. We further demonstrate that ShcA is upregulated in a reversible podocyte injury model coincident with relocalization of phosphorylated nephrin into endocytic structures, and that nephrin is removed from the cell surface in this model. Finally, we show increased expression of ShcA in humans with proteinuric
kidney disease. We thus conclude that ShcA is a central regulator of nephrin phospho-signaling dynamics, which in turn modulates filtration barrier function via direct control of nephrin turnover at the plasma membrane.

5.3 Results

5.3.1 ShcA is expressed in podocytes and interacts with nephrin via its SH2 domain

ShcA is among a select few SH2 domain-containing proteins predicted by DomPep modeling (199) to interact with phosphorylated nephrin (Supplemental Table 5.1). As ShcA is widely expressed, we first established the presence of this adaptor protein in podocytes via WB analysis of protein lysates obtained from cultured podocytes and neonatal and adult mouse kidney cortex. All ShcA isoforms are found within podocytes, and their expression declines once glomerulogenesis is complete, with the p46/52 isoforms predominating over the p66 isoform in both immature and mature kidney (Figure 5.1B). We also performed immunostaining of mouse kidney cryosections, where ShcA was distributed throughout the glomerulus and robustly localized with nephrin in podocytes (Figure 5.1C). To next investigate whether ShcA interacts with nephrin, FLAG-tagged p46/52 ShcA was coexpressed with Myc-tagged full-length nephrin in HEK293T cells. Nephrin co-precipitated with ShcA, and this effect was enhanced upon co-expression of constitutively active (CA) Fyn but not a kinase dead variant (Fyn-KD) of this SFK (Figure 5.1D), indicating a requirement for nephrin tyrosine phosphorylation in the nephrin-ShcA interaction. We confirmed that p66 ShcA could similarly associate with phosphorylated nephrin (Supplemental Figure 5.1A). ShcA contains both PTB and SH2 domains, thus we next wanted to determine which phosphotyrosine binding module might mediate interaction with nephrin. Pulldown assays of nephrin in the presence or absence of Fyn-CA using GST fused to each domain revealed that the SH2 domain but not the PTB domain is required for nephrin binding, and mutation of the phosphotyrosine binding pocket of the SH2 domain (R397K, SH2*) disrupted this interaction (Figure 5.1E).

5.3.2 Nephrin phosphotyrosine residues 1176, 1193 and 1217 mediate binding to ShcA

Nephrin possesses a series of intracellular tyrosine residues that are highly conserved between the human, mouse and rat protein sequences (Figure 5.2A). To identify which residues...
might mediate the interaction with ShcA, a spot peptide array harbouring phosphorylated and non-phosphorylated Y-based sequences from each species was incubated with purified GST-SH2 domain of ShcA. The SH2 domain bound consistently to phosphorylated Y1114, Y1176, Y1193 and Y1217, and not the non-phosphorylated peptide counterparts (Figure 5.2B). To validate this binding in the context of full-length nephrin, we performed pulldown assays of the ShcA GST-SH2 domain with nephrin variants bearing single or multiple Y-to-F mutations at these sites. The Y1193F and Y1217F mutations had the greatest reduction on ShcA binding compared to WT, while Y1114F surprisingly had no effect (Figure 5.2C). The nephrin-Y3F mutation completely abolished interaction with ShcA (Figure 5.2C). Lastly, we confirmed the specificity of ShcA binding to nephrin in vivo in mouse glomerular lysates, and determined that this interaction was disrupted in mice bearing the nephrin-Y3F mutation (Figure 5.2D). Altogether, these findings demonstrate that the ShcA adaptor interacts via its SH2 domain with multiple tyrosine phosphorylated sites on nephrin.

5.3.3 ShcA enhances Src-dependent nephrin phosphorylation and promotes nephrin endocytosis

We next sought to determine the functional consequences of the nephrin/ShcA interaction on downstream signaling. FLAG-tagged p46/52 ShcA was coexpressed with nephrin in HEK293T cells, and phosphorylation of several cellular targets was monitored. Intriguingly, nephrin tyrosine phosphorylation on Y1193 and Y1217 was enhanced in the presence of WT ShcA, and this effect was not seen with the ShcA-SH2* mutation which cannot bind nephrin (Figures 5.3A-C). The increase in nephrin tyrosine phosphorylation was accompanied by an increase in SFK activation (Figures 5.3A and D). Of note however, phosphorylation of ShcA itself as well as Akt downstream of nephrin remained unchanged (Figure 5.3A). We confirmed this effect on nephrin hyperphosphorylation within our documented inducible setting of nephrin activation (53), where ShcA was coexpressed with CD16-nephrin-GFP, and cells were stimulated with CD16 to promote nephrin clustering, phosphorylation and ShcA recruitment (Figure 5.3E and Supplemental Figure 5.1B). Inclusion of the SFK inhibitor PP2 but not vehicle (DMSO) alone suppressed the ShcA-mediated increase in nephrin tyrosine phosphorylation (Figure 5.3E). Nephrin signaling converges on the AP-1 transcription factor (52, 200), thus we investigated whether co-expression of ShcA with nephrin could affect nephrin-mediated AP-1
transactivation using a previously established luciferase reporter assay. We found that WT ShcA significantly suppressed AP-1 activity compared to cells without excess ShcA, and this effect was abrogated with the ShcA-SH2* mutation (Figure 5.3F). Furthermore, the effect of WT ShcA was comparable to that of β-arrestin (Figure 5.3F), which has been shown to attenuate nephrin signaling via induction of nephrin endocytosis (52). Accordingly, biotinylation assays revealed a significant reduction in nephrin cell surface expression with WT ShcA but not the SH2* mutation (Figure 5.3G). We thus conclude that Fyn-mediated hyper-phosphorylation of nephrin induced by high levels of ShcA promotes nephrin endocytosis and thereby suppresses nephrin signaling.

5.3.4 ShcA is upregulated during PAN nephrosis coincident with redistribution of tyrosine phosphorylated nephrin into early endosomes

We and others have previously demonstrated that nephrin tyrosine phosphorylation is perturbed in the PAN nephrosis rodent model of human MCD (95, 116), thus we next examined whether expression of ShcA might also be altered during the injury timecourse. Glomerular extracts were prepared from PBS-injected control rats and PAN-injected rats at the onset (Day 4) and peak (Days 7 and 14) of proteinuria (Supplemental Figure 5.2A). IB for ShcA revealed a rise in p46/52 ShcA expression, which reached a maximum on Days 7 and 14 (Figures 5.4A and B). Upregulation of ShcA was similarly seen in PAN-treated HPCs (Figures 5.4C and 4D). Intriguingly, the increase in ShcA expression in vivo correlated with a modest rise in nephrin phosphorylation on Y1193 and Y1217 at later points during the timecourse following the decrease (in Y1217) observed at Day 4 (Figure 5.4A). Immunostaining of glomeruli at Day 7 revealed a change in phospho-nephrin distribution compared to the control, where it appeared focal and punctate during injury, with areas of intense staining showing colocalization with the early endosome marker EEA1 (early endosome antigen marker 1) (Figure 5.4E). Podocin and EEA1 co-localization was also enhanced at this timepoint (Supplemental Figure 5.2B).

5.3.5 Nephrin is mislocalized from the membrane to the cytosol in response to podocyte injury

To further explore the nature of nephrin redistribution during PAN injury, we utilized SR-SIM to image nephrin-immunostained kidney sections prepared from PAN-injected rats and
controls. Continuous linear staining of nephrin can be observed along the capillary wall in control animals, while PAN injection leads to a punctate appearance, suggesting cytosolic accumulation of nephrin with injury (Figure 5.5A). To investigate this in more detail, we performed biotinylation assays on intact glomeruli isolated from PAN-injected rats and controls, followed by IB of enriched and lysate fractions to monitor changes in nephrin cellular partitioning (Figure 5.5B). Quantified streptavidin precipitation reveals a significant steady decrease in nephrin surface expression during the injury timecourse (Figures 5B and C), and a corresponding increase in cytosolic nephrin can be seen in the remaining supernatant (Figure 5.5B). This redistribution from the membrane to the cytoplasm correlates with the rise in ShcA expression shown in Figure 5.4. A transient decline in podocin surface expression was also observed (Supplemental Figures 5.2C and D), consistent with previous findings (201), and no surface labeling of the podocyte nuclear/cytosolic Wilms tumour (WT)-1 protein was detected, verifying the specificity of this approach (Supplemental Figure 5.2E). Together these results support the hypothesis that upregulation of ShcA triggers nephrin endocytosis and filtration barrier demise.

5.3.6 ShcA is overexpressed in human glomerulopathies associated with proteinuria

To determine whether the increase in ShcA expression within the rodent model of podocyte injury might be similarly observed in human glomerulopathies, we performed dual immunostaining of ShcA and nephrin on biopsies from patients with FSGS or healthy donor controls (Figure 5.6). In healthy adult controls, ShcA is expressed at low levels, similar to our findings in mature vs. neonatal mice (Figure 5.1B). However, a striking increase in ShcA was reproducibly observed in both MCD and FSGS patients, wherein it displayed robust colocalization with nephrin. Analysis of the open-source Nephroseq database (nephroseq.org) similarly shows upregulation of SHCA expression in FSGS and MCD, in addition to IgA nephropathy (IgAN) (Table 5.1). Of note, SCHA is among the top 2-4% of overexpressed glomerular genes in several of the datasets analyzed. These findings strongly imply that aberrant ShcA signaling is induced in human proteinuric glomerular diseases.
5.4 Discussion

Herein we have identified the ShcA adaptor protein as a putative novel regulator of nephrin surface expression and filtration barrier integrity. ShcA associates with tyrosine phosphorylated nephrin, and in the mature glomerulus, we propose that low levels of ShcA signaling contribute to a steady-state pool of phosphorylated nephrin within the SD and aid in nephrin recycling. Upon injury, ShcA expression increases, triggering a pathogenic positive feedback loop of nephrin hyper-phosphorylation and enhanced endocytosis. Concurrent loss of nephrin and podocin from the podocyte surface destabilizes the SD, leading to proteinuria. ShcA signaling thus provides a mechanism to control turnover and stabilization of this dynamic cell junction. Shc proteins possess a unique domain architecture that allows them to simultaneously engage multiple signaling pathways (192). The PTB domain is the most common mediator of these interactions; however, we have determined that recruitment of ShcA to phosphorylated nephrin is via its SH2 domain. The ShcA-SH2 domain-binding consensus is pY-ϕ-X-ϕ(202), which is in line with sequences surrounding nephrin Y1176, Y1193 and Y1217. Notably, these tyrosines were predicted by the DomPep method (199) to bind the ShcA-SH2 domain (Supplemental Table 5.1), and the peptide corresponding to ShcA’s SH2 domain was previously reported as an interaction partner of recombinant phospho-nephrin (54). The function of the ShcA PTB domain in podocytes remains to be determined, although an intriguing target is IQGAP1 (203), which can indirectly bind nephrin and regulate podocyte actin dynamics (204). Alternately, the ShcA PTB domain can bind acidic phospholipids, which may stabilize ShcA localization at the SD (205, 206). The central Grb2-binding phosphotyrosine residues on ShcA do not appear to be affected by nephrin signaling, consistent with reports that Grb2 does not bind nephrin and is not required for podocyte function (207, 208). These findings also support a non-canonical role for ShcA in nephrin signaling through additional elements such as the α-adaptin binding motif. Accordingly, our studies have revealed that ShcA promotes nephrin phosphorylation in a Fyn-mediated fashion, leading to nephrin endocytosis. ShcA may enhance activation of Src or Fyn directly (209) or indirectly via its interaction with the SHP-2 phosphatase, which in turn activates Fyn (108). Of note, SHP-2 overexpression was recently shown to enhance nephrin phosphorylation, and similar to ShcA, SHP-2 is overexpressed in a subset of human glomerular diseases (74).
Binding of ShcA to multiple phosphorylated tyrosine residues on nephrin appears to overlap with that of Nck (53), and both of these proteins can promote nephrin hyper-phosphorylation (106). However, the downstream consequences of nephrin hyper-phosphorylation are distinct between ShcA and Nck (106), and these findings implicate dual roles for nephrin tyrosine phosphorylation in healthy vs. injured podocytes. ShcA and Nck can both associate with Y1176, Y1193 and Y1217; however, ShcA shows preferential binding to Y1193 and Nck has the highest affinity for Y1217 (53). We have recently established that phosphorylation on these tyrosine residues is required throughout life to maintain podocyte function (118). Furthermore, we and others have shown transient changes in their phosphorylation patterns in PAN nephrosis, as well as in the PS model of rapid reversible FP effacement (118), a podocyte injury model associated with both cytoskeletal remodeling and nephrin endocytosis (123). Here, Y1217 phosphorylation decreases in conjunction with loss of nephrin-Nck binding during PS-induced damage, while nephrin phosphorylation and Nck binding are restored during FP recovery (118). Loss of Y1217 phosphorylation (and thus nephrin-Nck binding) was recently reported to induce β-arrestin binding to nephrin and subsequent endocytosis (91). By contrast, phosphorylation of Y1193 increases during PS-induced damage and declines again during recovery (74, 118). It is therefore tempting to speculate that in the prolonged PAN model, the rise in Y1193 (and Y1217) phosphorylation could promote recruitment of ShcA, triggering sustained removal of nephrin from the cell surface.

Consistent with this notion, we have shown that glomerular ShcA expression is induced in rodent exposed to PAN and humans with FSGS and MCD, coincident with nephrin mislocalization and proteinuria. Such atypical localization of nephrin is similarly seen in early stages of MN (117). We further demonstrate prominent internalization of phosphorylated nephrin, overlapping with EEA1 and podocin in the PAN model, as well as reduced surface expression of nephrin and podocin. Our findings provide compelling evidence to suggest that excessive nephrin endocytosis underlies the disease process. Moreover, the overall decrease in total nephrin phosphorylation reported previously in PAN, MCD and MN (53, 116, 117) may imply that internalized nephrin is subject to dephosphorylation. Intriguingly, nephrin dephosphorylation is accompanied by an increase in tyrosine phosphatase PTP1B expression in PAN (66). PTP1B is anchored in the membrane of the endoplasmic reticulum, and it has been
characterized to dephosphorylate the EGFR in endosomes and instruct receptor fate inside the cell (210). We postulate a similar mechanism within podocytes whereby ShcA upregulation leads to nephrin hyperphosphorylation and endocytosis, with a corresponding upregulation of PTP1B leading to dephosphorylation of nephrin in endosomes. This may prolong disruption of the filtration barrier and traffic internalized nephrin into recycling or destruction pathways (149).

Surface expression and intracellular trafficking of nephrin and podocin are intimately linked, as we observed throughout the PAN injury timecourse. Of note, missense mutations in podocin associated with SRN cause retention of both podocin and nephrin in the cytosol (121, 211). Furthermore, consistent with our findings, others have demonstrated mislocalization of podocin from the podocyte cell surface to the cytoplasm in PAN, and this report also identified podocin internalization in poor prognosis IgAN patients (201). Expression of SHCA is similarly upregulated in IgAN, as well as in other proteinuric kidney diseases including FSGS and MCD, and we propose that aberrant ShcA expression disrupts steady state recycling of nephrin and podocin, leading to destabilization of the SD. Further studies will reveal the potential for ShcA to serve as a biomarker for podocyte injury.
Table 5.1 *SHCA* gene expression in kidney disease patients. Gene rank and fold change *SHCA* expression in control (n=21, n=31, n=9) and focal segmental glomerulosclerosis (FSGS) (n=25, n=17), IgA nephropathy (IgAN) (n=27, n=25) or minimal change disease (MCD) (n=14, n=14, n=7) patients. ShcA overexpression is observed in both glomerular (Glom) and tubulointerstitial (TubInt) regions, but is greater in glomerular regions. OE: overexpressed.

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A

\[ \text{CH2} \rightarrow \text{CH1} \]

- ShcA
- PTB
- AD
- SH2
- p66
- p52
- p46

B

- podocytes
- cortex
- P4
- adult

IB: ShcA
IB: β-actin

C

- ShcA
- nephrin
- merge

adult mouse

D

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<tr>
<td>63</td>
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IB: Myc
IB: pTyr
IP: ShcA
IB: Myc
IP: ShcA
IB: pTyr

E

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IB: Myc (nephrin)
IB: pTyr
Figure 5.1 ShcA is expressed in podocytes and binds tyrosine phosphorylated nephrin. (A) Schematic of ShcA isoforms and signaling domains including an N-terminal PTB domain, a C-terminal SH2 domain, three central tyrosine residues (red circles), an alpha adaptin (AD) binding motif (yellow square) and a serine residue in p66 key for oxidative stress signaling (green circle). (B) Confirmation of ShcA protein expression in lysates prepared from cultured mouse podocyte cells and kidney cortex of postnatal day 4 (P4) and adult mice by immunoblotting (IB). (C) Dual immunofluorescence staining showing overlapping expression between ShcA (red) and nephrin (green) on kidney sections from adult mice. Scale bar: 10 μm. (D) Lysates from HEK293T cells transiently coexpressing p46/52 ShcA-Flag, Myc-nephrin and constitutively active (CA) or kinase dead (KD)-Fyn were immunoprecipitated (IP) with ShcA and immunoblotted (IB) as indicated. ShcA coimmunoprecipitated with phosphorylated nephrin. (E) Lysates from HEK293T cells overexpressing Myc-nephrin with or without constitutively active (CA) Fyn were incubated with immobilized GST fusion proteins corresponding to the PTB, SH2 or mutant SH2 (R397K, SH2*) domains of ShcA. Pulldown complexes immunoblotted for Myc indicate binding between the ShcA SH2 domain but not ShcA SH2* with phosphorylated nephrin. pTyr, phosphotyrosine.
A

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Overlay: GST-ShcASH2; IB: GST

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C

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Figure 5.2 ShcA binds multiple phosphotyrosine residues on nephrin in vitro and in vivo.
(A) Conserved tyrosine residues in human, mouse and rat nephrin. Boxes indicate residues demonstrated to be phosphorylated in vitro and/or in vivo. (B) Spot peptide arrays centred around tyrosine (Y) or phosphotyrosine (pY) of all phospho-sites in human, mouse and rat nephrin were incubated with purified GST-ShcA SH2 domain and immunoblotted with GST. Boxes indicate sites with positive anti-GST signal across all species. (C) Lysates from HEK293T cells overexpressing CA-Fyn with WT Myc-nephrin or variants with single, double or triple mutations at tyrosine sites identified in (B) were incubated with GST-ShcA SH2 domain. Pulldown complexes immunoblotted for Myc indicate reduced binding of the ShcA SH2 domain to nephrin variants lacking Y1193, and a complete loss of binding with the Y3F variant. (D) Immunoblot (IB) for nephrin and ShcA in anti-ShcA immunoprecipitates (IP) from nephrin-WT and nephrin-Y3F mouse glomerular lysates, confirming binding of ShcA and nephrin in vivo, and disruption of this interaction in mice lacking phosphorylated nephrin.
Figure 5.3 ShcA promotes tyrosine phosphorylation and endocytosis of nephrin. (A) Lysates from HEK293T cells transiently coexpressing Myc-nephrin with WT p46/52 ShcA-Flag or an SH2* variant that cannot bind nephrin were immunoblotted (IB) as indicated. Changes in phosphorylation in nephrin and Src but not ShcA or Akt are detected. (B-D) Densitometric quantitation of p-nephry1193 (n=3), p-nephry1217 (n=3) and p-SrcY416 (n=4) from (A), respectively. (E) Lysates from HEK293T cells transiently coexpressing CD16/7-nephrin-GFP with or without ShcA-Flag were stimulated with anti-CD16 antibody in the presence or absence of the SFK inhibitor PP2 or vehicle alone (DMSO), and immunoblotted (IB) as indicated. PP2 suppresses the ShcA-induced increase in nephrin and Src phosphorylation. (F) HEK293T cells transiently coexpressing ShcA-Flag or β-arrestin with Myc-nephrin, podocin, AP1-firefly luciferase and renilla luciferase were subject to a dual reporter assay. ShcA WT but not SH2* suppressed the nephrin/podocin-induced activation of AP-1, similar to β-arrestin (n=4). (G) HEK293T cells transiently coexpressing WT or SH2* ShcA-Flag with Myc-nephrin were subject to surface biotinylation. Densitometric comparison of streptavidin-precipitated biotinylated nephrin (surface) to total nephrin (input) indicates a reduction in surface nephrin with WT ShcA but not SH2* (n=3). All values were made relative to biotinylation result of cells transfected with Myc-nephrin alone. *P<0.05 and **P<0.01 by two-tailed Student’s t-test.
Figure 5.4 Puromycin aminonucleoside nephrosis induces upregulation of ShcA and nephrin endocytosis. (A) Glomerular lysates from puromycin aminonucleoside (PAN)-injected rats and controls were immunoblotted (IB) as indicated. Nephrin tyrosine phosphorylation on Y1217 decreases at Day 4 of the injury timecourse, and then both Y1193 and Y1217 increase at Day 7 coincident with ShcA expression. (B) Densitometric quantitation of p46/52 ShcA levels from (A), showing a significant increase in ShcA levels at Days 7 and 14 (n=6). *P<0.05 by two-tailed Student’s t-test. (C) Lysates from differentiated human podocytes treated with PAN (10 mg/mL) or vehicle control for 24 hours were immunoblotted for ShcA or β-actin loading control. (D) Densitometric quantitation of p46/52 ShcA levels from (C), showing a significant increase in ShcA following PAN exposure (n=4). *P<0.05 by two-tailed Student’s t-test. (E) Dual immunofluorescence staining for p-nephrinY1217 (green) and nephrin (red) or EEA1 (red) on kidney sections of control and PAN-injected Day 7 rats. Phosphorylated nephrin is decreased and relocalized at Day 7. Regions of intense p-nephrin signal show colocalization with the early endosome marker EEA1. Scale bar: 20 µm.
Figure 5.5 Puromycin aminonucleoside injury results in internalization of surface nephrin. (A) Super-resolution structured illumination microscopy for nephrin (green) on kidney sections of control and puromycin aminonucleoside (PAN)-injected rats at Day 7 post-injection. Scale bar: 20 µm. (B) Isolated glomeruli from PAN-injected rats and controls were subject to surface biotinylation, followed by lysis, streptavidin agarose precipitation (Ppt) and nephrin immunoblotting (IB). A portion of the initial lysate was saved to represent the total input, and the supernatant post-precipitation represents non-biotinylated (cytosolic) proteins. (C) Densitometric quantitation of streptavidin-precipitated biotinylated nephrin (surface) compared to total nephrin (input) from (B), showing a stepwise decrease in surface nephrin levels. All values were made relative to surface nephrin levels in control samples. *P<0.05 by two-tailed Student’s t-test.
Figure 5.6 ShcA is upregulated in human proteinuric kidney diseases. Representative confocal images following dual staining for ShcA (red) and nephrin (green) on kidney biopsies from control individuals (n=3) or patients with Focal Segmental Glomerulosclerosis (FSGS) (n=4) or Minimal Change Disease (MCD) (n=3). ShcA is prominently upregulated in FSGS and MCD compared to control biopsies, and is colocalized with nephrin. Biopsies shown were immunostained in parallel, and imaged at the same gain and exposure. Scale bar: 20 µm.
Supplemental Table 5.1 Dompep prediction of SH2 domain-containing binding partners for nephrin phospho-tyrosine residues (human nephrin numbering system).

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**Supplemental Table 5.2 Patient information at the time of biopsy.** FSGS= Focal Segmental Glomerulosclerosis; MCD= Minimal Change Disease; PCR= protein/creatinine ratio. Shaded rows signify samples used for representative image in Figure 5.6.

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Supplemental Figure 5.1 p66 ShcA also binds tyrosine phosphorylated nephrin and p46/52 ShcA recruitment to phosphorylated nephrin is disrupted via mutation to its SH2 domain.

(A) Lysates from HEK293T cells transiently coexpressing p66 ShcA-Flag, Myc-nephrin and constitutively active (CA) or kinase dead (KD)-Fyn were immunoprecipitated (IP) with Flag and immunoblotted (IB) as indicated. p66 ShcA coimmunoprecipitated with phosphorylated nephrin.

(B) Lysates from HEK293T cells transiently coexpressing WT p46/52 ShcA-Flag or an SH2* variant that cannot bind nephrin and CD16/7-neprhin-GFP. Stimulation with CD16 resulted in co-immunoprecipitation of nephrin and WT ShcA, but not the SH2* mutant.
Supplemental Figure 5.2 EEA1 and podocin colocalization is enhanced at the peak of puromycin aminonucleoside nephrosis. (A) Urinalysis of control (Cntl) and puromycin aminonucleoside (PAN)-injected (Day 4, 7, 14) rats showing the characteristic induction of proteinuria. (B) Dual immunofluorescence staining for EEA1 (red) and podocin (green) on kidney sections of control and PAN-injected rats at Day 7 post-injection. Merged images show increased colocalization of EEA1 and podocin at Day 7 compared to control. Scale bar, 20 µm. (C) Biotin-labelled and input samples from Figure 5B were immunoblotted (IB) with podocin. (D) Densitometric quantitation showing a transient decrease in surface podocin levels during the injury timecourse. (n=3). *P<0.05 by two-tailed Student’s t-test. (E) Samples from control and Day 7 of the PAN timecourse were immunoblotted (IB) with nephrin (as in Figure 5B) or WT-1, to verify that a podocyte-specific cytosolic marker is not labeled with biotin.
6.1 Summary of thesis work

The work outlined in this thesis has revealed a requirement for nephrin tyrosine phosphorylation in its endocytosis and that disruption of this mechanism perturbs the delicate balance of nephrin turnover required for maintenance of the filtration barrier (Figure 4.1). Aberrant activation of this machinery can also induce breakdown of the SD barrier, such as appears to occur in the NTS (Figure 4.5) and PAN (157) (Figure 5.4 and 5.5) rodent models of acute kidney injury. Collectively, this identifies tight regulation of phopho-dependent nephrin turnover as an important mechanism for regulation of podocyte function.

These investigations have identified the Nck (Chapter 4) and ShcA (157) (Chapter 5) adaptors as two novel mediators of nephrin endocytosis and the first known to do so by directly binding and modulating phosphorylation of nephrin tyrosine residues (106, 157) (Figure 5.3 and collectively 6.1). A role for the ShcA adaptor within the podocyte had not yet been demonstrated and we have now characterized its role as a novel nephrin phospho-tyrosine binding partner that works to internalize nephrin (Figure 5.1-5.3). We identified upregulated SHCA gene (Table 5.1) and/or protein expression (Figure 5.6) in FSGS, MCD and IgAN and demonstrated that this was associated with increased nephrin endocytosis in the PAN experimental model of MCD (157) (Figure 5.4 and 5.5). This potentially reveals ShcA as a new biomarker for kidney disease, although further analysis is required to confirm this as well as the mechanism by which ShcA induces nephrin’s internalization.

This work has also led to the expansion of Nck’s interactome within the podocyte to include dynamin2 (Figure 4.4). Nck is already well-established to be essential for podocyte FP formation and maintenance by connecting actin to nephrin (3, 53, 54, 75, 95) and we thereby propose that Nck may mediate complex formation between nephrin, actin and dynamin2 during endocytic scission. Actin-nephrin dynamics are essential for stabilization of the SD barrier and we have now demonstrated that they also manage its turnover. Unlike the minimum requirement for a single Y residue for actin tail formation at nephrin (97), however, phosphorylation at a single tyrosine residue appears insufficient for regular nephrin endocytosis (Supplementary figure 4.1); in the presence of dual or triple nephrinY-to-F mutations, nephrin accumulates in tubular structures at the cell surface. Dual mutants targeting Y1176/Y1217 lead to minimal
nephrin tubulation, indicating that phosphorylation of the Y1193 site is nearly sufficient to facilitate endocytosis, whereas loss of phosphorylation at Y1176 and Y1217 may be overcome (Supplementary figure 4.1). Interestingly, previous research has demonstrated that the Y1193F mutant actually allows for increased β-arrestin-nephrin interactions and thereby may promote endocytosis by alternate means (52). Future investigations with the single Y1193F mutant are required to clarify these disparities.

6.2 Why is actin so important? An emerging role for hemodynamic strain in progressive disease

Dysregulation of the actin cytoskeleton by mutations in proteins that link the SD to the actin cytoskeleton (α-actinin-4, FAT Atypical Cadherin (FAT1), CD2AP, PLC-ε1, nephrin and podocin) or that regulate actin polymerization (Inverted formin 2 (IFN2), myosin 1e (Myo1e), Rho GTPase activating protein (ARHGAP) 24, Rho GDP Dissociation Inhibitor Alpha (ARHGDIA) and TRPC6) or podocyte contractility (Myosin heavy chain 9 (Myh9), Myo1e, laminin subunit β2, integrin β4, integrin α3, Lamin A/C, tetraspanin (CD151) and several type IV collagens) give rise to spontaneous kidney disease in humans characterized by glomerular dysfunction (reviewed recently (212)). This thesis demonstrates that loss of nephrin tyrosine phosphorylation also weakens the linkage between the SD and actin cytoskeleton, rendering podocytes more vulnerable and less adaptable to damage (as observed in aging mice and in response to LPS and PS) (118) (Figure 3.1 and 3.2). A relationship between hemodynamic force regulation and actin plasticity has long been recognized (213, 214) and we propose that loss of nephrin tyrosine phosphorylation disrupts the podocyte’s ability to respond to changes in hemodynamic strain, a proposition that requires a thorough understanding of mechanical forces working on the podocyte.

6.2.1 The basis of mechanical strain in the podocyte

Blood filtration in the glomerulus is dependent on glomerular capillary pressure and the glomerular filtration rate (GFR), a metric of the amount of blood that passes through the nephron per minute and represents how well the kidneys are functioning. These two factors are critically regulated by contraction and stretching of the capillaries themselves. Since podocytes cover the outer aspect of the glomerular capillaries, mechanical forces that act on and within the
glomerular vasculature directly impact them (213, 214). These forces include tensile stress, generated by the pressure within the underlying vasculature (190, 215, 216), and fluid shear stress, caused by the passing filtrate at the SD (216, 217). Podocytes must respond accordingly to these multifaceted stressors and actin cytoskeletal restructuring is a well-documented response to both tensile and fluid shear stress in vitro and in vivo (218).

6.2.2 The importance of actin plasticity in resisting mechanical strain

The underlying tensile strain exerted on the podocytes is thought to be generated by the pressure difference between the glomerular capillary lumen underlying the podocyte FPs and the Bowman’s space, which lies behind the filtration barrier (216). In rat glomeruli, increasing the perfusion pressure from 65 to 105 mmHg results in an expansion of pericapillary (podocyte-covered) GBM area by 55% and the length of the SD increased by 59%, whereas there was no change in SD or FP width (215). As a logical consequence, FP must themselves lengthen and/or be newly formed to facilitate pericapillary expansion (217). Actin polymerization machinery presumably is required for these adaptations. In support of this cyclical stretching in vitro is well documented to induce reorganization of the actin cytoskeleton in podocytes (216, 219).

Podocytes appear to be sensitive to even slight changes in fluid shear stress at the SD. Primary filtrate passes through the open fenestrations of the glomerular endothelium and the GBM rather passively, but must squeeze through the narrow SD (190). The speed at which the filtrate passes the SD is relatively slow, but because the width is itself so small, it has been proposed that, even during healthy conditions, the podocytes and the SD components are faced with a high amount of shear stress. Any increases in flow above the normal rate results in a phenomenon known as hyperfiltration and this ultimately increases the shear stress experienced by podocytes (220). This pulls FPs toward the GBM and into the filtration slit and, even if this does not immediately affect podocytes pathologically, it is likely that these forces significantly impact actin’s ability to counteract the pull of FPs into the SD and thereby collapse of the podocytes refined architecture (177). Although it has been difficult to directly evaluate the role of shear stress on disruption of the unique 3-dimensional SD scaffold, reorganization of the actin cytoskeleton has been observed in podocytes exposed to fluid shear stress mimicking ultrafiltrate flow (221, 222), indicating that it is likely similar effects can be expected at the SD in vivo.
6.2.3 Beyond controlling foot process structure: effect of strain on slit diaphragm internalization?

As a function of its ability to disrupt the actin cytoskeleton, physical strain at the SD may play an essential role in the requirement for actin in nephrin’s endocytosis. The elongated endocytic pits observed in nephrin-Y3F mice (Figure 4.1) are reminiscent of those observed in yeast where membrane turgor dictates a requirement for actin during endocytosis (141, 223). This is in direct comparison to most other invaginations observed in mammalian cells, which remain roughly spherical in shape. The differences in the shape of endocytic invaginations has been proposed to be a direct result of differences in the surface tension of their membranes, which is dictated by their stiffness (133). More rigid membranes require actin in order to overcome heightened surface tension and aid in membrane invagination as well as vesicle neck constriction (134, 135), an order at the SD that cannot be filled in the absence of nephrin tyrosine phosphorylation.

By subjecting cells to hypotonic swelling (135) or mechanical stretch (135), it has already been demonstrated that hemodynamic strain can provoke a requirement for actin in endocytosis at the basolateral membrane of mammalian cells. It is likely that the immense tensile strain and shear stress present at the SD likewise dictates the requirement for actin in nephrin’s endocytosis. Nonetheless, it is important to determine experimentally whether hemodynamic strain directly affects nephrin’s internalization in vivo. One method would be to utilize pharmacological interventions to control blood pressure within mice (224) to determine whether high or low blood pressure impacts surface levels of nephrin. Further, it would be interesting to investigate whether chronic induction of high blood pressure, such as through use of the transverse aortic constriction model (225), would be sufficient to accelerate disease in C57BL/6 nephrin-Y3F animals. This would confirm an essential role for nephrin tyrosine phosphorylation in counteracting hemodynamic strain.

6.2.4 A potential role for surface tension of the blood?

Maintenance of appropriate surface tension is not only of fundamental importance in the dynamics of endocytosis, but it also directly affects the formation and function of several tissues including the kidney (226). Surface tension and cortical tension drive tissue morphogenesis (227)
by regulating actin at the cell surface (228). Along with proteins and lipids, the blood is comprised of surfactants that control the surface tension at the interface of the blood and the cell surface (226). Rounding of the cells and ultimately the tissue minimizes their exposure to and effects of surface tension strain and also allows for maximal cell-cell adhesions. When the surface tension of blood is increased, such as appears to be the case in instances of acute and chronic glomerulonephritis, it creates force on the surface of cells, leading to disruption of cortical actin and ultimately inducing flattening of the cell (226). In this manner, heightened surface tension of the blood, which has been documented in various instances of kidney disease (226), may be central to the development of FP effacement, and this may involve disruptions in nephrin’s internalization and SD turnover. Furthermore, the repercussion of increased surface tension on nephrin’s endocytosis may be additive, raising the requirement for actin to facilitate endocytosis while simultaneously disrupting its polymerization at the membrane.

6.3 Nck and ShcA proxies in nephrin endocytosis

The importance of actin polymerization to nephrin in podocyte function was established by studies that demonstrated that loss of nephrin (17), its cytoplasmic domain (17), Nck (53, 54) or various other actin mediators leads to widespread disruption of FP architecture and breakdown of the filtration barrier. The lack of similarly severe disruption until adulthood in nephrin-Y3F animals demonstrated that Y1176, Y1193 and Y1217 are not the only means to mediate normal actin polymerization to the SD (118). Nephrin interacts with several proteins at sites beyond Y1176, Y1193 and Y1217 that can influence actin polymerization at the SD including CD2AP (43, 229, 230), CIN85 (62, 231), IQGAP1 (49, 204), MAGI-1 (51, 232), TRPC6 (50) and Fyn (47, 48). These alternate interactions are proposed to allow for sufficient stabilization of the filtration barrier in the absence of conventional nephrin-Nck binding, indicating they may also act as partial proxies during nephrin endocytosis. However, over time the requirement for phosphorylation at these sites leads to barrier demise and FP disruption (118) associated with disrupted endocytosis (Figure 4.1), indicating their ultimate requirement for barrier maintenance.

Our data supports a role for Nck as central nexus between nephrin, actin and dynamin during endocytosis, evident by the rescue of tubulation with the nephrinY3F-Nck2SH3X3 fusion protein (Figure 4.3). However, in the absence of Nck (Nck null MEFs), internalization of
wildtype nephrin appears to be largely normal (Figure 4.2), again specifying that alternate means of connection between nephrin, actin and dynamin exist during internalization. Whether these are fully compensatory, however, is unknown and, under increased tension induced by hypertonic strain or stretch (135), defects in nephrin endocytosis may be revealed in Nck-null MEFs. Besides the loss of all 3 Nck binding sites, dual mutations involving Y1193 lead to the greatest tubulation of nephrin (Supplemental figure 4.1). We wonder whether this supports the premise that the alternative mechanism must rely on binding to nephrin at this Y1193 site. PLC-γ1, a well-documented binding partner of phospho-nephrin Y1193 (61), can also bind dynamin2 (186), identifying it as a candidate substitute to Nck. Confirmation of this proposed alternate mechanism would require generating triple Nck1, Nck2 and PLC-γ1 knockdown or knockout cells to investigate their collective role in mediating nephrin’s endocytic scission by repeating biotinylation experiments with and without rescue by Nck1/2 and/or PLC-γ1. Importantly, however, in the presence of NckSH3*x3, nephrin endocytosis is again disrupted (Figure 4.2). This dominant negative effect indicates that Nck must act as a preferential binding partner for nephrin tyrosines in that the expression of this mutant blocks that ability of unidentified alternate mechanisms.

Unlike Nck, a requirement for ShcA within podocytes has not yet been demonstrated and disruption of nephrin endocytosis with knockout or knockdown of ShcA in cells and animals may further support a role for ShcA in nephrin internalization. Moreover, the mechanism by which ShcA promotes nephrin’s endocytosis is unknown and it is thereby difficult to hypothesize whether proxies exist in ShcA-mediated disease. Previous studies have demonstrated that ShcA contains an α-adaptin binding domain, which has been shown to bind the AP-2 protein complex, thereby facilitating the recruitment of clathrin to endocytic pits (194). Our group recently demonstrated that disruption of this site through mutagenesis affects ShcA localization to the membrane as well as the internalization of the receptor tyrosine kinase EGFR (233). This mutant may likewise disrupt nephrin’s internalization, demonstrating its dependence on ShcA’s AP-2 site. However, several other podocyte proteins can also bind and recruit clathrin through direct interactions with AP-2, including β-arrestin (234, 235) and even nephrin itself (122), indicating that, in the healthy podocyte, low levels of ShcA may be dispensable for nephrin’s internalization. In the diseased podocyte, however, heightened levels of ShcA may override these normal pathways, leading to aberrant nephrin endocytosis.
6.4 Re-visiting the nephrin Y1193 endocytic switch model

Early characterization of nephrin internalization focused on a key role for β-arrestin in nephrin endocytosis through competition for nephrin with podocin. Loss of nephrin Y1193 phosphorylation promotes β-arrestin binding over podocin and promotes nephrin endocytosis (52). These findings led to the development of the ‘switch model’ which proposes that loss of phosphorylation at Y1193 signals nephrin’s endocytosis, although it should be noted that neither β-arrestin (90) or podocin (44) actually bind the Y1193 site. Theoretical estimations may support the hypothetical role for de-phosphorylation at Y1193 further in that the YDEV residues may act as a YxxØ motif in the unphosphorylated format, allowing for the recruitment of the µ2 subunit of AP-2 and thus clathrin directly to nephrin (122). However, the YDEV motif is also present at the Y1176 site, which should remain available to bind AP-2 in the absence of phosphorylation at Y1193, at least in mice and humans, suggesting that AP-2 recruitment to nephrin may not be the deciding factor in the switch mechanism. Further, because ShcA, which binds preferentially to phosphorylated Y1193, can also recruit AP-2 through its α-adaptin motif, should also be able to mediate AP-2- nephrin interactions in the presence of phosphorylated Y1193.

More recently, podocin was also reported to mediate nephrin endocytosis, although in a phospho-dependent manner through CIE, raft-mediated means (123). This has remained a somewhat confusing concept for several reasons including the phospho-independent nature of podocin-nephrin interactions (44). It is possible, however, that the phospho-dependency of ‘podocin-induced’ nephrin endocytosis is less-so related to competition for nephrin and more-so related to the ability of phosphorylation at these sites to lead to the recruitment of previously unidentified endocytic modulators. It is tempting to propose Nck as one of these candidate molecules. Nck’s recruitment to nephrin may signal for nephrin internalization in the absence of β-arrestin and podocin-mediated retention of nephrin at the invaginating membrane can then facilitate its endocytosis through clathrin-independent mechanisms. Further investigation is required, however to determine whether Nck even mediates nephrin endocytosis through the CME and/or CIE pathways and whether it works in concert with podocin-dependent, raft-associated endocytic mechanisms or possibly via one of the other many clathrin dependent and independent mechanisms that have been identified (126, 236).
It is difficult to consolidate how Y-to-F mutation of residue 1193 can both promote nephrin endocytosis through β-arrestin and also inhibit endocytosis through podocin, Nck and/or ShcA. Research has demonstrated that β-arrestin-induced internalization of nephrin is a CME event, which happens quickly over a short-term timeframe, whereas podocin-mediated internalization occurs via CIE mechanisms and works on a slower-paced, long timeframe (123). It is possible that the Y1193 model can now be re-envisioned as a mechanism to switch between clathrin-dependent and -independent endocytosis of nephrin rather than simply turning endocytosis ‘on’ or ‘off’. These mechanisms may have a direct impact on the outcome of nephrin turnover in either maintaining the barrier, or mediating its breakdown, an area of nephrin trafficking that is poorly understood. In the classical sense, CME occurs constitutively and largely as a means to regularly recycle components at the membrane. Conversely, CIE is often as the result of stimulation, and its long-term nature (and thereby delay in recovery to the surface) may provide the window of opportunity for prolonged protein leakage to occur, as is observed in CKD.

PLC-γ1 signaling dynamics may also fit into this newly proposed switch-like mechanism. It was recently demonstrated that PLC-γ1 can promote phosphorylation of nephrin T1120/T1125, leading to nephrin internalization through β-arrestin (91). However, PLC-γ1 is also recruited to nephrin once it is phosphorylated at Y1193 (61), which appears to inhibit β-arrestin-mediated endocytosis (52). It is possible that phosphorylation of nephrin at Y1193 is a mechanism to suppress PLC-γ1-mediated promotion of β-arrestin mechanisms. This may contribute to the switch-like mechanism that facilitates conversion between CIE and CME-mediated endocytosis of nephrin.

Further investigation into the role of CME versus CIE in these processes is clearly required. This may be aided by the use of various endocytic pathway inhibitors, although they have been characterized to be notoriously non-specific (237, 238). The interplay and potential redundancy of these differential mechanisms represent a major gap in our understanding of nephrin endocytosis. Surface expression of nephrin T-to-A (T1120A/T1125A) (90) and Y3F mutants have not yet been directly compared, and this simple study might help to determine if a disproportionate blockage of endocytosis is observed in one mutant over the other. Further, it would also be interesting to generate a novel compound mutant protein containing both the
T1120A/T1125A and Y3F mutations. This compound mutant may lead to even greater disruption of nephrin internalization, thereby indicating that these two mechanisms are complementary means for nephrin turnover.

6.5 Emerging players in podocyte trafficking

During the course of this work, roles for several other molecules in podocyte trafficking were identified by others, including for CIN85, PACSIN2 and Vps34. These investigations are largely preliminary in nature and future investigations into their relevance and interplay in nephrin turnover in vivo is essential to understanding their role in the podocyte.

6.5.1 CIN85

A role for CIN85/RukL, a homolog of CD2AP (239), in nephrin and podocin endocytosis was recently identified in podocytes (62). Although the precise site of interaction is unknown, CD2AP binds the cytoplasmic tail of nephrin and CIN85 is believed to interact by similar means. CIN85 expression seems to be negatively regulated by CD2AP (240) and depletion of CIN85 in diabetic mice preserves nephrin expression at the SD and reduces proteinuria (178). Of note, reduced nephrin tyrosine phosphorylation at all 3 residues is induced by high blood glucose (73) and it would be interesting to investigate the affect of nephrin’s tyrosine phosphorylation on CIN85-mediated endocytosis. The precise mechanism of CIN85-mediated induction of nephrin endocytosis is unknown. Although some propose CIN85-nephrin interactions to lead to nephrin endocytosis by blocking CD2AP’s stabilization of nephrin through actin (239), other studies have shown that CIN85 can also interact and modulate actin (231) and can even work in concert with CD2AP in actin bundling through Src activation (231). No information on whether CIN85 induces CME or CIE is yet available and it would be interesting to determine the clathrin dependency of these events as well as the impact of nephrin tyrosine and threonine phosphorylation on this mechanism.

6.5.2 PACSIN2

Protein kinase C and casein kinase substrate in neurons (PACSIN)2 is a recently identified, novel nephrin binding partner shown to induce nephrin’s endocytosis. PACSIN2 is upregulated in aging rats in the ZDF obesity/typeII diabetes model, as was the endocytic
trafficking protein Ras-related protein (Rab)5. Although PACSIN and Rab5 appeared to facilitate nephrin endocytosis in culture, their role in the induction of nephrin endocytosis in vivo has not yet been demonstrated. Interestingly, PACSIN 2 can also bind dynamin (241), and, similarly to Nck, it thereby may mediate complex formation between nephrin and dynamin during endocytosis. However, PACSIN has been previously shown to inhibit endocytosis through recruitment of dynamin (241). Further investigations will aid in elucidating PACSIN2’s role in nephrin’s turnover in the future.

6.5.3 Vps34

Unlike CIN85 and PACSIN2, the class III PI3K vacuolar protein sorting 34 (Vps34) protein appears to play a role in modulating endosomal trafficking rather than endocytic initiation within podocytes. In mice, podocyte-specific conditional knockout of Vps34 results in proteinuria, effacement, glomerular scarring, and death within 3-9 weeks of age (242, 243). This is associated with severe disruption of Rab5-positive endosomal compartments and overall endocytosis in Vps34-deficient podocytes. Further, Vps34 deficiency in nephrocytes, the podocyte-like cells of Drosophila, results in a block between Rab5- and Rab7-positive endosomal compartments indicating a disruption in the progression from early to late endosomes. In summary, these data identify Vps34 as a major regulator of endosomal trafficking pathways in podocytes, although a direct role in nephrin turnover has yet to be investigated.

6.6 A new archetype for nephrin internalization

With the emergence of key mechanisms involved in nephrin trafficking, and a potential re-calculation of the Y1193 switch model, we have begun to generate an updated picture of how nephrin turnover is facilitated in the podocyte (Figure 6.2). Both Nck and ShcA appear to work in a feed-forward mechanism whereby, when available to bind to nephrin through their SH2 domains, they facilitate Fyn-mediated hyper-phosphorylation of nephrin Y’s 1176, 1193 and 1217 (106, 157) (Figure 5.3). This may allow for Nck and ShcA to outcompete other phospho-independent nephrin interactions, such as β-arrestin, podocin, Vangl2, CD2AP, CIN85 and PACSIN2, many of which also influence nephrin internalization. Alternatively, because nephrin molecules cluster at the SD, it is also possible that the pool of nephrin molecules is able to bind a multitude of binding partners simultaneously and that competitive nephrin binding is not a major
factor in determining internalization cues. This balance may be tipped with the up- or
downregulation of certain proteins, however, as appears to be the case with ShcA in kidney
disease (Figure 5.4-5.6).

Of note, Nck and ShcA are the only two proteins currently demonstrated to bind multiple
residues at Y1176, Y1193 and Y1217. ShcA prefers binding Y1193, but can also bind Y1217
(157) (Figure 5.2), whereas Nck can bind all 3 residues, although it appears to show a preference
for Y1217 (53). This allows for flexibility in a newly emerging model of nephrin internalization,
whereby maintenance of phosphorylation on least Y1193 or Y1217 for ShcA, and seemingly any
two sites for Nck, is sufficient for their participation in nephrin internalization. The redundancy
of these interactions allows for broader availability of nephrin to Nck and ShcA, regardless of
site-specific phosphorylation. In the case of Nck, this may allow for the inclusion of actin and
dynamin in the endocytic process, regardless of their dependence on clathrin, consistent with a
paradigm in which Nck is central to nephrin endocytosis.

6.7 Impact on human health

The ultimate goal of our investigations is to move towards improving diagnostics and
interventions for individuals suffering with kidney disease. We have identified several exciting
new pathways that influence maintenance of filtration selectivity by directly modulating nephrin
turnover, which seems to be important in maintaining the barrier as well as inducing its
breakdown after aberrant activation.

6.7.1 An overlooked potential for disordered nephrin tyrosine phosphorylation in the
pathogenesis of congenital disease

The discovery of the Fin\textsuperscript{minor} R1109X nonsense mutation initiated intense investigations
into the role of nephrin’s cytoplasmic region in podocyte function nearly twenty years ago (17).
This mutation causes severe congenital disease and the lack of development of SD structures
(27). Until recently, it was largely believed that interactions between phosphorylated nephrin and
Nck were central to the importance of nephrin’s cytoplasmic tail (3, 53, 54, 75). However, the
\textit{NPHSI} mutation R1160X, which causes a smaller truncation of nephrin still inclusive of the
Nck-binding residues, causes childhood disease that is delayed and less severe as compared to
R1109X (81). The disease phenotype displayed by these patients is more directly relevant to the role of Y’s 1176, 1193 and 1217 than full deletion of nephrin’s cytoplasmic region. Not surprisingly, nephrin-Y3F mice display a disease phenotype that is strikingly similar to patients harbouring the R1160X mutation (118) and we have now re-evaluated the role of phosphorylation at Y1176, Y1193 and Y1217 to hold the greatest importance after the completion of barrier development.

Because mutations of NPHS1 Y1176, Y1193 or Y1217 have not been directly identified in human instances of disease, a significant role for nephrin tyrosine phosphorylation in the pathogenesis of CNS has been largely overlooked. Hundreds of NPHS1 missense mutations have been identified in CNS patients (28), but characterization of their effects on nephrin phosphorylation is scarce. Early characterizations of CNS-causing NPHS1 mutations preceded the development of antibodies that could detect tyrosine phosphorylated nephrin. Many of these initially identified mutations afflicting the extracellular IgG-like motifs or their linker domains, causing retention of nephrin in the endoplasmic reticulum of cultured cells (22). Similar mutations with similar patient outcomes were later identified and they were reported to likely lead to similar patterns of disruption, and were not characterized further. More recently, protein folding and pathogenicity prediction models (244-246) have begun to be more widely utilized, although these models are not capable of predicting the impact of mutations on cellular signaling events such as phosphorylation. Now that phospho-nephrin antibodies have been developed, it would be interesting to re-evaluate some of these deleterious mutations to directly determine their impact on nephrin phosphorylation and to determine whether localization of the mutant is disrupted. The importance of these studies is made evident by the sole report where the impact of a nephrin mutation on its tyrosine phosphorylation was investigated, the nephrin-V822M mutation. Similar to other mutations, nephrin-V822M causes a missense mutation in the extracellular region of nephrin, and this leads to congenital disease (149). However, rather than disrupting trafficking to the cell membrane, nephrin-V822M causes disrupted trafficking from the cell membrane and this is accompanied by decreased nephrin tyrosine phosphorylation. Revisiting the characterization of other mutants may similarly unearth a previously disregarded mechanism by which mutations in nephrin’s extracellular domain can disrupt nephrin’s tyrosine phosphorylation and internalization, leading to podocyte dysfunction.
Overall, the role of nephrin tyrosine phosphorylation in instances of disease caused by \textit{NPHS1} mutations is an area that merits further investigation. This may show particular significance in patients in which \textit{NPHS1} mutations result in disease of a more delayed type (during late adolescence and even into adulthood) similar to nephrin-Y3F mice (118).

\textbf{6.7.2 Nephrin tyrosine phosphorylation in non-hereditary disease}

The data presented in this thesis has broadened our knowledge about the role of hypo- and hyper-phosphorylation of nephrin in the pathogenesis of kidney disease of a non-hereditary nature (grey font, Table 1.4). Decreased nephrin phosphorylation is observed in non-heritable MCD (116) and MN (117) as well as during disease initiation in the PAN (95, 116, 157) (Figure 5.4), PS (54, 118) (Figure 3.1), LPS (118) (Figure 3.2) and DN (73, 113) animal models. Increased nephrin phosphorylation can also be observed in disease initiation, including in the PS (54, 118) (Figure 3.2) and NTS (Figure 4.5) models, although disruption of nephrin tyrosine phosphorylation by introduction of the nephrin-Y3F mutation only blocks initiation of NTS-induced damage (Figure 4.6) and not PS-induced injury (Figure 3.1), indicating that the increase in phosphorylation in the PS model may not be directly causative of FP effacement.

ShcA-mediated endocytosis of nephrin, which we hypothesize is induced in the PAN model of injury, requires nephrin tyrosine phosphorylation (Figure 5.3). It would therefore be interesting to repeat PAN experiments in nephrin-Y3F mice to determine the effect of loss of nephrin tyrosine phosphorylation on the induction of disease. Nephrin endocytosis is similarly observed in diabetic models of disease and it would likewise be interesting to examine whether induction of type II diabetes through STZ injections in nephrin-Y3F mice, or by crossing nephrin-Y3F mice onto the Akita type I diabetes model, would further support a role for tyrosine phosphorylation in deleterious nephrin endocytosis during DN.

The recovery period in these acute models is commonly accompanied by normalization of nephrin tyrosine phosphorylation, as in the case of spontaneous recovery from PAN (95, 116, 157) (Figure 5.4), LPS (118) (Figure 3.2) or by administration of HS after PS (118) (Figure 3.1). However, hyper-phosphorylation of nephrin tyrosines may drive recovery in some instances, such as in NTS in which a spike in tyrosine phosphorylation during disease initiation (Figure 4.5) is followed by a prolonged decline below baseline coincident with proteinuria (119) (Figure 3.3).
until hyper-phosphorylation of nephrin is initiated during the 48 hour timepoint, which is consistent with the recovery period (74).

This work has demonstrated that nephrin tyrosine phosphorylation cannot be stringently classified as deleterious or protective, although the nephrin-Y3F mouse model has provided us with convincing evidence that nephrin tyrosine phosphorylation is generally protective, preventing barrier breakdown during aging and promoting recovery from multifarious injuries (118). Aberrant phosphorylation does appear to play a role in the initiation of some forms of disease (Figure 4.5 and 4.6), however, cautioning us on drawing broad conclusions about the role of tyrosine phosphorylation at any given moment. Future investigations should focus on the co-signaling mechanisms that lead to these differential outcomes for nephrin tyrosine phosphorylation, allowing us to gain a better understanding into their role in health and disease.

6.7.3 Is Nck a target for aberrant nephrin signaling?

The role of Nck in modulating nephrin tyrosine phosphorylation has been established in vitro and in vivo and loss of Nck leads to reduced nephrin phosphorylation in mice (106). Modulation of Nck may thereby allow us to manipulate nephrin signaling within the podocyte in a two-pronged manner: 1) by directly disrupting nephrin’s tyrosine phosphorylation; and 2) by blocking its own ability to mediate nephrin signaling through its SH3 domains. However, loss of Nck1/2 is itself deleterious and leads to major disruption of podocyte FP structure and function beyond inducing disrupted nephrin signaling (53, 95, 118), making it a difficult proposition to investigate. Recently, however, the successful use of a cell-permeable chemical inhibitor for Nck’s 1st SH3 domain, AX-024, was described in a mouse model (247). Among other identified binding partners, this is the site of recruitment of the WASP, a relative N-WASp, which also acts as a potent inducer of actin polymerization (248). Interestingly, administration of AX-024 in mice does not appear to significantly affect kidney function, nor does it appear to cause deleterious disruptions to other organs in which Nck expression is known to be essential, such as the heart. Instead, it successfully hampered the immune response in a mouse model of autoimmune disease by blocking Nck’s central role in T-cell activation (247). The level of inhibition exerted by AX-024 thereby seems sufficient to exert significant biochemical effects without disrupting the minimum requirement for Nck. This may be because only a single SH3
domain is targeted, leaving adequate means for the other domains to carry out basic cellular functions.

It is interesting to postulate that use of AX-024 may be a mechanism by which over-activation of nephrin signaling can be modulated within the podocyte. To test this theory, AX-024 could be administered transiently to mice undergoing NTS or PAN-mediated injury. This may disrupt Nck-mediated nephrin endocytosis by disrupting the recruitment of actin that is required for internalization. This may also exacerbate disease, indicating that the ultimate requirement for Nck for podocyte function circumvents its role in nephrin endocytosis during disease initiation.

**6.7.4 Can ShcA serve as a novel biomarker?**

A major shortcoming in the podocyte field remains our ability to identify those patients that will progress to CKD. Identification of novel biomarkers is a great asset in our ability to identify individuals requiring increased monitoring and early intervention. These investigations have revealed that heightened expression of ShcA accompanies disease in a broad range of patients, including those with FSGS, MCD and IgA nephropathies (Table 5.1). This was validated at the level of protein expression in FSGS and MCD patients, who also display abnormal nephrin localization (Figure 5.6). This increase in ShcA was reciprocated in the rat PAN model of MCD (Figure 5.4), in which nephrin and its SD binding partner podocin are endocytosed (Figure 5.5 and 5.6). In the future, it would be beneficial to determine whether increased levels of ShcA are likewise apparent in other models such as NTS, a more acute model of MCD, or DN, both of which have been characterized to initiate nephrin’s endocytosis (73, 111) (Figure 4.5).

It is an exciting possibility that ShcA could be a valuable biomarker for the development of kidney diseases, which are commonly idiopathic in nature (ie: no underlying mutation known). Further characterization of ShcA’s role in barrier demise is required, however, as it is unknown whether ShcA upregulation initiates disease or occurs as a consequence of it and therefore whether it can be a good predictor of the disease progression or, potentially, if it could become a target for therapy in the future. In support of a role for ShcA in kidney damage, previous work has demonstrated that levels of ShcA’s p66 isoform are heightened in
hyperglycemia, which induces oxidative stress (249). Inhibition of upregulated p66 ShcA is sufficient to protect podocytes from oxidative-stress induced damage (249), a result paralleled in mesangial cells (198, 250). Importantly, p66 ShcA is upregulated in experimental DN models (198, 251), and genetic ablation of p66ShcA is sufficient to block the development of DN in vivo through protection of both podocyte and mesangial cell survival (198, 251). Although these studies did not investigate the role of the p46/52 ShcA isoforms in barrier demise, unpublished data from a previous graduate student in our lab has demonstrated that p46/52 ShcA isoforms are similarly upregulated in podocytes cultured in high glucose. This remains an exciting avenue of investigation for the future and collectively supports a role for ShcA in the pathogenesis of podocyte dysfunction.

Consistent with reports concerning p66 ShcA, increased levels of total ShcA were not limited to kidney podocytes in our study, and we observed increased staining for ShcA in the mesangium and tubules of several kidney disease sections (Figure 5.6). Expression profiles from the nephroseq.org database likewise identified heightened SHCA expression in the tubules of diseased patients, although the increases in the glomeruli were consistently greater than that in the tubules (Table 5.1). To directly test whether increased p46/52 ShcA levels within podocytes are sufficient to induce spontaneous disease in vivo, podocyte-specific p46/52 ShcA overexpressing mice should be generated. To separate the contribution of upregulated ShcA in different renal cell types, overexpression studies exploiting tissue-specific promoters could also be employed to allow for the targeted deletion or overexpression of ShcA in podocytes (18), the mesangium (252) or tubules (253, 254) respectively, using standard transgenesis. In addition, using an inducible promoter, such as those sensitive to doxycycline (eg: reverse tetracycline trans-activator (rtTA)), overexpression could be turned ‘on’ or ‘off’. This manipulation would allow for the determination of the impact of ShcA overexpression during development or in adulthood and would also provide the flexibility to modulate ShcA expression during disease initiation or recovery.

Finally, it would be of interest to investigate the mechanism of heightened SHCA gene expression in response to injury. Our finding that ShcA upregulation is similarly seen in PAN-treated podocytes in culture allows us to employ this model as a screen for such a mechanism. A High-Throughput Screen (HTS) using lentiviral- mediated RNA interference (RNAi) is a
sophisticated means that can be used to not only identify the mechanism by which protein expression is altered, but also to simultaneously test potential pharmacological interventions to counteract these alterations (255). Briefly, a library of pooled small interfering (siRNA)-containing lentiviruses targeting thousands of ‘druggable’ genes are screened for their ability to disrupt the upregulation of a protein during some sort of stimulation, in this case, upregulation of p46/52 ShcA induced by PAN treatment of podocytes. Once candidate targets are identified, the accompanying drug can be tested to determine whether it can successfully revert upregulation of p46/52 ShcA. Promising therapies can then be tested within the PAN model, which will allow for determination of the treatment’s efficacy in protecting from kidney disease in vivo. Ultimately, this workflow may lead to the development of treatments for human patients that display heightened levels of ShcA, an exciting prospect for the future.

6.8 Determining nephrin’s fate: the next frontier in nephrin trafficking

Several kidney diseases are characterized by declining NPHS1 expression and reduced levels of nephrin protein, presumably resulting in greater barrier instability. The fate of nephrin after its internalization remains largely a mystery and the majority of studies thus far, including those discussed within the thesis, have focused on mechanisms impacting nephrin’s internalization alone. Trafficking of nephrin to lysosomes post-endocytosis may be a mechanism for this downregulation that has yet to be considered. In fact, decreased levels of nephrin are observed in DN (31-35), MCD (36-38), FSGS (39) and MN (36, 39, 40), all of which are also characterized by nephrin mislocalization. It is logical that the decrease in nephrin levels in these models may occur through the degradation of post-endocytic nephrin. Investigation of nephrin trafficking via IF experiments in which nephrin can be co-stained with endocytic trafficking markers such as EEA1 (256)/Rab5 (257), Rab11 (258) and Rab7 (259, 260) which are widely established markers for early, recycling and late endosomes respectively, may help to unravel nephrin endosomal trafficking post-endocytosis. The role of nephrin tyrosine and threonine phosphorylation post-endocytosis could likewise be characterized using these methods, which may reveal roles for site-specific phosphorylation in nephrin trafficking. This would be consistent with an important role for tyrosine phosphorylation in the trafficking of other receptors post-internalization, such as EGFR (233).
6.9 Towards better models to study nephrin signaling

Although there have been significant advancements in recent years in our ability to characterize cell signaling cascades, unique hurdles remain to furthering our understanding of podocyte cell biology.

6.9.1 Phenotyping tools

A major barrier in the exploration of nephrin dynamics is a lack of tools to investigate site-specific phosphorylation of nephrin tyrosines and threonines throughout signaling events, such as endocytosis. Several groups have developed antibodies that reportedly target phosphorylated nephrin, but only our group’s antibodies have been appreciably characterized for site-specific reactivity and, further, they are the only commercially available antibodies, making it difficult to compare results across studies (Table 1.4). An antibody that detects phosphorylation at T1120/T1125 (90) has also been generated and, although it also is not commercially available, we are currently coordinating its procurement for future studies. Hopefully, future investigations will better characterize the interplay between phosphorylation at these individual sites with respect to their effect on nephrin trafficking.

6.9.2 Cell models

Much of our understanding about the role of ShcA, Nck and other players in nephrin endocytosis has been generated through inferences gained using easily manipulated cell lines such as HEK293T and MEF cells rather than podocytes. HEK and MEF cells lines are easy to culture, transfect and to perform gene silencing in, and they are readily available in nearly every lab. Although these have proven to be valuable tools, podocytes are a highly unique cell type and they express proteins generally absent in other cell types including WT-1, podocin, nephrin and synaptopodin. Further, podocytes are distinctively actin-rich, which we expect to greatly impact endocytosis. Several limitations exist in the culturing of these cells, however, which has made it difficult to use podocyte cell lines thus far. Podocyte cells are terminally differentiated and it is only possible to culture podocyte in vitro through the generation of temperature sensitive conditionally immortalized mouse podocyte cells (MPCs) (261). Unfortunately, even MPCs lack the arborized organization of FP projections observed in vivo and, although several recent
attempts have been made to model the in vivo environment, such as 3-dimensional apparatuses (262, 263) and even a microfluidic glomruli-on-a-chip (264), these models are cumbersome to use and the possibility of their use for routine investigations of gene or protein expression appear far from reality. Of most immediate concern to the use of podocytes for investigation of nephrin signaling is that podocyte cells lose nephrin expression once cultured, and, whereas HEK and MEF cells can easily be transfected with recombinant nephrin to overcome this limitation, podocyte cells are highly resistant to chemical transfection or electroporation (265). This likewise makes it difficult to perform knockdown experiments in MPCs. Collectively this has made it virtually impossible to use standard podocyte cell lines for our study of nephrin signaling and endocytosis to this point.

In an attempt to overcome some of these difficulties, we have begun to generate several tools including an Nck knockout, conditionally immortalized MPC line (265). We have also begun generating adenoviruses to overcome transfection difficulties, a newly emerging technique in the study of podocyte cell biology that is highly efficient (266). Adenoviruses expressing wildtype and mutant nephrin are currently being characterized and will provide us the valuable ability to model nephrin signaling within podocytes. The use of these tools should allow for investigation of nephrin trafficking directly within podocytes and it is important to repeat our previous findings to confirm their relevance within the native cell type. Ultimately, however, limitations in our ability to model the complexity of the glomerular environment in cell culture are unlikely to change, hampering our ability to model the most multifarious aspects of podocyte biology in vitro. It is unlikely that trafficking mechanisms uncovered in cell culture always parallel the same events in podocytes within the complex organism, or that cell signaling alterations in vitro always impact glomerular filtration in vivo. The use of both cell culture and animal models, in concert with data from human patients where possible, remains an important strategy for future studies (265).

6.9.3 Animal models

The emergence of animal models as a means to study human disease has been a valuable tool in the study of CKD (156). Unfortunately, animal models too have limitations in their ability to replicate the human environment including CKD (267). CKD is a diverse group of diseases
and patients suffering with even the same diagnosis or same stage of disease display a range of traits and, presumably, underlying signaling signatures. Even within subtypes, disease etiology is often unknown and it is believed that disease origins are diverse and multifactorial. When combined with the vast differences in underlying genetics and health histories amongst individual patients, it is not surprising that disease progression, response to treatment and eventual outcomes are likewise variable between patients. It is not possible to represent the diversity of CKD patients with the uniform pathomechanisms of the disease models we employ and the inbred mice that we tend to use. In addition, disease models are often based on genetic mutations or chemical exposures that do not even afflict humans, bringing into question how well they can model human disease. Further complicating matters, analogous genes even appear to have different impacts in rodents and humans. For example, mutations in PLCε1 underlie CNS in humans, while PLCε1-null mice do not display any overt phenotype (268). Although undeniably useful, these examples show that there is room for improvement in our ability to use rodents to model human disease.

In recent years, a movement towards using ‘humanized’ animal models has emerged, improving the ability to model human diseases in mice (269). For example, mice lack the NADPH oxidase (Nox) 5 enzyme, which is a significant source of reactive oxygen species and appears to contribute to podocyte dysfunction in human DN, a disease that is severely muted in mice as compared to instances in human patients (270). ‘Humanizing’ mice through podocyte-specific expression of human Nox5 led to a striking exacerbation of DN in these mice (270), making them more akin to their human counterparts. This not only again demonstrates the limitations of using animals to model human disease, but also highlights our ability to devise means to overcome these limitations to allow rodent models to better replicate human conditions.
Figure 6.1 Contributions of Nck and ShcA adaptor proteins in nephrin’s internalization.
Upon tyrosine phosphorylation by the Src family kinase Fyn, nephrin can recruit the SH2-containing adaptors Nck and ShcA to the indicated residues on its cytoplasmic tail, which facilitates nephrin’s internalization. ShcA’s adaptin binding domain facilitates recruitment of clathrin to the invaginating membrane, while Nck’s 3 SH3 domains facilitate actin recruitment through proline rich regions (PRR) on PAK-1 and N-WASp, which facilitates pit elongation, as well as the GTPase dynamin, which facilitates scission of the nascent vesicle. Both Nck and ShcA work in a feed-forward mechanism (red and green arrows) whereby they promote tyrosine phosphorylation of nephrin by hyper-activation of Fyn.
Figure 6.2 Representation of our current understanding of the network responsible for regulation of nephrin endocytosis. Nephrin’s internalization is regulated by a variety of binding partners that interact with nephrin by phospho-dependent and independent means on a multitude of tyrosine and threonine residues. Clathrin-dependent nephrin endocytosis (black) is facilitated through β-arrestin and ShcA, and this pathway is supported by proteins associated with cell polarity. Alternatively, podocin promotes clathrin-independent nephrin endocytosis (white), mediated by lipid raft and caveolae-associated mechanisms. CIN85 competes with CD2AP for nephrin to induce its internalization. Nck adaptors facilitate the recruitment of actin and dynamin to nephrin irrespective of clathrin (speckled). The ability of Nck to bind 3 distinct nephrin residues allows Nck, actin and dynamin to participate throughout endocytosis, independent of site-specific phosphorylation status. Competition for nephrin between β-arrestin and podocin, PLC-γ1 and Nck is dictated by the phosphorylation status of Y1193. In this manner, Y1193 acts as the switch between clathrin-dependent and independent mechanisms and this can impact the speed of nephrin internalization and possibly also its downstream trafficking.
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