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Alpha-Actinin-4 and the Podocyte - Implications for Focal and Segmental Glomerulosclerosis

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α-Actinin-4 and the podocyte – Implications for focal segmental glomerulosclerosis

Jean-Louis R. Michaud

Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
In partial fulfillment of the requirements
for the Ph.D. program in Cellular and Molecular Medicine

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Abstract

Focal and segmental glomerulosclerosis (FSGS) is a common glomerular lesion and a significant cause of end-stage renal disease (ESRD). FSGS lesions result from damage to glomerular epithelial cells called podocytes, a key cell type involved in glomerular filtration. Mutations in the *ACTN4* gene, encoding an actin-crosslinking protein, are causative of late-onset familial FSGS in humans. We have developed a mouse model of FSGS by expressing a high-affinity variant of α-actinin-4 (K256E) in a podocyte-specific manner. Transgenic mice display podocyte damage, subsequent loss of serum proteins into the urine (proteinuria), and glomerular sclerosis, similar to that observed in human patients. In cultured podocytes, α-actinin-4^{K256E} is mislocalized to stable actin-rich structures, which impairs cytoskeletal-dependent processes such as cell spreading and migration. We have also demonstrated that binding of α-actinin-4 to filamentous actin is regulated by calcium and phosphoinositides. Binding of calcium decreases the association of α-actinin-4 with actin, while binding of PIP_2 and PIP_3 enhances this interaction. High-affinity variants of α-actinin-4 are very static compared to the wildtype protein, and are insensitive to regulation by calcium or phosphoinositides. Such cytoskeletal dysfunction severely impairs the podocyte's ability to withstand mechanical stretch, a mimic of the distensive forces exerted on the podocytes *in vivo*. Although much insight has recently been gained into the biology of the podocyte, treatments for FSGS and other glomerular disorders remain few and ineffective. Further studies into the mechanisms involved in regulating the podocyte cytoskeleton in healthy and diseased states will surely lead to novel therapeutic interventions.

Table of contents

Table of contents

List of tables

Chapter 1

List of figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

List of abbreviations

ABD : actin binding domain
ABS : actin binding site
ACE : angiotensin-converting enzyme
ANOVA : analysis of variance
AT1 : angiotenin II type 1
BCA : bicinchoninic acid
BSA : bovine serum albumin
CD2AP : CD2-associated protein
CH : calponin homology
CIHR : Canadian Institute of Health Research
CNF : congenital nephrotic syndrome of the Finnish type
CORR : Canadian organ replacement register
DAPI : 4'-6-diamidino-2-phenylindole
ECL : enhanced chemiluminescence
ECM : extracellular matrix
EDTA : ethylene glycol tetraacetic acid
EGTA : ethylene diamine tetraacetic acid
EM : electron microscopy
ES : embryonic stem
ESRD : end-stage renal disease
F-actin : filamentous actin
FAK : focal adhesion kinase
FBS : fetal bovine serum
FRAP : fluorescence recovery after photobleaching
FSGS : focal segmental glomerulosclerosis
G-actin : globular actin
GBM : glomerular basement membrane
GDIa : GDP dissociation inhibitor α
GFP : green fluorescent protein
GLEPP1 : glomerular epithelial protein 1
GPCR : G-protein-coupled receptor
GST : glutathione S-transferase
HA : hemagglutinin
HIV : human immunodeficiency virus
HRP : horseradish peroxidase
ILK : integrin-linked kinase
JAM4 : junctional adhesion molecule 4
MAGI-1 : MAGUK with inverted orientation-1
MAGUK : membrane-associated guanylate kinase

MOI : multiplicity of infection
mut : mutant
NHERF : Na^+/H^+ exchanger-regulatory factor
NP : nephrin promoter
NSAID : non-steroidal anti-inflammatory drug
ORF : open reading frame
PAS : periodic acid Schiff
PBS : phosphate-buffered saline
PCR : polymerase chain reaction
PIP_2 : phosphatidylinositol (3,4)-bisphosphate
PIP_3 : phosphatidylinositol (3,4,5)-trisphosphate
PMSF : phenylmethylsulfonyl fluoride
RAS : renin angiotensin system
ROS : reactive oxygen species
RT-PCR : reverse transcriptase PCR
S.E.M. : standard error of the mean
SDS-PAGE : sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA : short hairpin loop RNA
SPARC : secreted protein acidic and rich in cysteine
STZ : steptozotocin
TI : Triton X-100 insoluble
TRPC6 : transient receptor potential channel 6
TS : Triton X-100 soluble
USRDS : United States Renal Data System
UTR : untranslated region
VEGF : vascular endothelial growth factor
wt : wildtype
WT1 : Wilms' tumour suppressor 1
ZO-1 : zonula occludens-1

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Thirdly, I would like to thank various collaborators for donating reagents used in these studies. After nearly a year of unsuccessfully attempting to transfect podocyte cell lines, the adenoviruses developed by Robin Parks (Ottawa Health Research Institute) finally provided a tool for efficient gene expression in cultured podocytes. The conditionally-immortalized podocyte cell lines were kindly donated by Peter Mundel (Mount Sinai School of Medicine, New York, USA) and Karlhans Endlich (University of Greifswald, Greifswald, Germany); the A7r5 cell line was obtained from Carol Otey (University of North Carolina at Chapel Hill, Chapel Hill, USA).

Lastly, I would like to thank the members of the Kennedy lab, the Animal Care and Veterinary Services staff, and the entire Kidney Research Centre staff for their help and ongoing support.

Chapter 1

General introduction

1.1 Focal and segmental glomerulosclerosis

Second only to diabetes, focal and segmental glomerulosclerosis (FSGS) has become a leading cause of end-stage renal disease (ESRD) (Kerjaschki 2001). ESRD is characterized by a dramatic loss in renal function requiring organ replacement therapy, and has thus become a major public health concern. According to the Canadian Organ Replacement Register (CORR) 2006 Annual Report, 18,827 patients were on dialysis and 12,099 patients were living with a functional kidney transplant at the end of 2004. These numbers amount to a total of 30,926 Canadians with kidney failure registered in the CORR. According to data from the United States Renal Data System (USRDS), the proportion of ESRD attributed to FSGS has increased considerably to 2.3% in 2000. However, patients classified as having acquired immunodeficiency syndrome nephropathy were excluded from this study. A recent review of available data suggests that FSGS may account for up to 20% of dialysis patients (Daskalakis and Winn 2006). To date, most treatment strategies other than organ transplantation and dialysis have proven to be ineffective at preventing FSGS. A better understanding of the molecular events involved in the progression of FSGS is needed in order to develop more effective therapies.

1.1.1 Etiology and manifestations

FSGS is not a particular disease, but rather represents a heterogeneous pattern of glomerular lesions (scarring) associated with nephrotic syndrome (Figure 1.1) (D'Agati 1994). The term "focal" implies that lesions are observed only in some glomeruli of a

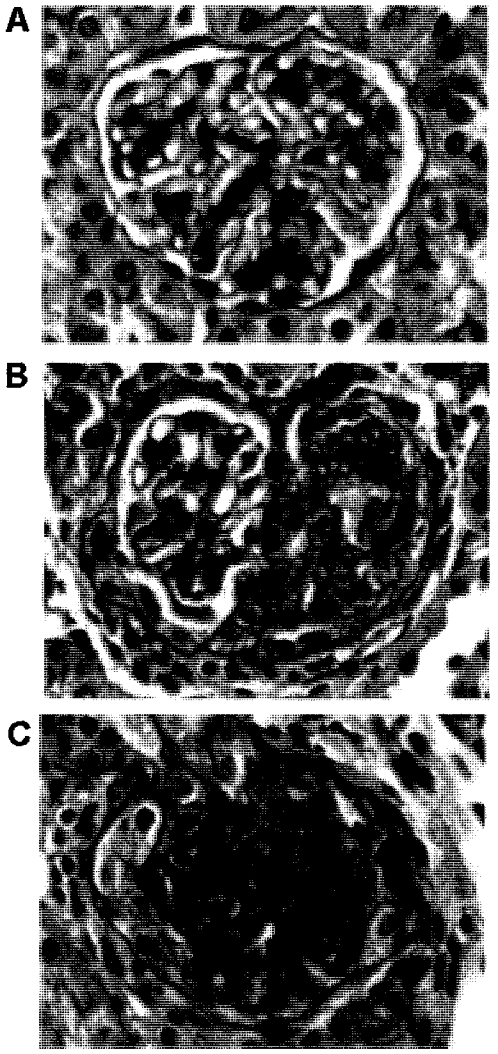

Figure 1.1 Ultrastructure of renal glomeruli.
The glomerulus is a capillary bed essential for the filtration of plasma and subsequent removal of toxic molecules from the body. Damage to the glomerular epithelial cells known as podocytes initiates the progression from healthy glomerulus **(A)** to segmental glomerulosclerosis **(B)**, a common lesion observed in many renal disorders. Such lesions often progress to global sclerosis **(C)**, a state in which the capillary loops are completely obstructed by extracellular matrix components (scar tissue). Stain: periodic acid Schiff (PAS); magnification: 200X.

given renal biopsy. The term "segmental" implies that these lesions only affect regions or segments of a given glomerulus. Due to the heterogeneous nature of these lesions, the classification of FSGS is not as straightforward as one would think. However, the widely accepted classification separates FSGS based on its etiology (detailed in Table I) (D'Agati 1994; Meyrier 2005). Primary (idiopathic) FSGS is a disease of unknown cause, possibly due to the actions of a yet unidentified circulating glomerular "permeability" factor. Secondary FSGS results from structural and/or functional glomerular changes arising from other conditions such as diabetes, obesity, and hypertension. Lastly, familial FSGS results from genetic mutations in a small number of genes important for glomerular function (Table II) (Daskalakis and Winn 2006).

The major clinical manifestations of FSGS include the leakage of protein into the urine, a condition known as proteinuria (or albuminuria), and declining renal function (D'Agati 1994; Cameron 2003). Excessive loss of protein into the urine can in turn lead to reduced vascular oncotic pressure and edema. Hypertension is also commonly associated with FSGS. The pathology of FSGS is characterized by segmental glomerular sclerosis and loss of normal glomerular epithelial cell (podocyte) structure. It is now widely accepted that injury to the podocyte plays a crucial role in the initiation and progression of FSGS lesions (Kriz 2002).

1.1.2 Models of progression

The decline in renal function observed in renal failure is attributed to the progressive loss of viable nephrons, the functional units of the kidney (Kriz and LeHir 2005; LeHir and Kriz 2007). It is hypothesized that most glomerular diseases are

Table I. Classification of focal segmental glomerulosclerosis *

Type	Etiology
Primary (idiopathic) FSGS	
	Unknown
	Circulating permeability factor
Secondary FSGS	
	Renal dysplasia
	Reflux Nephropathy
	Surgical ablation
	Diabetes mellitus
	Hypertension
	Obesity
	Sickle cell disease
Familial FSGS	
	Mutations in podocyte-related genes
HIV-associated FSGS	
	HIV infection
Heroin nephropathy	
	Heroin and cocaine abuse

* Adapted from D'Agati 1994.

Table II. Genes associated with familial FSGS

Gene	Protein product	Inheritance	Effect of mutations
NPHS2	Podocin	Autosomal recessive	Failure of nephrin recruitment to lipid rafts
		Compound heterozygous	Decreased nephrin binding
ACTN4	α-Actinin-4	Autosomal dominant	Increased affinity for filamentous actin
CD2AP	CD2-associated protein	Autosomal dominant (haploinsufficiency)	Severe truncation resulting in impaired intracellular trafficking
		Autosomal recessive	Slight truncation causes decreased affinity with filamentous actin
TRPC6	Transient receptor potential channel 6	Autosomal dominant	Increased calcium influx

initiated within the podocyte and progress to glomerular sclerosis via common pathways. Three pathways, each involving podocyte dysfunction, have been proposed – the degenerative, the inflammatory, and the dysregulative pathways (Kriz and LeHir 2005).

In the degenerative pathway, podocytes are exposed to persistent long-term challenges, including glomerular hypertension and metabolic disturbances, leading to a progressive loss of podocytes (via apoptosis or detachment). The remaining podocytes will undergo adaptive hypertrophy in order to maintain the integrity of the filtration barrier, resulting in an increased workload on these cells. Once a threshold of podocytes has been lost, naked areas of glomerular basement membrane (GBM) come into contact with the parietal epithelial cells that line Bowman's capsule. This interaction triggers the formation of an adhesion to the glomerular tuft as the parietal cells produce excessive extracellular matrix (ECM) components.

As its name implies, the inflammatory pathway results from an endocapillary inflammation which induces a stereotypical pattern of morphologic alterations in podocytes. The most remarkable feature of such inflammatory diseases is the presence of coarse and irregular microprojections on the apical aspect of the podocyte cell body, which form bridges with the parietal basement membrane. Cells adjacent to a podocyte bridge develop further bridges, and also deposit ECM components between the cells.

In the dysregulative pathway, podocyte injury (e.g. via HIV infection) leads to loss of specific cell shape accompanied by uncontrolled proliferation, a process known as dedifferentiation. The cells adopt a cuboidal shape and fill Bowman's space, causing endothelial damage followed by the collapse of the glomerular capillaries.

1.1.3 Treatment

Parallel to the degree of heterogeneity of FSGS lesions is the variability in the course of the disease. While some patients can have relatively mild, subnephrotic-range proteinuria and stable renal function, others develop severe nephrotic syndrome with progressive renal failure (D'Agati 1994). During early stages of the disease, therapies are aimed at limiting proteinuria in order to slow the progression to ESRD, which requires renal replacement therapy (dialysis or organ transplant) (Korbet 2002; Cameron 2003). Treatment strategies often include dietary modifications (e.g., restriction of protein intake) and antihypertensive therapy using angiotensin-converting enzyme (ACE) inhibitors, which block angiotensin-induced post-glomerular vasoconstriction. Supplementing ACE inhibition with non-steroidal anti-inflammatory drugs (NSAIDs) may exert an additive effect by countering prostaglandin-induced pre-glomerular vasodilation. Corticosteroid therapy has been effective in some patients; however, many forms of FSGS are steroid-resistant.

Interestingly, recent studies using a chemically-induced model of FSGS report that podocyte depletion below 20% results in transient proteinuria and normal renal function. Conversely, podocyte depletion greater than 40% results in sustained high-grade proteinuria, global glomerulosclerosis and reduced renal function (Wharram, Goyal et al. 2005). These studies suggest that therapeutic interventions during the early stages of the disease are imperative for the maintenance of glomerular integrity and sustained renal function. However, these studies also highlight the fact that a better understanding of the mechanisms involved in the progression of podocyte injuries is needed in order to develop optimal therapeutic strategies for various stages of the disease.

1.2 Structure and function of the podocyte

The filtering capacity of the kidney resides in a specialized part of the nephron called the glomerulus. The glomerulus is a capillary bed home to three different cell types – endothelial cells, mesangial cells, and epithelial cells (podocytes) – each contributing to the integrity and function of the glomerular filtration barrier. This barrier acts to restrict the passage of blood cells and large proteins while permitting the unimpeded flow of fluid and electrolytes into the nephron. Podocytes are now considered the most important cell type in glomerular filtration, and injury to these cells is the key event which initiates the progression to FSGS lesions.

1.2.1 Podocyte architecture

The podocyte is a peculiar, arborized cell which lines the outer aspect of the glomerular capillaries. Structurally, podocytes consist of a large cell body, from which emanate major processes that branch into numerous foot processes (Figure 1.2) (Pavenstadt, Kriz et al. 2003). Foot processes from a given podocyte interdigitate with those of its neighbors, providing an interface with a large surface area where filtration may occur. The two principal functions ascribed to the podocytes are to counteract the distensive forces of the capillary wall and contribute to the size-selective nature of the filtration barrier.

Podocytes are attached to the underlying GBM via the action of $\alpha_3\beta_1$ integrins and the dystroglycan complex (See Figure 2.1) (Kretzler 2002). Expression of these adhesive components is limited to the sole plate of the foot processes, which securely anchor the

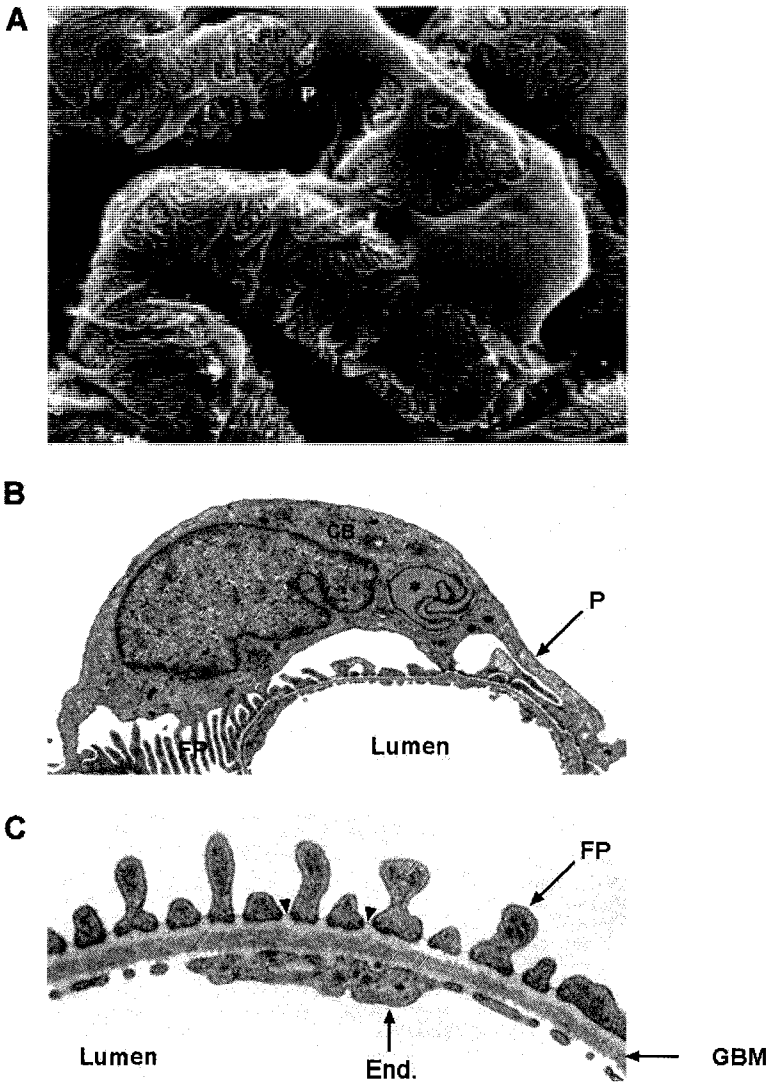

Figure 1.2 Architecture of the glomerular podocyte.
(**A**) Podocytes are epithelial cells which line the outer aspect of the glomerular capillaries. They consist of a cell body (CB), from which emanate primary processes (P) that separate into interdigitating foot processes (FP). (**B**) Cross-section of a podocyte, illustrating the relationship between the cell body, primary processes and foot processes. (**C**) Cross-section of podocyte foot processes illustrating the layers of the glomerular filtration barrier: endothelial cells (End.), glomerular basement membrane (GBM), and foot processes. The space between adjacent foot processes is bridged by a molecular sieve called the slit diaphragm (arrowheads). Adapted from Pavenstädt et al., *Physiological Reviews*, 2003.

podocyte to the GBM, while the voluminous cell body appears to "float" in the urinary space. Decreased expression of podocyte adhesion molecules is disastrous, leading to the loss of podocytes via detachment and ensuing glomerular sclerosis (Adler 1992).

1.2.2 The podocyte cytoskeleton

The unique morphology of the glomerular podocyte is highly dependent on its cytoskeleton, a cytoplasmic system of fibers which provides rigidity while remaining pliable. Three basic types of filaments exist in eukaryotic cells; microfilaments (7-9 nm diameter), intermediate filaments (10-12 nm), and microtubules (24 nm). These filaments each contribute to the structural integrity of the podocyte and display several levels of organization.

Microfilaments, composed of actin monomers (G-actin) organized into filamentous structures (F-actin), are the predominant cytoskeletal constituent of the foot processes (Pavenstadt, Kriz et al. 2003). These filaments provide structural integrity to the foot process and are tethered to cell-matrix contacts and cell-cell contacts. At the base of the foot processes, microfilaments are coupled to the intermediate filaments and microtubules of the major processes (Drenckhahn and Franke 1988).

Intermediate filaments are commonly thought to provide mechanical strength to cells. Podocytes express the intermediate protein vimentin (Holthofer, Miettinen et al. 1984), along with the intermediate filament-associated protein plectin (Yaoita, Wiche et al. 1996). Plectin acts as a link between the three components of the cytoskeleton, and may therefore play an important role in stabilizing the transition between primary processes and foot processes.

Microtubules, made of α- and β-tubulin, are stiff tubular structures that originate in the perinuclear region of the podocyte cell body. Microtubules are essential for the structure of major processes and provide a connection between the cell body and the foot processes (Kobayashi and Mundel 1998). Since trafficking of proteins from the Golgi apparatus to the cell periphery is a major role of microtubules, they are likely of critical relevance for process formation and maintenance, ensuring adequate delivery of proteins to the foot processes.

As mentioned above, podocyte injury often results in a structural alteration of foot processes, known as foot process effacement. Effacement involves the fusion of two or more foot processes and is initiated at the cytoskeleton, resulting in the alteration of both cell-matrix contacts and cell-cell contacts, each contributing to the progression of glomerular sclerosis.

1.2.3 Components of the slit diaphragm

The interdigitating pattern of the podocyte foot processes provides a very high surface area where filtration can occur. The spaces between adjacent foot processes – the filtration slits – are bridged by a molecular diaphragm which allows passage of small molecules such as water and electrolytes, while effectively retaining larger molecules such as albumin in the blood stream. The structure of the slit diaphragm was first described in 1974, when Rodewald and Karnovsky used electron micrography to observe a zipper-like substructure with alternating cross bridges extending from the podocyte plasma membrane (Rodewald and Karnovsky 1974). This structure was eventually further resolved, with the discovery of nephrin (Kestila, Lenkkeri et al. 1998) – a key

component of the slit diaphragm – paving the way to the identification of numerous other slit diaphragm proteins (Figure 2.1). For further details on the discovery of individual slit diaphragm components and their function, see Chapter 2.

While the slit diaphragm effectively functions as a physical sieve, it is also believed that some of its components may participate in outside-in signaling, allowing the podocyte to respond to changes in the extracellular environment (Benzing 2004). For this to be true, the slit diaphragm must be stabilized at the plasma membrane of the podocyte foot process and securely anchored to intracellular components of the cytoskeleton. Reduced expression of many slit diaphragm proteins and cytoskeletal alterations are observed in many glomerular diseases, accompanied invariably by proteinuria, supporting the notion that preservation of slit diaphragm integrity is critical for glomerular filtration.

1.3 The α-actinin family

The podocyte cytoskeleton is responsible for the unique morphology of this cell type and is also crucial for the organization and integrity of the slit diaphragm. Working in concert with the various cytoskeletal components are numerous cytoskeleton-associated proteins (Figure 2.1). One such protein is α-actinin-4, an actin crosslinking protein of the spectrin superfamily. α-Actinin-4 is highly expressed in the podocyte foot processes; its putative functions include F-actin bundling, stabilizing slit diaphragm components, and linking adhesive structures to the cytoskeleton.

1.3.1 Structure of α-actinins

The α-actinins are a family of highly conserved actin-binding proteins belonging to the spectrin protein superfamily. To date, four different isoforms with unique tissue-expression profiles have been described (Otey and Carpen 2004). α-Actinin-2 and -3 are components of the Z-line of striated muscle, while α-actinin-1 and -4 are expressed in non-muscle cells.

α-Actinins are cytosolic proteins (MW of ~ 105 kDa) that exist as head-to-tail homodimers with an actin-binding domain (ABD) at the N-terminus of each monomer (Figure 1.3) (Matsudaira 1991). The ABD is composed of two calponin homology (CH) domains, and contains 3 specific actin-binding sites (ABS1 -3). Under normal conditions, the ABD adopts a "closed" conformation, in which ABS2 and 3 are exposed and able to bind F-actin, while ABS1 remains buried within the ABD and unavailable for F-actin binding (Franzot, Sjoblom et al. 2005). A phosphoinositide-binding site is also present within the second CH domain, suggesting that the binding of α-actinin to actin may be regulated by phospholipids (Honda, Yamada et al. 1998).

The central portion of α-actinins, also known as the rod domain, consists of four tandem spectrin-like repeats. These repeats were initially regarded as simple spacers for building extended, rod-shaped molecules. However, beyond their role in dimerization, recent data suggest that the spectrin-like repeats also serve as docking sites for other molecules (Otey and Carpen 2004). Furthermore, the spectrin-like repeats may confer a degree of flexibility to the α-actinin molecule, allowing it to resist mechanical strain (Ylanne, Scheffzek et al. 2001).

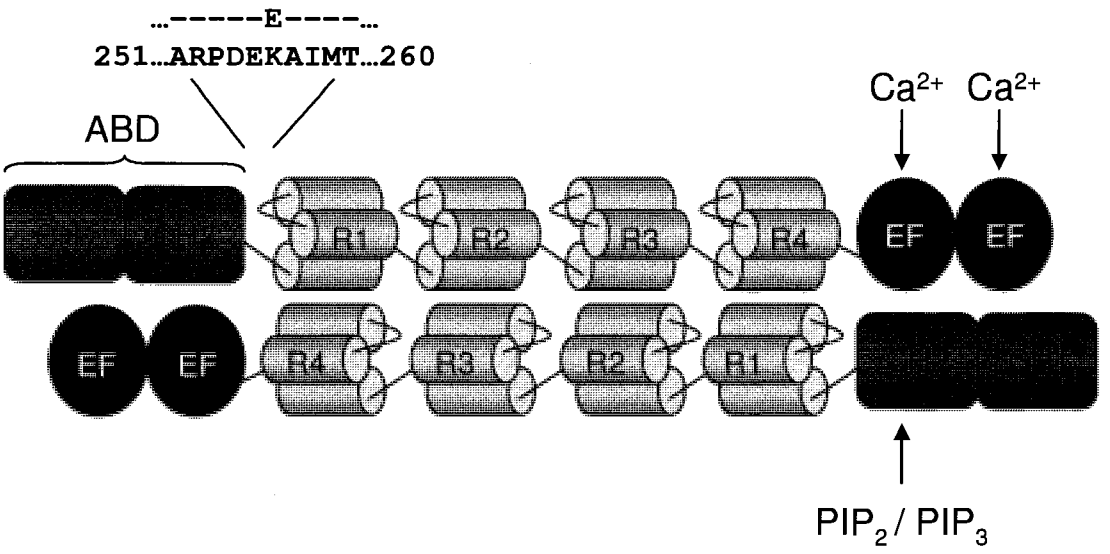

Figure 1.3 Domain structure of α-actinins.
α-Actinins are cytosolic proteins existing as head to tail homodimers. Each monomer contains an actin-binding domain (ABD), four central spectrin-like repeats (R1-R4), and two calcium sensing EF-hand motifs (EF). A phosphoinositide (PIP_2/PIP_3)-binding site is also found within the second calponin-homology (CH) domain of the actin-binding domain. Mutations in α-actinin-4, such as the K256E mutation shown in red, are associated with a familial form of FSGS.

The C-terminal portion of α-actinins contains two Ca^{2+}-sensing EF-hand motifs. Although these motifs are present in all four isoforms, only the non-muscle isoforms have retained sensitivity to Ca^{2+}, suggesting that regulation by Ca^{2+} may play an important regulatory role in non-muscle cells.

1.3.2 Sarcomeric (muscle) α-actinins

In skeletal muscle, α-actinin-2 and -3 constitute the predominant protein component of the sarcomeric Z-line, where they form a lattice structure that anchors together actin-containing thin filaments and stabilize the muscle contractile apparatus (MacArthur and North 2004). In addition to their mechanical role, the sarcomeric α-actinins interact with proteins involved in a variety of signaling and metabolic pathways. The expression pattern of the two sarcomeric α-actinins has diverged, with α-actinin-2 becoming the predominant isoform in heart and oxidative skeletal muscle fibers, while α-actinin-3 expression is largely restricted to fast glycolytic skeletal muscle fibers (Beggs, Byers et al. 1992).

Given the localization of the sarcomeric α-actinins, the *ACTN2* and *ACTN3* genes appeared to be excellent candidates for genes affected in human muscle disease. Initial screening of a number of congenital muscular dystrophy patients showed a striking deficiency of α-actinin-3 in a subset of patients (MacArthur and North 2004). However, it was later established that the deficiency was completely unrelated to muscle disease in many patients, as linkage analysis ruled out the *ACTN3* gene as the causative locus. Subsequent sequencing of the entire coding region of the *ACTN3* cDNA from an α-actinin-3-deficient patient revealed a premature stop codon mutation (R577X) in one allele, resulting in an unstable protein that was rapidly degraded (North, Yang et al.

1999). However, approximately 18% of humans of various ethnic groups are homozygous for this polymorphism, indicating that the complete deficiency of α-actinin-3 does not result in a disease phenotype.

Interestingly, the frequency of the R577X null allele was found to be significantly lower in elite sprint/power athletes, suggesting that α-actinin-3 is required for optimal muscle performance at high velocity (Yang, MacArthur et al. 2003). Conversely, a higher frequency of the null allele was found in endurance athletes, suggesting that the absence of α-actinin-3 may actually be of some benefit for endurance performance.

The recent development of α-actinin-3-deficient mice has provided mechanistic insights into the association between α-actinin-3 deficiency and human athletic performance. Muscle from *ACTN3*$^{-/-}$ mice displays reduced force generation, consistent with results from human association studies (Macarthur, Seto et al. 2008). Detailed analysis of *ACTN3*$^{-/-}$ mice revealed a reduction in fast fiber diameter, altered contractile properties, and enhanced recovery from fatigue, suggesting a shift in the properties of fast fibers towards those of slow fibers. In sum, the two *ACTN3* alleles may each provide advantages for different types of muscle performance, a possibility with obvious implications in the understanding of the genetic influences on human physical performance.

1.3.3 Cytoskeletal (non-muscle) α-actinins

α-Actinin-4, the newest member of the α-actinin family, was identified following its upregulation in highly motile cells (Honda, Yamada et al. 1998). α-Actinin-4 shares a high degree of homology with α-actinin-1 (86% similarity at the amino acid level), both of which are expressed ubiquitously. In non-muscle cells, α-actinins are thought to

crosslink actin filaments and connect the actin cytoskeleton to the cell membrane. α-Actinin-1 has been reported to associate with cell adhesion molecules and is thought to play an important role in stabilizing cell adhesions (Rajfur, Roy et al. 2002) and regulating cell shape and motility.

Upregulation of α-actinin-4 has been reported in various colorectal, lung, ovarian and breast cancer cell lines (Honda, Yamada et al. 1998; Honda, Yamada et al. 2004; Honda, Yamada et al. 2005; Yamamoto, Tsuda et al. 2007). The increased expression of α-actinin-4 seems to be involved in the infiltrative growth of some cancers, leading Yamagata et al to conclude that α-actinin-4 may be a significant predictor of metastatic potential (Yamagata, Shyr et al. 2003). Intriguingly, α-actinin-4 has also been reported to shuttle between the cytoplasm and the nucleus. Cytoplasmic localization was associated with an infiltrative phenotype and correlated significantly with a poorer prognosis in many cases of breast cancer (Honda, Yamada et al. 1998). The relevance of nuclear α-actinin-4 remains unknown, though it has been suggested that transfer to the nucleus may abolish the metastatic potential of human cancers.

Alternative RNA splicing has gained considerable attention, especially in the context of cancer-related genes. Consistent with its apparent role in cell motility and tumorigenicity, a novel splice variant of α-actinin-4 was identified in human small cell lung cancer (Honda, Yamada et al. 2004). Alternative splicing of exon 8 encodes a variant with three amino acid substitutions which increase its binding to F-actin. Interestingly, these mutations in exon 8 are adjacent to those identified in three families affected with FSGS (see section *1.3.5*). The authors conclude that dysregulation of the actin cytoskeleton may explain the small cell size, cell fragility, and the aggressive behavior of these cancerous cells. However, the effects of alternatively-spliced α-actinin-

4 may be cell specific, as expression of the splice variant led to a less severe phenotype in a non-cancerous cell line (NIH 3T3).

Non-muscle α-actinins are often regarded as simple actin filament crosslinking proteins. Expression of these isoforms at the cytoplasmic face of several types of cell adhesion sites – including focal adhesions, adherens junctions, and hemidesmosomes – suggests a higher degree of versatility. The first α-actinin binding partners identified were the β subunit of integrins and intercellular adhesion molecule-1 (ICAM-1) (Otey and Carpen 2004). Numerous proteins have since been added to the list (see Table III), many of them implicated in cell-cell or cell-matrix interactions. The interaction between α-actinin and adhesion receptors may provide structural stability for the adhesion sites. Since α-actinins also bind to various regulatory molecules, it may also serve as a scaffolding protein to integrate signaling molecules at various adhesion sites.

α-Actinins therefore appear to be much more than simple actin crosslinking proteins. The list of interacting proteins is likely to grow, and will provide a better understanding of the diverse functions of α-actinins in the many non-muscle tissues where it is abundantly expressed.

1.3.4 Regulation of cytoskeletal α-actinin function

A fascinating property of the actin cytoskeleton is its ability to provide structural stability, while retaining a degree of plasticity. Following various extracellular cues such as mechanical stimulation or growth factor receptor activation, a cell can actively undergo cytoskeletal remodeling, allowing it to respond and cope with the demands of its environment. The presence of regulatory domains in the structure of α-actinin-4 suggests that it may be a key participant in cytoskeletal remodeling, allowing assembly and

Table III. Binding partners for cytoskeletal α-actinins *

Binding partner	Category
CLP36	stress fiber
Palladin	stress fiber
	focal adhesion
Zyxin	stress fiber
	focal adhesion
CRP	focal adhesion
Vinculin	focal adhesion
β_1, β_3 integrins	focal adhesion
β_2 integrins	lymphocyte targeting
ICAM-1	lymphocyte targeting
ICAM-2	lymphocyte targeting
L-selectin	lymphocyte targeting
GpIb-IX	platelet adhesion
ADIP	adherens junction
α-catenin	adherens junction
BP180	hemidesmosome
MAGI-1	tight junction
Syndecan-4	cell-matrix adhesion
ADAM12	cell-cell adhesion
NMDA receptor	synapse
Densin	synapse
A2A receptor	synapse
Rabphilin 3A	synapse
E3KARP	regulatory
MEKK1	regulatory
PIP_2	regulatory
PIP_3	regulatory
iNOS	regulatory
PI 3-kinase	regulatory
PKN	regulatory
CaMKII	regulatory

* Adapted from Otey and Carpen 2004.

disassembly of actin-based structures in response to various stimuli. Four different mechanisms have been proposed to contribute to the regulation of α-actinin function: processing by proteases, phosphorylation by tyrosine kinases, binding of phosphoinositides, and binding of Ca^{2+} (Otey and Carpen 2004). Amid various and sometimes contradicting evidence, a picture of the mechanisms involved in the regulation of α-actinin-1 is beginning to emerge. However, very little is known about the mechanisms involved in the regulation of α-actinin-4 function.

Cell motility is a cytoskeletal-dependent process that involves protrusion of the cell's leading edge, formation of new focal adhesions at the front of the cell, followed by detachment at the rear of the cell. Although numerous studies demonstrate a role for α-actinin in cell motility, the precise mechanism remains unclear. The protease calpain cleaves a number of focal adhesion proteins (vinculin, talin), and also has a potent effect on the recruitment of α-actinin to focal adhesions (Cuevas, Abell et al. 2003). Recent evidence also suggests that α-actinin is cleaved by calpain, thereby contributing to the orderly disassembly of focal adhesions required for cell migration.

Phosphorylation of α-actinin-1 has been reported in activated platelets (Izaguirre, Aguirre et al. 1999). Tyrosine phosphorylation at position 12 was found to be dependent on focal adhesion kinase (FAK), supporting a role for α-actinin in cell adhesion. Phosphorylation of α-actinin-1 reduced its association with F-actin *in vitro*, suggesting that α-actinin mediates FAK-dependent cytoskeletal remodeling (Izaguirre, Aguirre et al. 2001). The amino acid sequence of α-actinin-4 reveals a corresponding tyrosine residue at position 32, suggesting that phosphorylation may also regulate the binding of α-actinin-4 to F-actin. Similar experiments were carried out in our laboratory, but we were unable

to demonstrate tyrosine phosphorylation of α-actinin-4 (unpublished observations), suggesting that their modes of regulation are not completely identical.

The ABD of α-actinin contains a phophoinositide-binding site within the second CH domain. Extensive biochemical studies on the effect of phosphoinositide binding to α-actinin-1 have been performed. Binding of phosphatidylinositol (3,4,5)-trisphosphate (PIP_3), and to a lesser extent phosphatidylinositol (4,5)-bisphosphate (PIP_2), decreases the association between α-actinin-1 and F-actin, suggesting that phosphoinositides may be key mediators of cytoskeletal remodeling (Fraley, Tran et al. 2003; Fraley, Pereira et al. 2005). To date, no studies have assessed the role of phosphoinositides on α-actinin-4 function.

As stated previously, the structure of α-actinin also reveals the presence of two EF-hand motifs at the C-terminus of each monomer, although only isoforms 1 and 4 demonstrate sensitivity to Ca^{2+} (Burridge and Feramisco 1981; Burridge and Feramisco 1982). For these non-muscle isoforms, the binding of Ca^{2+} is thought to reduce α-actinin's affinity for actin, providing yet another potential mechanism for α-actinin-dependent cytoskeletal remodeling.

1.3.5 α-Actinin-4 and FSGS

Mutations in a growing number of podocyte-related genes – including podocin, CD2-associated protein, transient receptor potential channel 6 (TRPC6), and α-actinin-4 – underlie several familial forms of FSGS (Daskalakis and Winn 2006). Point mutations in the *ACTN4* gene are causative for late-onset FSGS in several families (Kaplan, Kim et al. 2000). Furthermore, α-actinin-4-deficient mice display progressive proteinuria and podocyte foot process effacement, resulting in glomerular disease (Kos, Le et al. 2003).

Contrary to humans, mouse podocytes express both α-actinin-1 and -4, although the former is unable to rescue the aberrant podocyte morphology in α-actinin-4-deficient mice. This observation suggests that the two non-muscle isoforms of α-actinin have evolved specific and non-redundant functions in spite of their high degree of sequence identity.

FSGS-associated mutations in the *ACTN4* gene give rise to protein variants with increased F-actin-binding activity *in vitro*, suggesting a gain-of-function mechanism. In support of this, disease severity correlates directly with mutant α-actinin-4 expression in transgenic mice (Michaud, Lemieux et al. 2003). Knock-in mice harboring two copies of a disease-associated mutant α-actinin-4 display a phenotype very similar to that observed in knock-out mice. The authors of this study also provide evidence that mutant α-actinin-4 aggregates and is rapidly degraded by the ubiquitin-proteasome pathway, suggesting a loss-of-function mechanism (Yao, Le et al. 2004).

Despite these seemingly contradictory findings, it is evident that α-actinin-4 dynamics are crucial for the health of the podocyte. It is likely that the phenotype in mice and humans involves both gain-of-function and loss-of-function mechanisms. A better understanding of the biology of FSGS-associated mutations will undoubtedly provide novel insights into the dynamic role of α-actinin-4 in glomerular podocytes.

1.4 Research question and objectives

Advances in the field of podocyte biology have clearly identified a critical role for the cytoskeleton in the maintenance of foot process structure and glomerular filtration barrier integrity. Indeed, cytoskeletal alterations are observed in numerous glomerular

diseases, parallel to foot process effacement and disorganization of the slit diaphragm. The identification of FSGS-associated mutations in the *ACTN4* gene has further supported the notion that injury to or within the podocyte triggers a series of events culminating in glomerular dysfunction and subsequent scarring. However, the molecular mechanisms mediating *ACTN4*-dependent podocyte injury remain unclear.

The general hypothesis of this thesis is that **altered α-actinin-4 dynamics lead to podocyte dysfunction and subsequent glomerular sclerosis.** The results presented herein will address this hypothesis by meeting the three following objectives:

Objective 1 – Develop a mouse model of familial FSGS using a missense mutation in the *ACTN4* gene analogous to that identified in human patients.

Objective 2 – Determine the effects of a gain-of-affinity mutation in the *ACTN4* gene on podocyte dynamics in culture.

Objective 3 – Explore the mechanisms involved in the regulation of α-actinin-4, and determine the effects of gain-of-affinity mutations on such regulatory elements.

Thesis content

The first chapter of this thesis (Chapter 1) contains a general introduction describing current knowledge of podocyte biology and α-actinins. Chapter 2 comprises a detailed look at the identification and function of various podocyte molecules, as well as recent methods used for targeted gene expression in mice. The development of a mouse model of FSGS is described in Chapter 3, while the effects of gain-of-affinity mutations on podocyte dynamics are detailed in Chapter 4. The role of α-actinin-4's regulatory domains and their implications for podocyte dynamics form the basis of Chapter 5. The final chapter (Chapter 6) is dedicated to a general discussion of these results and their relevance to the field of podocyte biology and glomerular diseases.

Chapter 2

The podocyte in health & disease – Insights from the mouse

The podocyte in health & disease – Insights from the mouse

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Description and author contribution

The recent identification of numerous podocyte-specific gene products has refocused investigations towards the podocyte and its role in health and glomerular disease. The first half of this review describes various strategies used to manipulate the mouse genome. The strategies described include transgenic mice using tissue-specific promoters, global gene-deficient mice, as well as tissue-specific gene-deficient mice using the Cre-lox system. Novel gene-targeting approaches using inducible expression systems are also described. The second half describes how such strategies have furthered our understanding of the glomerular filtration barrier, with respect to the specific function of individual genes.

The introduction and the description of gene-targeting strategies were written by Chris Kennedy. The description of individual podocyte-related genes and their relevance for podocyte function was written by myself. Both Chris Kennedy and I developed the figures for the article and performed the required revisions prior to publication.

Abstract

The glomerular filtration barrier consists of the fenestrated endothelium, the glomerular basement membrane, and the terminally-differentiated visceral epithelial cells known as podocytes. It is now widely accepted that damage to, or originating within the podocytes is a key event that initiates progression towards sclerosis in many glomerular diseases. A wide variety of strategies have been employed by investigators from many scientific disciplines to study the podocyte. While invaluable insights have accrued from conventional approaches, including cell culture and biochemical-based methods, many renal researchers continue to rely upon the mouse to address the form and function of the podocyte. This review summarizes how genetic manipulation in the mouse has advanced our understanding of the podocyte in relation to the maintenance of the glomerular filtration barrier in health and disease.

Podocyte structure and function

Podocytes are highly differentiated epithelial cells that contribute to the size-selective nature of the glomerular filtration barrier (reviewed in [1]). Major processes extend from the cell body and branch out as foot processes which adhere to the glomerular basement membrane (GBM) and establish an interdigitating pattern with a foot process from a neighboring cell. In 1974, the elegant studies of Karnovsky et al., first described a zipper-like molecular diaphragm that spans the gap between opposing foot processes [2]. Almost 25 years passed until Tryggvason's seminal work identified nephrin as the integral protein that accounted for the filtration slit [3]. Following this discovery, an explosion of work has refocused attention upon the podocyte and its role within the glomerular filtration barrier. Indeed, we now know the identity of a number of gene products that participate in the assembly of the foot processes and slit diaphragm (Figure 2.1). In addition to nephrin, other proteins such as CD2-associated protein (CD2AP), the canonical transient receptor potential 6 (TRPC6) ion channel, podocin, P-cadherin, α- and β-catenin, ZO-1, and NEPH1, colocalize within this unique and highly specialized subcellular domain to form a modified adherens junction to establish the molecular sieve known as the slit diaphragm.

The maintenance of the slit diaphragm complex is critical to the sieving qualities of the glomerular filter. Indeed, a large volume of data has emerged indicating reduced expression of these proteins associated with the onset of many glomerular diseases. Furthermore, the health of the slit diaphragm complex is inextricably tied to the internal scaffolding network of the podocyte – the actin cytoskeleton. This cytoskeleton not only

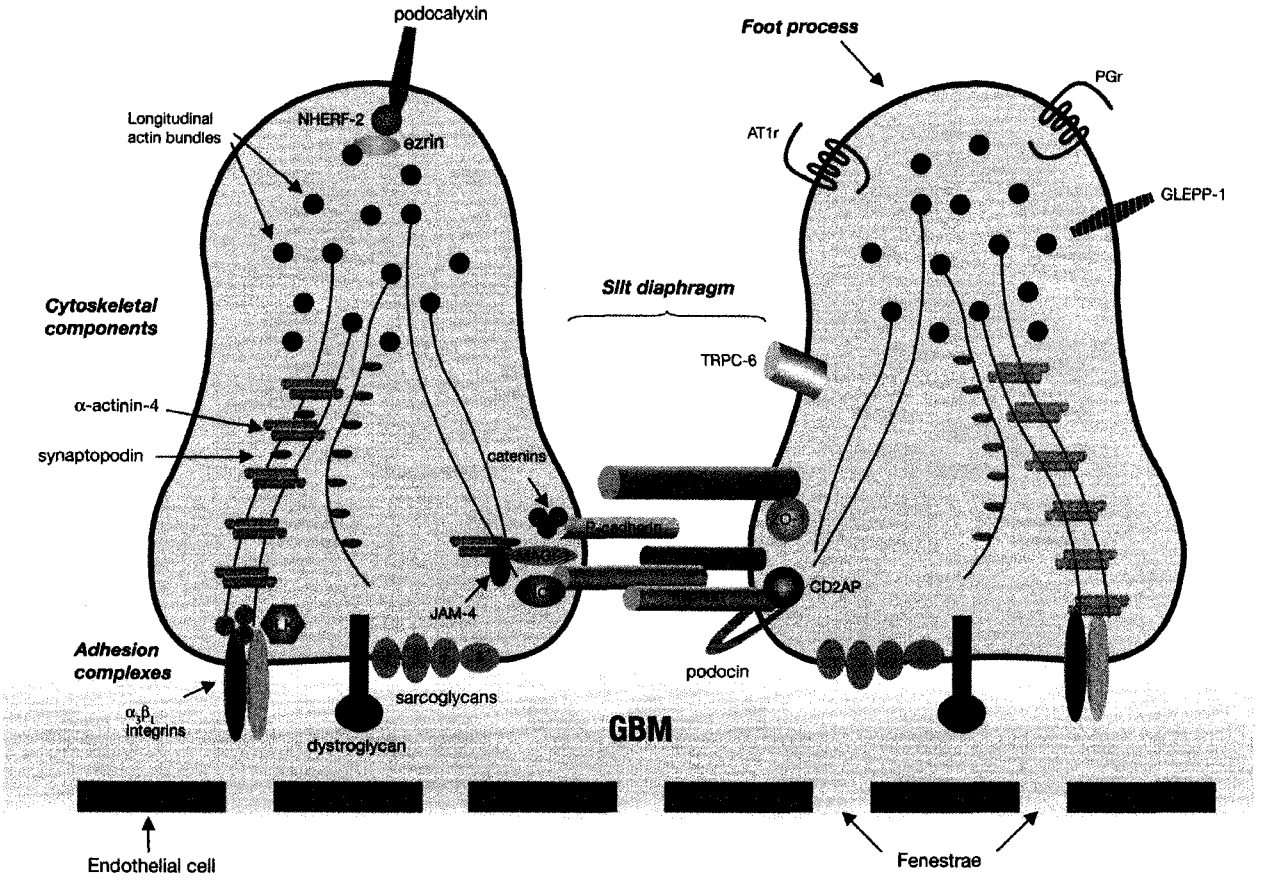

Figure 2.1 Key molecular components of the podocyte.
Shown are two interdigitating foot processes emphasizing the following structural and functional regions of interest - cytoskeletal components, adhesion complexes and slit diaphragm. Additional gene products include: angiotensin II receptor subtypes (ATr), prostaglandin receptor subtypes (PGr), and podocalyxin, along with its interacting partners – NHERF-2 and ezrin. Other abbreviations: ILK (integrin-linked kinase); V (vinculin); T (talin); P (paxillin).

establishes and maintains podocyte morphology, but anchors the slit diaphragm complex via a network of proteins - the intermediate filament proteins vimentin [4-7] and desmin [8] in the cell body and a microfilament-based contractile apparatus composed of actin, myosin-II, α-actinin, synaptopodin, paladin, talin, and vinculin in the foot processes. This cytoskeleton is anchored to focal adhesions along the sole of the foot process via an $\alpha_3\beta_1$-integrin complex, which in turn fastens the podocyte to the underlying GBM [9-11]. Alterations in cytoskeletal function may underlie characteristic changes to podocytes observed in the earliest stages of many glomerular diseases including: foot process effacement, cell hypertrophy, pseudocyst formation, cytoplasmic overload with resorption droplets, apoptosis and detachment from the GBM. The retraction and effacement of the podocyte foot processes yields a diffuse cytoplasmic sheet along the GBM. The slit diaphragm ceases to exist when foot processes are effaced. Thus, the podocyte cytoskeleton determines the unique morphology of these cells and is critically related to their sieving function.

Treatment options for those affected by glomerular disease are few. Once the initial podocyte modifications have progressed to sclerotic lesions, therapies are aimed at limiting proteinuria and slowing the progression of renal fibrosis in order to avoid end stage renal disease (ESRD). Current treatment may entail corticosteroids, diet modification, ACE inhibition or immunosuppression [12] to reduce hypertension/proteinuria encountered with some individuals. However, many forms of glomerular disease are steroid-resistant and dietary compliance is often unreliable. Moreover, recent evidence from genetically-induced rodent FSGS models suggests that podocyte loss exceeding 40% signals irreversible progression, while lower attrition (20%) yields chronic albuminuria without scarring [13]. This suggests that early intervention is

imperative for prevention (i.e., at the initial stages of podocyte damage) while the limited options for treatment demand that a more substantial understanding of how podocytes become damaged be provided.

Manipulating the mouse podocyte

The podocyte exhibits a remarkably unique gene expression profile - one that greatly facilitates the generation of transgenic and/or gene-targeted mice. This has allowed investigators to harness a variety of promoters to drive transgene expression in a podocyte-specific manner, with relatively insignificant activity elsewhere. In contrast, consider the glomerular mesangial cell which has long been associated with the progression of numerous glomerular disease processes (e.g., diabetic nephropathy). The identification of promoters offering mesangium-specific activity has consistently eluded the renal researcher. As a result, identifying the role(s) of countless gene products in the context of the mesangial cell in health and disease, has proved challenging.

During the last two decades we have witnessed the emergence and subsequent refinement of strategies for manipulating the mouse genome. The associated technical skills, initially available to relatively few researchers, have become widespread, affordable and even routine, as evidenced by the existence of core transgenic mouse facilities at virtually every research institution. A thorough discussion of the technical aspects of murine gene-targeting and transgenics is beyond the scope of this review; however, a brief discussion of a few approaches is warranted (Figure 2.2).

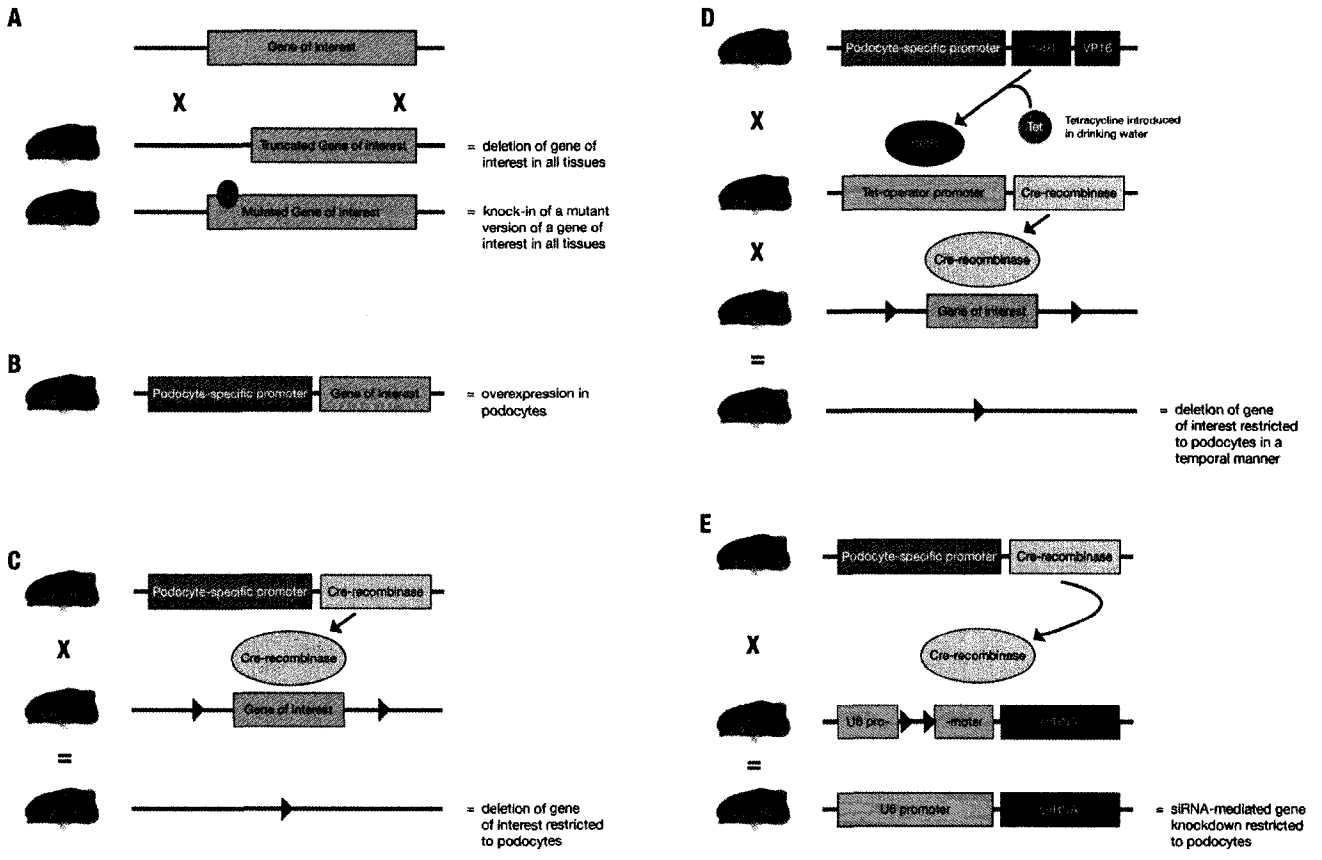
A
Gene of interest
X
X
Truncated Gene of interest
= deletion of gene of interest in all tissues
Mutated Gene of interest
= knock-in of a mutant version of a gene of interest in all tissues
B
Podocyte-specific promoter
Gene of interest
= overexpression in podocytes
C
Podocyte-specific promoter
Cre-recombinase
X
Cre-recombinase
Gene of interest
=
= deletion of gene of interest restricted to podocytes
D
Podocyte-specific promoter
VP16
X
Tet
Tetracycline introduced in drinking water
Tet-operator promoter
Cre-recombinase
X
Cre-recombinase
Gene of interest
=
= deletion of gene of interest restricted to podocytes in a temporal manner
E
Podocyte-specific promoter
Cre-recombinase
X
Cre-recombinase
U6 pro-
-moter
=
U6 promoter
= siRNA-mediated gene knockdown restricted to podocytes

Figure 2.2 Strategies for manipulating gene expression in the mouse.
A) Conventional gene-targeting by homologous recombination. For the 'knock-out' of a gene, typically the exon containing the start codon is replaced by a shortened construct sequence lacking the start codon. For a 'knock-in' strategy, a construct harbouring a mutation (e.g., reflecting a genetic disease) replaces the gene sequence. **B**) Classic transgenic overexpression. For this strategy, a line of transgenic mice is engineered using a construct harbouring a tissue-specific promoter (e.g., that of nephrin or podocin) to drive the overexpression of a gene in the podocytes. **C**) Spatially-restricted gene-targeting using the Cre/loxP strategy. Two lines of mice are required: 1) a transgenic mouse expressing Cre-recombinase under the control of a podocyte-specific promoter; 2) a gene-targeted mouse with loxP sequences inserted by homologous recombination into 2 introns which flank a key exon within the gene of interest – to yield a 'floxed' gene. When the two mice are intercrossed, the podocyte-restricted expression of Cre-recombinase catalyzes the excision of the 'floxed' gene sequence, effectively disabling its expression in the podocytes. **D**) Spatial and temporal gene-targeting in the mouse using the 'Tet-on' system. Three lines of mice are required: 1) a gene-targeted mouse harbouring a 'floxed' gene; 2) a transgenic mouse expressing a transactivator fusion gene composed of the reverse tetracycline repressor (rTetR) and the acidic domain of the herpes simplex viral protein 16 (VP16) under control of a podocyte-specific promoter; 3) a transgenic mouse expressing Cre-recombinase under the control of a promoter containing Tet-operator sequences. When the three lines are intercrossed, tetracycline (Tet) is given to the mice by drinking water. In the presence of Tet, a transactivator becomes active and induces Cre-recombinase expression in the podocytes to excise the 'floxed' gene sequence. **E**) shRNA-mediated gene knockdown in the mouse. In this approach, transgenic mice are developed using a construct that contains an shRNA designed against the gene of interest cloned downstream of the U6 promoter. This promoter is inactive since it contains an intervening 'stuffer' sequence flanked by two loxP sites. The mice are bred to a second transgenic line (described in C) expressing Cre-recombinase driven by a podocyte-specific promoter. With the expression of Cre-recombinase, the intervening sequence is excised and the promoter becomes active and able to drive shRNA expression and knockdown the expression of the target gene.

The so-called 'gene–targeted' mouse (Figure 2.2A) involves the insertion of a fragment of a gene of interest into the allelic locus by homologous recombination in murine embryonic stem (ES) cells. In this way a gene can either be interrupted (i.e., knocked-out) or a mutation introduced into its coding sequence (i.e., knocked-in) and the impact upon a relevant biological endpoint assessed. The podocyte-specific promoters have also been harnessed to generate transgenic mice which overexpress genes of interest (Figure 2.2B). A clear disadvantage of the conventional gene-targeting approach is that deletion of the gene of interest (or insertion of the mutation) is accomplished in all tissues and may thus prove to be embryonic lethal if its function is essential for early development. To circumvent this problem, conditional gene-targeting has been adopted to allow for gene manipulation in mice in a tissue-specific manner (Figure 2.2C). Indeed, a number of labs have used promoter sequences of the nephrin or podocin genes to generate podocyte-specific Cre-recombinase mouse lines [14, 15]. Others have refined the Cre/lox system to allow for temporal control over gene expression/activity by engineering doxycycline-inducible podocyte-specific expression of Cre recombinase (Figure 2.2D) [16].

Finally, novel strategies may be amenable to the podocyte, including shRNA (short hairpin loop RNA) -mediated knockdown of gene products *in vivo*. While this elegant method has seen widespread use in cell culture, it appears that the Cre/lox system may be adapted to allow for tissue-specific shRNA expression in mice (Figure 2.2E) [17, 18]. A shRNA-based approach obviates the need for time consuming gene-targeting steps and instead relies upon the relatively straightforward generation of two separate transgenic mouse lines. Other methods on the horizon include infection of mice with adeno-associated viruses or lentiviruses which could encode small interfering RNAs

(siRNAs). However, a number of technical hurdles must be cleared before such reagents find a use for the manipulation of podocyte genes in rodents. Foremost among these is the accessibility of the podocyte within the glomerular capillary to virus. Using this growing list of strategies to study gene function *in vivo,* it will be exciting to see how the podocyte researcher will advance our understanding of disease processes.

Podocyte molecules – Insights from mice

The recent identification of podocyte-specific gene products has refocused investigations towards the podocyte and its role in health and glomerular disease. The objective of the next section of this review is to highlight how animal models have contributed to shaping our understanding of the podocyte.

Slit diaphragm and associated proteins

The role originally ascribed to the slit diaphragm was that of a physical sieve able to prevent passage of protein (i.e, albumin) into the urine. Ample evidence in humans and in rodents supports this view; however, recent findings suggest that the slit diaphragm's role may be more complex as signaling mechanisms originating from this structure have been identified which could regulate podocyte function.

Nephrin

Nephrin, encoded by the *NPHS1* gene, is a member of the Ig superfamily of proteins. The eight extracellular IgG-like domains of nephrin facilitate protein-protein interactions within the slit diaphragm. Tryggvason identified mutations in the *NPHS1*

gene as the underlying cause of congenital nephrotic syndrome of the Finnish type (CNF) [3]. The indispensable role of nephrin at the slit diaphragm was further evidenced by the development of nephrin-null mice, which displayed massive proteinuria and died within 24 hours of birth [19]. Nephrin-null mice exhibit podocyte foot process effacement and a complete loss of a functional slit diaphragm. Elegant studies using electron tomography later demonstrated that the slit diaphragm is a network of molecular strands, which is completely lost in patients with CNF and in nephrin-null mice [20]. Furthermore, nephrin expression is reduced in many human diseases and in animal models of podocyte injury.

Fyn and Nck

Accumulating evidence suggests that nephrin offers not only a structural contribution to filtration barrier, but can mediate signal transduction from the slit diaphragm in the podocytes [21]. The intracellular domain of nephrin, which contains six conserved tyrosine residues, is phosphorylated by the Src-family kinase Fyn. Phosphorylation of nephrin by Fyn greatly enhanced its association with the slit diaphragm protein podocin, and significantly augmented downstream signaling through activation of the AP-1 promoter [22]. Fyn-null mice did not exhibit proteinuria, but exhibited subtle morphologic changes within their foot processes [23]. Concomitant deletion of Fyn and the Src-family kinase Yes led to proteinuria, endothelial swelling, and podocyte foot process effacement, suggesting at least some level of redundancy and requirement for Src kinases [23]. More recently, two independent groups provided evidence that phosphorylation of nephrin by Fyn recruits the adapter protein Nck, which then leads to actin reorganization [24, 25]. The relevance of Nck recruitment *in vivo* was demonstrated by podocyte-specific deletion of Nck in transgenic mice; these mice displayed defects in foot process formation leading to congenital nephrotic syndrome.

These exciting findings provide evidence that the slit diaphragm can trigger intracellular signaling pathways that mediate cytoskeletal dynamics. Future studies will undoubtedly move further downstream of the slit diaphragm and focus on the roles played by the various signaling cascades in the podocyte.

Podocin

Podocin, the protein encoded by the *NPHS2* gene, is an integral membrane protein which binds to nephrin. Podocin was identified by positional cloning and is mutated in patients with autosomal-recessive steroid-resistant nephrotic syndrome [26]. Podocin-null mice develop severe proteinuria and die a few days after birth from renal failure [27]. Podocin displays a short hairpin-like structure with both termini inside the podocyte cytoplasm. Podocin was shown to interact directly with nephrin, and this interaction is enhanced following nephrin phosphorylation [22]. These findings suggest that podocin may act to stabilize nephrin at the slit diaphragm and facilitate out-to-in signaling.

CD2-Associated Protein

CD2-associated protein (CD2AP) is an SH3-containing protein originally identified in T-cells as an adapter between adhesion receptors and the cytoskeleton [28]. CD2AP-null mice were originally generated to assess the role of this protein in the immune system. Indeed these mice displayed a compromised immune function, but surprisingly they died at 6 to 7 weeks of age from renal failure [29]. It was then that CD2AP was localized in the podocytes, where it interacts with nephrin. Further studies with CD2AP heterozygous mice, which are otherwise healthy, revealed a defect in the formation of multivesicular bodies in the podocytes, suggesting a defect in the endocytic pathway [30]. These studies of CD2AP-null mice prompted investigators to search for mutations in human patients with glomerular disease. Accordingly, mutations in the

CD2AP gene leading to haploinsufficiency have been identified in several families with FSGS [30]. Interestingly, compound heterozygous-null CD2AP / Fyn mice are proteinuric and exhibit FSGS lesions [31]. This suggests that the effects of mutations in these genes may combine to promote a common etiology for glomerular dysfunction.

The Neph Family

The Neph proteins (Neph1, Neph2 and Neph3) are transmembrane proteins of the Ig superfamily with a structure similar to that of nephrin. Neph1 is crucial for the integrity of the slit diaphragm since mice lacking Neph1 exhibit proteinuria and die before the age of 8 weeks [32]. The extracellular domains of Neph1 and Neph2 heterodimerize with nephrin, and form homodimers with themselves [33, 34]. Furthermore, all 3 Neph proteins can bind to the slit diaphragm proteins podocin and zonula occludens-1 (ZO-1) [35, 36]. The Neph proteins may therefore be ligands for nephrin or each other within the plane of the slit diaphragm and participate in out-to-in signaling events. Podocyte-specific gene targeting approaches are needed in order to elucidate the roles of Neph2 and Neph3 in filtration barrier integrity.

P-Cadherin and FAT

The slit diaphragm spanning the gap between adjacent foot processes shares morphological features with adherens junctions. Indeed, P-cadherin, an adherens junction protein, was detected at cell-cell contacts in cultured podocytes and at the slit diaphragm *in situ* [37]. P-cadherin is not an essential component of the slit diaphragm since P-cadherin-null mice do not exhibit a renal phenotype [38]. Another set of adherens junction components, the FAT family, are giant protocadherins with 34 extracellular cadherin-like repeats. FAT1 was isolated from glomerular cDNA using primers for conserved cadherin sequences and the protein colocalized with slit diaphragm

components by immunohistochemistry [39]. Unlike P-cadherin, FAT1 is an essential component of the filtration barrier. FAT1-null mice exhibit perinatal lethality caused by loss of glomerular slit junctions and podocyte foot process effacement [40]. More recently, FAT2 has also been identified as a component of the slit diaphragm. Though FAT1 binds to Ena/VASP and participates in actin dynamics and cell polarity [41], further research is needed to define the exact role(s) of FAT proteins in maintenance of the glomerular filtration barrier.

ZO-1, MAGI-1 and JAM4

ZO-1 is a member of the membrane associated guanylate kinase (MAGUK) family. Within the glomerulus, ZO-1 is predominantly expressed in the podocyte foot processes and is concentrated at the level of the slit diaphragm [42]. Expression levels of ZO-1 may vary following podocyte injury, but its role in the slit diaphragm complex has not been fully elucidated. Membrane-associated guanylate kinase with inverted orientation-1 (MAGI-1) is a tight junction protein expressed in the podocytes. MAGI-1 interacts with nephrin [43] and with the cytoskeletal proteins synaptopodin and α-actinin-4, suggesting that it may play a role in linking the slit diaphragm to the podocyte cytoskeleton [44]. MAGI-1 also interacts with junctional adhesion molecule 4 (JAM4), the significance of which is not yet known. Altered expression of JAM4 was reported in a model of podocyte injury and may be a useful marker of injured podocytes [45]. Further research into these tight junction proteins may uncover novel characteristics and roles of the podocyte slit diaphragm.

Transient Receptor Potential Channel 6

The transient receptor potential channel 6 (TRPC6) is part of the transient receptor potential (TRP) family of cation-selective channels. TRPC6 belongs to a subgroup of calcium-permeable channels responsible for intracellular calcium increases following activation of G-protein coupled receptors and receptor tyrosine kinases. Mutations in the TRPC6 cause an autosomal dominant kidney disease termed focal segmental glomerulosclerosis (FSGS) [46, 47]. A subset of disease-causing mutations enhance TRPC6-mediated calcium influx following receptor activation by agonists. Furthermore, TRPC6 interacted directly with the slit diaphragm proteins nephrin and podocin, as evidenced by co-immunoprecipitation studies [47]. TRPC channels may therefore be key players in mediating the podocyte's response to slit-diaphragm-induced signals. Undoubtedly, a number of mouse models will likely be developed, including TRPC6-null mice and perhaps transgenic mice with podocyte-specific overexpression of the disease-causing mutant TRPC6 protein. Such mice should facilitate our understanding of this channel in the podocyte.

Podocyte adhesion molecules

The foot processes of the glomerular podocytes are tethered to the underlying GBM by transmembrane adhesion molecules. These podocyte-matrix contacts help preserve the structure of the foot processes. Mutant forms of GBM components such as collagen IV and laminin, or defects in the expression of podocyte adhesion molecules ($\alpha_3\beta_1$-integrins, α- and β- dystroglycan) can lead to podocyte injury or loss, and failure of the filtration barrier.

Integrins

Integrins are heterodimeric transmembrane receptors that bind to their ligands in the GBM, anchoring a cell to its underlying matrix. The major integrin expressed on the sole of the foot process is the $\alpha_3\beta_1$ heterodimer. The physiological relevance of podocyte-matrix interactions was illustrated by the development of proteinuria, foot process fusion, and podocyte detachment following injection of rodents with anti-β_1-integrin antibodies [48]. Genetic deletion of α_3-integrins in mice yielded a disorganized GBM and podocytes which failed to form mature foot processes [49]. Genetic deletion of β_1-integrins in mice is embryonic lethal as homozygous null embryos develop to the blastocyst stage but die shortly after implantation [50]. The Cre/loxP approach will likely be very informative if used to ablate β_1-integrin from the podocyte. Meanwhile, other research is aimed at elucidating the intracellular signals triggered by integrins and their impact on podocyte function and morphology (see *Integrin-Linked Kinase* below).

α- and β- Dystroglycans

Components of the dystroglycan complex are also expressed in the basal cell membrane of the podocyte foot processes. The dystroglycan complex is known to provide a link between the underlying extracellular matrix and the cytoskeleton. Podocyte injury induced by protamine sulfate and the generation of reactive oxygen species (ROS) led to a disruption of α-dystroglycan-matrix complexes, endocytosis of the liberated receptor, and disorganization of the GBM [51]. Furthermore, deglycosylation of α-dystroglycan by ROS led to disruption of the dystroglycan-matrix complex [52], which may contribute to podocyte detachment *in vivo*. Further studies using temporal deletion

of dystroglycan chains in podocytes may uncover the link between dystroglycan complexes and the actin cytoskeleton and its relevance to podocyte biology.

Integrin-Linked Kinase

Beyond their role as cellular anchors, integrins are also involved in signal transduction. Engagement of integrins by extracellular matrix can trigger intracellular signaling pathways (outside-in signaling), and the adhesive characteristics of integrins can be modified in response to intracellular cues (inside-out signaling). Integrin-linked kinase (ILK), a serine/threonine kinase, has emerged as a key mediator of podocyte-matrix interactions. ILK mRNA induction was demonstrated in glomeruli of three different renal diseases characterized by a defective filtration barrier [53]. The involvement of ILK in outside-in signaling was demonstrated by its inhibition in response to fibronectin. Furthermore, overexpression of ILK caused a decrease in integrin-matrix adhesion, illustrating its involvement in inside-out signaling. Following puromycin-induced podocyte injury, ILK induces the nuclear translocation of β-catenin, podocyte detachment, cell proliferation and repression of the slit diaphragm molecules P-cadherin and CD2AP; these effects were blocked by a small molecular inhibitor of ILK [54]. To further evaluate the role of ILK *in vivo*, mice with Cre-mediated podocyte-specific ILK inactivation were generated. These mice developed progressive focal segmental glomerulosclerosis and died of renal failure [55]. GBM alterations at the onset of proteinuria were followed by foot process effacement and loss of slit diaphragm. Consistent with previous observations in ILK-deficient podocytes, α_3-integrins relocalized into a granular pattern along the GBM. These recent findings suggest that ILK and podocyte-matrix interactions play an essential role in podocyte function.

Cytoskeletal components

The unique architecture of the podocyte is achieved largely through the assembly of an elaborate cytoskeleton. The actin filament bundles of the foot processes are not only responsible for maintaining the cell's shape, but also connect the slit diaphragm on the lateral sides of the foot processes to the adhesion complexes on the foot process sole. Furthermore, the cytoskeleton may also contribute to counteract the distensile forces of the capillary wall. To carry out these roles, a highly ordered cytoskeleton must provide a balance of structural strength and pliability in order for the podocyte to cope with changes in glomerular capillary pressure as well as being responsive to extracellular stimuli. A growing list of cytoskeletal components accounts for the dynamic nature of the podocyte – some of which are discussed below.

Synaptopodin

Synaptopodin is an actin-associated protein highly expressed in podocyte foot processes and telencephalic dendrites [56]. The observation that synaptopodin expression is restricted to areas of high synaptic plasticity in the brain, along with results from cultured cells, suggested that synaptopodin may play an important role in modulating actin-based shape and motility of cells. Synaptopodin-null mice lack the dendritic spine apparatus but do not display ultrastructural defects within the podocytes [57]. However, the recovery from chemically-induced foot process effacement was impaired in synaptopodin-null mice, highlighting a defect in podocyte cytoskeletal dynamics. Furthermore, the authors showed that synaptopodin regulates the actin-bundling activity of α-actinin-4 in highly dynamic areas such as podocyte foot processes. The involvement of synaptopodin in cytoskeletal dynamics and podocyte motility was recently supported

as gene silencing of synaptopodin in cultured podocytes caused a loss of stress fibers, formation of aberrant filipodia, and impaired podocyte migration [58]. Taken together, these findings show that synaptopodin is essential for cytoskeletal dynamics and podocyte migration.

α-Actinin-4

α-Actinin-4, an actin crosslinking protein, is expressed in the foot processes where it is responsible for bundling longitudinal actin fibers. Mutations in the *ACTN4* gene cause a late-onset autosomal dominant form of focal segmental glomerulosclerosis (FSGS) [59]. A subset of FSGS mutations located just outside the actin binding domain, increases the affinity of α-actinin-4 for actin, suggesting that the podocyte cytoskeleton may be dysregulated. In mice, podocyte-specific expression of a high-affinity variant of α-actinin-4 caused proteinuria, foot process effacement, and glomerular sclerosis [60]. Consistent with such podocyte damage, transgenic kidneys exhibited significantly reduced mRNA and protein levels of the slit diaphragm component, nephrin. Genetic deletion of α-actinin-4 in mice or replacement of the endogenous *ACTN4* gene with copies harboring a disease-associated mutation leads to a similar phenotype, characterized by proteinuria and the onset of glomerular disease [61, 62]. A fraction of FSGS-associated α-actinin-4 was found to form large aggregates, which in turn target the protein for degradation via the ubiquitin-proteasome pathway. In contrast, our lab has discovered that gain-of-affinity mutations in α-actinin-4 confer mislocalization of the protein in cultured podocytes, which adversely affects cytoskeletal dynamics and podocyte motility (Kidney International, in press). Taken together, these findings clearly

indicate that the cytoskeleton plays a crucial role for the structure of podocytes, and that a dynamic cytoskeleton is required for podocyte dynamics.

Other podocyte molecules

In addition to the myriad of slit diaphragm components, adhesion molecules and cytoskeletal proteins described above, several other proteins that are required for podocyte form and function have been described. Many of these proteins are expressed on the apical membrane of the podocytes, and their function ranges from generation of a charged glycocalyx to intracellular signaling.

Podocalyxin

Podocalyxin, a sialomucin, is the major sialoglycoprotein expressed in podocytes and is responsible for generating a negative charge on the surface of the podocyte [63]. It is thought that the negative charge of podocalyxin helps repel proteins from passing through the GBM and serves as a spacer between adjacent foot processes. The cytoplasmic domain of podocalyxin interacts with two cytosolic adapter proteins, ezrin and Na+/H+ exchanger-regulatory factor 2 (NHERF2), providing a link to the actin cytoskeleton [64]. Disruption of podocalyxin/NHERF2/ezrin interactions in pathologic conditions associated with changes in foot process structure provides evidence of its relevance *in vivo*. Furthermore, podocytes of podocalyxin-null mice fail to form foot processes and the slit diaphragm is replaced by tight and adherens junctions; these mice die within 24 hours of birth from anuric renal failure [65]. It was later demonstrated that podocalyxin activates RhoA, a small-GTPase, through NHERF and ezrin, leading to a redistribution of actin filaments [66]. Taken together, these results indicate that

podocyalyxin is essential for the formation of the filtration barrier, and that podocalyxin may mediate signals affecting the cytoskeletal organization of the foot processes.

Glomerular Epithelial Protein 1

A candidate for cytoskeletal regulation in the podocyte is glomerular epithelial protein 1 (GLEPP1), a podocyte-specific receptor tyrosine phosphatase. In mice, GLEPP1 is first expressed at the capillary loop stage of glomerular development and persists at high levels in fully developed glomeruli. GLEPP1-null mice do not exhibit proteinuria, but podocytes display morphological changes such as broadening of the foot processes [67]. Since no ligand or substrates have been described for GLEPP1, its exact function in glomerular filtration remains unknown.

Rho GDP Dissociation Inhibitor α

Members of the Rho family of small GTPases are important mediators of actin cytoskeleton reorganization leading to changes in morphology, adhesion and motility. Rho GTPases cycle between their active GTP-bound and inactive GDP-bound states. Rho GDP dissociation inhibitor (GDI) α is a negative regulator of Rho, keeping it in its inactive GDP-bound state within the cytosol. The involvement of Rho in podocyte cytoskeletal dynamics was illustrated by the development of massive proteinuria and nephrotic syndrome in Rho GDIα-null mice [68]. Since Rho participates in the formation of actin fibers, uncontrolled formation and bundling of actin filaments most likely impairs cytoskeletal dynamics and foot process structure.

Wilms' Tumor Suppressor-1

The Wilms' tumor suppressor 1 (WT1) protein is a key transcription factor involved in podocyte function. WT1 is a zinc finger transcription factor that is required

for podocyte maturation but remains highly expressed into adulthood. Dominant mutations in the WT1 gene cause Denys-Drash syndrome and Frasier's syndrome, both characterized by progressive glomerulopathy and male pseudohermaphroditism. Disruption of the WT1 gene in mice is embryonic lethal owing to the failure of kidney and gonad development . Heterozygous mice ($WT1^{+/-}$) appear normal during early stages of life but develop adult-onset nephrotic syndrome with mesangial sclerosis [69]. The WT1 gene is very complex; alternative start sites, alternative splicing and RNA editing can result in at least 24 different isoforms [70]. Alternative splice donor sites in exon 9 can lead to the lack of a 3 amino acid sequence (lysine, threonine, and serine) termed the KTS motif [71]. Homozygous mice lacking the -KTS variant die shortly after birth and display hypoplastic kidneys and streak gonads, indicating a role for the WT1 (-KTS) variant in embryonic kidney and gonad survival. Similarly, homozygous mice lacking the +KTS variant also die shortly after birth but display reduced podocyte differentiation and XY sex reversal. Heterozygous mice with reduced expression levels of the +KTS variant develop glomerulosclerosis and represent a model of Frasier's syndrome. Podocyte specific genes that may be targets of WT1 are beginning to emerge. Binding of WT1 to conserved elements within the podocalyxin gene promoter results in potent transcriptional activation [72]. Furthermore, WT1 may be required for regulation of the NPHS1 gene and the intermediate filament protein nestin [73]. Further studies are clearly needed in order to define the physiological roles of the numerous WT1 isoforms and their relevance to podocyte biology.

Secreted Protein Acidic and Rich in Cysteine

Elevated intraglomerular pressure is a common occurrence in many renal disorders and promotes podocyte injury and glomerulosclerosis. Increased mechanical

strain on the podocytes can initiate maladaptive responses such as apoptosis and podocyte detachment from the extracellular matrix. Secreted protein acidic and rich in cysteine (SPARC) is a matricellular protein with anti-proliferative effects in various tissues. SPARC is activated following complement-mediated podocyte injury [74]. SPARC-null mice are less sensitive to streptozotocin (STZ)-induced diabetic nephropathy, further illustrating the deleterious effects of SPARC activation on podocytes [75]. In cultured podocytes, mechanical strain increases both intracellular and secreted levels of SPARC, while experimental glomerular hypertension increases podocyte SPARC levels *in vivo* [76]. Taken together, these results suggest that SPARC activation promotes podocyte injury and the development of glomerulosclerosis.

Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF) is a growth factor involved in angiogenesis. VEGF can regulate vascular permeablility, endothelial cell migration, proliferation and survival. Within the glomerulus, VEGF is expressed on podocytes, while its receptors (VEGFR1/Flt-1 and VEGFR2/Flk-1) are expressed on endothelial cells, suggesting that VEGF functions in juxtacrine/paracrine signaling pathways [77]. Elegant genetic studies have illustrated an exquisite dosage-sensitivity for VEGF within the glomerulus [78]. Homozygous VEGF-null mice die during embryogenesis from failed vascularization. Podocyte-specific deletion of one VEGF allele leads to proteinuria and endotheliosis, while deletion of both VEGF alleles resulted in perinatal lethality and the absence of a filtration barrier due to defects in endothelial cell migration, differentiation, and survival. Furthermore, podocyte-specific overexpression of VEGF causes collapsing glomerulopathy. These results demonstrate that VEGF signaling is critical for the formation of a functional filtration barrier. In a recent study, VEGF

production by the podocyte was also shown to be required for mesangial cell migration and survival [79]. Numerous studies have shown that VEGF is indispensable for glomerular capillary growth. However, the role of sustained VEGF expression following nephrogenesis is not clearly defined. Conditionally-immortalized glomerular endothelial cells have recently been described; these cells demonstrate fenestrations, which increase strikingly in number following stimulation with VEGF [80]. These results suggest that podocyte-derived VEGF may play a key role in maintaining endothelial fenestrations within the glomerulus. Further studies are needed to elucidate the signaling pathways triggered by VEGF and its receptors.

G-Protein Coupled Receptors and Gαq

A role for the renin angiotensin system (RAS) in modulating podocyte function in disease has been implied by numerous studies showing that angiotensin converting enzyme (ACE) inhibition can blunt albuminuria in many glomerular disease states [81-84]. Although the source and actions of angiotensin II in the pathogenesis of glomerular disease remain incompletely resolved, cell culture studies of conditionally immortalized podocytes revealed the presence of the major members of the RAS including AT1 receptors and ACE [85]. It is thought that enhanced intraglomerular capillary pressure (Pgc) and AT1 receptor activity combine to drive the podocyte along an apoptotic pathway, thereby damaging the glomerular filter and contributing towards a sclerotic phenotype according to the Kriz hypothesis [86]. While podocyte-specific deletion of the AT1 receptor (or ACE) in mice awaits investigation, transgenic rats overexpressing the human angiotensin AT1 receptor under the control of the nephrin promoter exhibit several defects in podocyte function, including effacement, pseudocyst formation and

detachment from the GBM – all of which result in significant albuminuria by 8 weeks of age [87]. The AT1 receptor is a member of the G-protein coupled receptor (GPCR) superfamily and signals from the cell surface via the Gq protein to activate phospholipase C. Transgenic mice bearing podocyte-specific expression of a constitutively-active Gαq subunit, were recently generated [88]. These mice developed albuminuria along with reduced renal mass and nephron number coupled with diminished expression of podocalyxin and nephrin, and eventually exhibited FSGS lesions. Additionally, the susceptibility of such mice to glomerular injury with puromycin aminonucleoside was increased. These findings strongly suggest that GPCR-induced signaling in the podocyte may contribute to the early damage seen in at least some glomerular diseases. However, additional studies are needed to investigate the roles played by the numerous GPCRs expressed by the podocyte, as they respond to their relevant ligands.

Conclusions and outlook

Targeting and modification of genes in mice and rats has greatly facilitated the study of gene function *in vivo*. The application of these powerful techniques to the podocyte has provided invaluable information about the complexity of this essential cell type in its role as a central player for the maintenance of the glomerular filtration barrier. Undoubtedly, future studies will continue to advance our knowledge of the podocyte as more rodent models are developed to complement the wealth of biochemical, cell biological, genomic and proteomic based approaches that together, will lead towards a more comprehensive appreciation of this fascinating cell type and its role in health and disease.

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Chapter 3

Focal and segmental glomerulosclerosis in mice with podocyte-specific expression of mutant α-actinin-4

Focal and segmental glomerulosclerosis in mice with podocyte-specific expression of mutant α-actinin-4

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Description and author contribution

This manuscript describes the development of a mouse model of FSGS. Using a mutation (K256E) previously described in human patients affected with this disorder, we confirm that mutations in the *ACTN4* gene can severely injure podocytes *in vivo* and lead to glomerular scarring.

Most of the experiments described in this manuscript were performed by myself. Our laboratory technician, Lyne Lemieux, performed genotyping, urine collections and urinary analyses while overseeing breeding of the mouse colony. Micronuclei injection of transgenic DNA was performed by Manon Dubé in Barbara Vanderhyden's laboratory. Renal pathology and disease severity was assessed by Susan Robertson, a renal pathologist at The Ottawa Hospital. The manuscript was written by myself (Materials and methods, Results, and Figures) and Chris Kennedy (Introduction and Discussion). Both myself and Chris Kennedy contributed to the revision of the manuscript prior to publication.

Abstract

Mutations in the gene encoding α-actinin-4 (*ACTN4*), an actin crosslinking protein, are associated with a form of autosomal dominant focal segmental glomerulosclerosis (FSGS). To better study its progression, we developed a transgenic mouse model by expressing murine α-actinin-4 containing a mutation analogous to that affecting a human FSGS family, in a podocyte-specific manner using the murine nephrin promoter. Consistent with human *ACTN4*-associated FSGS, which shows incomplete penetrance, a proportion of the transgenic mice exhibited significant albuminuria (8 of 18) while the overall average systolic blood pressure was elevated in both proteinuric and non-proteinuric *ACTN4*-mutant mice. Immunofluorescence confirmed podocyte-specific expression of mutant α-actinin-4, while real-time RT-PCR revealed that HA-*ACTN4* mRNA levels were higher in proteinuric versus non-proteinuric *ACTN4*-mutant mice. Only proteinuric mice exhibited histological features consistent with human *ACTN4*-associated FSGS, including: segmental sclerosis and tuft adhesion of some glomeruli, tubular dilatation, mesangial matrix expansion, as well as regions of podocyte vacuolization and foot process fusion. Consistent with such podocyte damage, proteinuric *ACTN4*-mutant kidneys exhibited significantly reduced mRNA and protein levels of the slit diaphragm component - nephrin. This newly developed mouse model of human *ACTN4*-associated FSGS suggests a cause and effect relationship between actin cytoskeleton dysregulation by mutant α-actinin-4 and the deterioration of the nephrin-supported slit diaphragm complex.

Introduction

Focal segmental glomerulosclerosis (FSGS) is a syndrome of primary glomerular lesions with a population incidence of approximately 2 per million (1). FSGS is a significant cause of end-stage renal disease (ESRD), comprising up to 5% of adults and 20% of children with ESRD (2, 3). The clinical hallmarks of FSGS routinely include proteinuria, hypertension, and the progression to ESRD. Renal biopsy often reveals areas of solidification, glomerulosclerosis and tuft collapse that are both focal (not all glomeruli are affected) and segmental (only a part of each glomerulus is damaged). The accumulating evidence suggests that either damage to, or within glomerular visceral epithelial cells (podocytes) plays a key role in the development of these lesions (4-10).

Podocytes are highly differentiated epithelial cells that stabilize the glomerular basement membrane (GBM) and contribute to the formation of the glomerular filtration barrier. Podocytes possess foot processes originating from the cell body that interdigitate over each capillary, forming the filtration barrier and counteracting the distensive forces along the GBM. This unique morphology is supported by a complex network of spatially-regulated cytoskeletal proteins dividing podocytes into cell body, major processes, and foot processes. Each foot process is equipped with a microfilament-based contractile apparatus composed of actin, myosin-II, α-actinin, talin, paxillin and vinculin (11, 12). This complex is fixed to focal adhesions at the basal cell membrane of foot processes via an $\alpha_3\beta_1$-integrin complex, which in turn anchors the entire foot process to the underlying GBM (13-16). Key components of the slit diaphragm, including nephrin – which maintains the barrier to protein, are indirectly tethered to this actin cytoskeleton through intermediate molecules such as CD2AP (17, 18). This highly ordered architecture

is critically related to podocyte function in the glomerulus and the unique location of these cells render them susceptible to damage in many nephropathies, including congenital nephrotic syndrome and FSGS. Indeed, mutations in *NPHS1*, the gene encoding for nephrin, are associated with Finnish-type nephrotic syndrome (19). Recent studies have linked mutations in a gene encoding for α-actinin-4 (*ACTN4*), an actin-filament crosslinking protein, with a familial form of autosomal dominant FSGS (20-22). Such mutations increase the affinity of α-actinin-4 for filamentous actin (F-actin). Affected individuals may therefore develop sclerotic lesions as a direct result of dysregulation of the actin cytoskeleton. An elucidation of how increased affinity of α-actinin-4 for actin leads to podocyte damage and subsequent sclerosis would be facilitated by a mouse model for this clinical entity.

We now report the development of mice with podocyte-specific expression of a mutated/high-affinity form of α-actinin-4. These mice develop characteristic FSGS-like features reminiscent of human cases of *ACTN4*-associated FSGS, including proteinuria in some but not all mice, systolic hypertension that does not correlate with proteinuria, podocyte foot process fusion, glomerular tuft adhesion and collapse, focal and segmental sclerosis, and renal tubular dilatation. Furthermore, we demonstrate that proteinuric *ACTN4*-mutant mice have reduced nephrin mRNA expression, suggesting a close relationship between the regulation of the actin cytoskeleton and the maintenance of key components of the slit diaphragm complex.

Materials and Methods

Construction of the Targeting Vector

The murine *ACTN4* cDNA was cloned into pCDNA3 (Invitrogen, Carlsbad, CA) as follows: Two overlapping I.M.A.G.E. clones (GenBank accession AI119186 and W57010) with > 95% homology to the human *ACTN4* sequence were identified through the I.M.A.G.E. Clone Consortium (http://image.llnl.gov) and purchased from ResGen/Invitrogen (Carlsbad, CA). An A766G point mutation (underlined in AI119186 reverse primer) analogous to A763G in the human *ACTN4* open reading frame (ORF) was introduced by PCR mutagenesis, resulting in a K256E amino acid substitution. (Note - the original sequence reported for human *ACTN4* (gi 4826638) indicating an A682G mutation was recently replaced in the NCBI database by a sequence (gi 12025677) that adds 81 nucleotides to the initial 5' region, thereby rendering the mutation at position 682 as reported by Kaplan et al., to nt 763 (20)). The untranslated region (UTR) was removed from each I.M.A.G.E. clone by PCR (AI119186 forward primer: 5'-GCG CGA ATT CAT GGT GGA CTA CCA CGC A-3'; reverse primer: 5'-ATA TGT CAT TAT GGC CTC CTC G-3'; W57010 forward primer: 5'-GCT ATG ACG TGG AGA ATG AC-3'; reverse primer: 5'-TGC GGC CGC TCA CAG GTC GCT CTC CCC ATA-3'). A double 5' hemagglutinin (HA) epitope tag was introduced using overlapping and complimentary oligonucleotides (5'-GAT CCA TGT ATC CAT ATG ACG TCC CAG ACT CTG CCT ATC CAT ATG ACG TCC CAG ACT CTG CCG-3' and 5'-AAT TCG GCA GAG TCT GGG ACG TCA TAT GGA TAG GCA GAG TCT GGG ACG TCA TAT GGA TAC ATG-3'). The HA-*ACTN4* mutant ORF construct was generated by

ligating 5'-*ACTN4* and 3'-*ACTN4* I.M.A.G.E. clone sequences digested at a common *DraIII* site (nt 1485 of the murine *ACTN4* ORF). A similar construct was engineered for the wt form of *ACTN4* (HA-*ACTN4* wt). The *ACTN4*-pCDNA3 constructs were sequenced to rule out the introduction of mutations other than the desired substitution at nt 766.

Actin-Binding Experiments

The actin-binding assay was performed as described by Kaplan et al. (20). Mutant and wt *ACTN4* constructs were *in vitro*-translated using a TnT T7 Coupled Reticulocyte Lysate kit (Promega, Madison, WI). The *in vitro*-translated products were incubated with 2.5 μM actin (Cytoskeleton, Inc., Denver, CO, USA) and 10 μM cold α-actinin (Cytoskeleton, Inc.) for 1 hr at room temperature (RT). Samples were centrifuged at 10^5 *x g* for 1 hr to pellet the F-actin-α-actinin complexes. The supernatants containing unbound α-actinin were removed and the pellet was resuspended in the initial reaction volume (80 μL). Supernatants and resuspended pellets were resolved by 10% SDS-PAGE and transferred to Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech Inc., NJ). The western blots were probed with a polyclonal anti-HA tag antibody (diluted 1:500, CLONTECH Laboratoires, Inc., Palo Alto, CA) followed by an HRP-conjugated secondary antibody (1:1000, Amersham Pharmacia Biotech Inc., Piscataway, NJ) and developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). The membranes were then exposed to film and images captured using a Kodak DC290 ZOOM Digital Camera. Blots were then stripped with Pierce IgG elution

buffer and reprobed with a rabbit anti-actin antibody (1:1000) (Sigma A-2066) and processed as described above.

Transfection of COS-7 Cells, Cytoskeleton Pulldown, and Western Blotting

COS-7 cells were transfected with either pCDNA3 vector, HA-*ACTN4* wt, or HA-*ACTN4* mutant using the Effectene Transfection Reagent (QIAGEN Inc., Mississauga, ON, Canada) according to the manufacturer's instructions. Transfected cells were lysed in buffer (50 mM Tris, 150 mM NaCl, 2 mM EDTA and 1% Nonidet P40, pH 7.4) containing 1mM PMSF and protease inhibitor cocktail (Sigma-Aldrich, Inc., Saint Louis, MO) by passing them twelve times through 26G½ and 30G½ needles followed by centrifugation for 1 hr at 10^5 *x g* and 4°C to pellet the insoluble cytoskeleton. Lysates (10 µg total protein), supernatants (10 µg total protein) and resuspended pellets (10 µg total protein) were resolved by 10% SDS-PAGE and transferred to Hybond ECL Nitrocellulose membranes (Amersham Pharmacia Biotech Inc., NJ). The western blots were probed with a polyclonal anti-HA tag antibody (diluted 1:1000, CLONTECH Laboratoires, Inc., Palo Alto, CA) followed by an HRP-conjugated secondary antibody (1:1000, Amersham Pharmacia Biotech Inc., Piscataway, NJ) and developed using SuperSignal Chemiluminescent Substrate (Pierce, Rockford, IL). The membranes were then exposed to film and images captured using a Kodak DC290 ZOOM Digital Camera. Densitometry of the 103 kDa band was measured using Kodak 1D 3.5 software. Blots were then stripped with Pierce IgG elution buffer and reprobed with a rabbit anti-actin antibody (1:1000) (Sigma A-2066) and processed as described above.

Generation and Genotypic Analysis of Transgenic Mice

An 8.3 kb fragment of the murine nephrin promoter (*NP*) (cloned from a BAC library) which included the 5' untranslated and 5' flanking regions of the murine *NPHS1* gene was cloned immediately upstream of the HA-*ACTN4*-mutant ORF. The *NP*-HA-*ACTN4*-mutant transgene was linearized with *HindIII/RsrII* then microinjected into B6C3F1 (C57Bl6/C3H) mouse embryos at a concentration of 1.758×10^{-13} M and incubated overnight to assure viability. Embryos were then surgically transferred to the oviduct of pseudopregnant CD1 recipient female mice. Both the embryo donor mice and the recipient mice were purchased from Charles River Laboratories, Inc. (Wilmington MA). Genotyping of resulting pups (founders) was performed by Southern analysis of *BamHI*-digested tail DNA using an *NP* probe (an *EcoRI/StuI* fragment). *BamHI*-digestion of the endogenous nephrin gene yields a 3.6 kb fragment while the *NP*-HA-*ACTN4*-mutant transgene yields a 1.7 kb fragment. Two founders exhibiting significant proteinuria were backcrossed with purebred C3H mice to generate the N_1 mice reported in this study.

Proteinuria, Albuminuria and Serum Analysis

Individual mice were housed overnight in diuresis cages (Nalge Nunc International, USA) and urine collected after 24 hrs. Urine samples (5 µL) were mixed with sample loading buffer, incubated at 95°C for 5 min and resolved by 10% SDS-PAGE. The gels were stained with Coomassie Brilliant blue for 30 min, destained for 4 hrs, and images captured with a Kodak DC290 ZOOM Digital Camera. Urine samples were also assayed for total protein using Bio-Rad Protein Assay reagent (Bio-Rad

Laboratories, Hercules, CA). Urinary albumin content was assayed using the Albuwell M Test Kit (Exocell, Inc., Philadelphia, PA) according to the manufacturer's protocol. At 10 weeks of age, mice were anesthetized with halothane, sacrificed and blood was collected in heparinized syringes by cardiac puncture. Serum samples were analyzed for creatinine, urea, and albumin using a Beckman LX-20 instrument.

Blood Pressure Studies

Systolic blood pressure was measured daily by tail-cuff plethysmography (BP-2000, Visitech Systems, Apex, NC). Mice were trained for an initial period of seven consecutive days and measurements were subsequently collected for an additional 5 days. Values were compared between wt (n=11), non-proteinuric *ACTN4*-mutant (n=10) and proteinuric *ACTN4*-mutant mice (n=8).

Structural Analysis by Light and Electron Microscopy

Kidneys from wt and *ACTN4*-mutant mice were resected immediately following blood collection. Kidneys to be used for light microscopic analysis were incubated overnight in 4% paraformaldehyde/PBS, subsequently dehydrated and paraffin-embedded. Sections were cut at 5 μm and stained with either periodic acid Schiff (PAS), trichrome or silver stain. For electron microscopy, kidneys were incubated overnight in 2.7% gluteraldehyde. Specimens were then rinsed in sodium cacodylate buffer, followed by OsO_4, water, uranyl acetate, and then dehydrated in ethanol and acetone and finally embedded in spurr resin. Sections were then cut and visualized with a Hitachi 7100 transmission electron microscope. Each section and electron micrograph were examined

by a renal pathologist, in a blinded manner with regard to genotype. Trichrome-stained sections were used to quantify glomerular interstitial and tubular damage for each mouse with respect to % glomeruli sclerosed, % segmental sclerosis, tubular dilatation (none, mild, moderate), and interstitial fibrosis (none, mild, moderate).

Immunofluorescence of Tissue Sections

Tissues for immunohistochemical analysis were embedded in Cryomatrix (Shandon, Pittsburgh, PA) and immediately frozen in a bath of 2-butanol cooled in liquid nitrogen. Embedded tissues were sectioned at 10 μm with a cryostat and sections were washed 3 times in PBS and blocked with 5% normal goat serum (Vector Laboratories Inc., Burlingame, CA) in PBS for 1 hr at RT. Following 3 washings in PBS, the sections were incubated with the primary antibodies (mouse 12CA5 anti-HA 1:1000, rabbit anti-HA 1:1000, rabbit anti-WT-1 (Zymed, San Francisco, CA) 1:500, or rabbit anti-nephrin 1:1000, a kind gift from Dr. Tomoko Takano, McGill University) for 1 hr at RT, washed 3 times and incubated with the secondary antibodies (anti-mouse-FITC or anti-rabbit-Cy3) for 1 hr at RT. Sections were washed 3 times in PBS and mounted with fluorescent mounting media (Vector Laboratories). Sections were visualized using a Zeiss Axioskop 2 fluorescence microscope (Zeiss Axioskop 2 MOT, Zeiss Germany) and images captured with a Zeiss AxioCam.

RNA Extraction and Real-Time RT-PCR Analyses

Kidneys were snap-frozen in liquid nitrogen and total RNA was extracted using an RNeasy Kit (QIAGEN Inc., Valencia, CA). *NPHS1*, *HA-ACTN4,* endogenous *ACTN4*

and *WT-1* mRNA levels were determined by real-time RT-PCR using TaqMan One-Step RT-PCR Master Mix Reagents (Applied Biosystems, Branchburg, NJ) and an ABI Prism 7000 Sequence Detection System. Reactions were carried out using 50 ng of total kidney RNA under the following conditions: 48°C for 30 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. Primers and TaqMan probe for *NPHS1*: forward primer 5'-AAG CTG GAC GTG CAT TAT GCT-3', reverse primer 5'-CGG TGC AGA CTA TAT CCA CAG AAC-3' and probe 6FAM-TGC CCT GAA GGA CCC TAC TGA GGT GAA-TAMRA. Primers and TaqMan probe for *WT-1*: forward primer 5'-TCT TCC GAG GCA TTC AGG AT-3', reverse primer 5'-TGC TGA CCG GAC AAG AGT TG-3' and probe 6FAM-TGC GGC GTG TAT CTG GAG TGG C-TAMRA. Primers and MGB probe for *HA-ACTN4* were designed to overlap the hemagglutinin tag and the 5' terminal *ACTN4* sequence in order to exclusively recognize the transgene: forward primer 5'-CAT ATG ACG TCC CAG ACT CTG C-3', reverse primer 5'-TGG TTC GCT GCG TGG TAG T-3' and probe 6FAM-ACT CTG CCG AAT TCA TGG T-MGBNFQ. Primers and TaqMan probe for endogenous *ACTN4*: forward primer 5'-TGA TCA GGT CAT CGC CTC CTT-3', reverse primer 5'-GGC AGC TCT CTC CGC AGT TC-3' and probe 6FAM-AGG TCC TGG CAG GAG ACA AGA ACT TCA TCA C-TAMRA. Values were normalized to *GAPDH* mRNA levels in each sample as determined by a TaqMan Rodent *GAPDH* Control Reagent kit (Applied Biosystems, Foster City, CA). To account for potential differences in podocyte number between wt, proteinuric *ACTN4*-mutant and non-proteinuric *ACTN4*-mutant animals, we normalized relative nephrin mRNA values to the total number of podocytes counted per tissue section as assessed by counting the number of *WT-1* positive cells per glomerulus (wt, 11.2 ± 0.2

podocytes/glomerulus; non-proteinuric, 11.1 ± 0.2 podocytes/glomerulus; proteinuric, 11.5 ± 0.1 podocytes/glomerulus).

Statistics

Values reported are the means ± S.E.M.. Appropriate statistical comparisons were made using either the Student's t-test or ANOVA followed by the Newman-Keuls multiple comparison test.

Results

Cloning and Mutagenesis of Murine ACTN4 cDNA

The murine *ACTN4* cDNA was cloned by homology to the human form using the mouse EST database. Two overlapping I.M.A.G.E. clones (GenBank accession AI119186 and W57010) demonstrating >95% homology to the human *ACTN4* sequence were obtained. The authenticity of these sequences was later confirmed by alignment with the recently submitted mouse *ACTN4* sequence (GenBank accession NM021895 and AJ289242). In the Kaplan & Mathis studies of familial FSGS, an A-G substitution at nt 763 in the *ACTN4* ORF resulted in a K255E substitution in a highly conserved region immediately distal to the actin-binding domain of α-actinin-4 (20-22). Alignment to the human sequence revealed significant homology at both nucleotide and amino acid levels in this affected region (Figure 3.1). Using PCR-based mutagenesis, we introduced an analogous A766G substitution in order to obtain a K256E mutation in the translated product. Additionally, an N-terminal double-HA epitope tag was introduced to facilitate analysis of transgene expression *in vivo*.

The α-Actinin-4 Mutant Protein Exhibits Increased Affinity for F-Actin In Vitro

Kaplan et al observed enhanced *in vitro* binding to actin for the 3 mutant human α-actinin-4 proteins (20). To verify that the K256E murine α-actinin-4 protein exhibits similar properties, we performed an actin pull-down assay. *In vitro*-translated wt or mutant α-actinin-4 (with or without HA tag) were incubated in the presence of 10 μM cold actinin and 2.5 μM actin under polymerizing conditions, followed by high-speed

α-actinin-4		A	R	P	D	E	**K/E**	A	I	M	T
hACTN4	748-GCC	CGG	CCC	GAC	GAG	**A/G**AG	GCC	ATA	ATG	ACC-777	
mACTN4	751-GCC	CGG	CCC	GAC	GAG	**A/G**AG	GCC	ATA	ATG	ACA-780	

Figure 3.1 Homology between human and mouse α-actinin-4.
Amino acid sequence comparison reveals the highly conserved region containing one of three point mutations identified by Kaplan et al (20). An A→G mutation at nucleotide 763 of the human ORF results in a K255E substitution immediately distal to the actin binding site. An analogous mutation was engineered into the mouse ORF at position 766 by PCR-based mutagenesis.

centrifugation to pellet F-actin/actinin complexes. As shown in Figure 3.2a, the inclusion of 10 μM cold α-actinin, while able to displace wt α-actinin-4, was unable to displace the mutant α-actinin-4 from F-actin complexes. Similar results were obtained when using [^{35}S]-methionine-labeled *in vitro*-translated wt and mutant α-actinin-4, thereby confirming that the N-terminal double-HA tag did not alter the co-sedimentation of either wt or mutant α-actinin-4 with F-actin (data not shown). We next confirmed the increased affinity of mutant α-actinin-4 for F-actin in a cell culture model. COS-7 cells were transiently transfected with pCDNA3 vector, HA-*ACTN4* wt, or HA-*ACTN4* mutant. The actin cytoskeleton was pelleted from cell lysates at 10^5 *x g* (23) and the amount of HA-α-actinin-4 assessed by western blot as analyzed with a polyclonal anti-HA tag antibody (1:1000). As shown in Figure 3.2b, compared to the wt form, a significantly greater proportion of mutant murine α-actinin-4 associated with the insoluble cytoskeletal fraction containing an equal amount of pelleted actin. These results confirm that like the mutant variant of human α-actinin-4, the mutant form of murine α-actinin-4 exhibits abnormally high affinity for filamentous actin thereby suggesting that even low expression levels of the mutant transgene may be sufficient to dysregulate the actin cytoskeleton.

Development of ACTN4-Mutant Transgenic Mice

The lesions associated with many forms of FSGS are thought to occur following damage to the podocytes. To address whether the A763G *ACTN4* point mutation associated with a familial form of FSGS exerts direct influence in podocytes, we developed mice with podocyte-specific expression of the α-actinin-4 K256E mutant. An

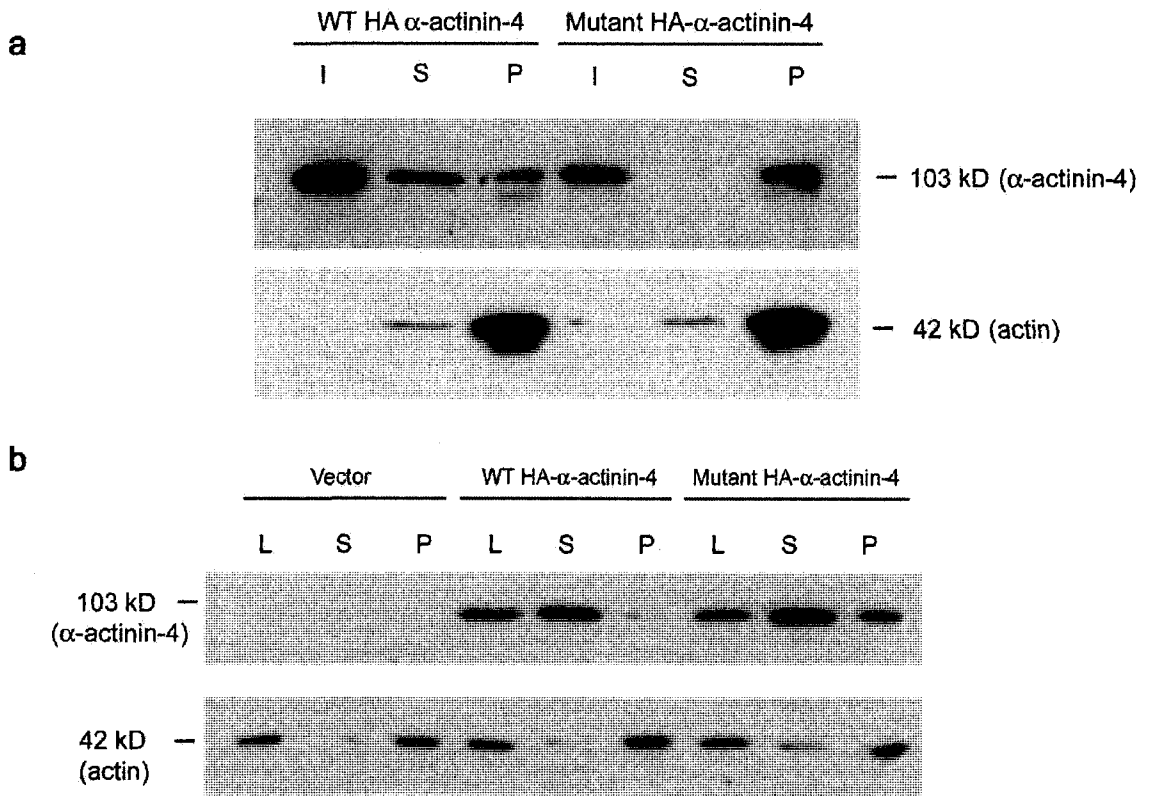

Figure 3.2 Mutant murine α-actinin-4 exhibits increased affinity for actin.
(**a**) Similar quantities of wt and mutant *in vitro*-translated α-actinin-4 were incubated with 2.5 μM actin and 10 μM cold α-actinin for 1 hr. Samples were centrifuged to pellet α-actinin-actin complexes. *In vitro*-translated products (input, I), supernatants (S) containing unbound α-actinin, and pellets (P) were resolved by SDS-PAGE. Western blotting with an anti-HA tag antibody (a, upper panel) revealed that mutant-HA α-actinin-4 was not competed from actin by cold α-actinin. The blot was reprobed with an anti-actin antibody (a, lower panel). (**b**) COS-7 cell lysates transfected with either vector alone, HA-*ACTN4* wt or HA-*ACTN4* mutant were centrifuged at 10^5 *x g* and the lysates (L), supernatants (S) and pellets (P) resolved by SDS-PAGE. Western blots were probed with an anti-HA tag antibody (b, upper panel). Blots were then reprobed with an anti-rabbit actin antibody (b, lower panel) to verify that comparable amounts of actin were pelleted from both wt and mutant lysates. Each experiment was performed 3 times.

8.3 kb fragment of the murine nephrin promoter (*NP*) which included the 5' untranslated and 5' flanking regions of the murine *NPHS1* gene was chosen to drive the expression of the HA-*ACTN4* mutant ORF in a podocyte-specific manner (Figure 3.3a). Fragments of both the human and mouse nephrin promoter region were recently used to generate mice with podocyte-specific expression of a LacZ reporter gene (24) (25) and Cre-recombinase (26). More recently, a similar mouse promoter fragment was employed to generate mice with podocyte-specific expression of mutant *WT-1* (27). In the present study, transgenic mice were obtained by pronuclear injection and founders were identified by Southern analysis of genomic DNA derived from tail biopsies (Figure 3.3b). 12 transgenic founders out of 48 pups (25%) were identified carrying the *NP*-HA-*ACTN4*-mutant transgene (designated - *ACTN4*-mutant mice). Urinalysis for 2 male founders revealed significant albuminuria (#6445, 105 μg/ml/g body weight; #7180, 72 μg/ml/g body weight) and these were subsequently backcrossed with C3H mice to establish the experimental lines and provide the N_1 generation mice used in the present study.

ACTN4-Mutant Mice Exhibit Proteinuria

Moderate proteinuria is a common clinical finding in many, but not all *ACTN4*-associated FSGS patients (20, 21). The *ACTN4*-mutant mice from both founder lines could likewise be divided into proteinuric (> 4 μg albumin/ml/g body weight, mean 40.8 ± 11.9 μg albumin/ml/g body weight, n=8; $P < 0.001$ vs wt and non-proteinuric) and non-proteinuric (0.5 ± 0.1 μg albumin/ml/g body weight, n=10) groups based upon urinalysis at 10 weeks of age (Figure 3.4). Of the 8 proteinuric animals, 4 were derived from founder 6445 and 4 from founder 7180. The onset of albuminuria appeared to precede

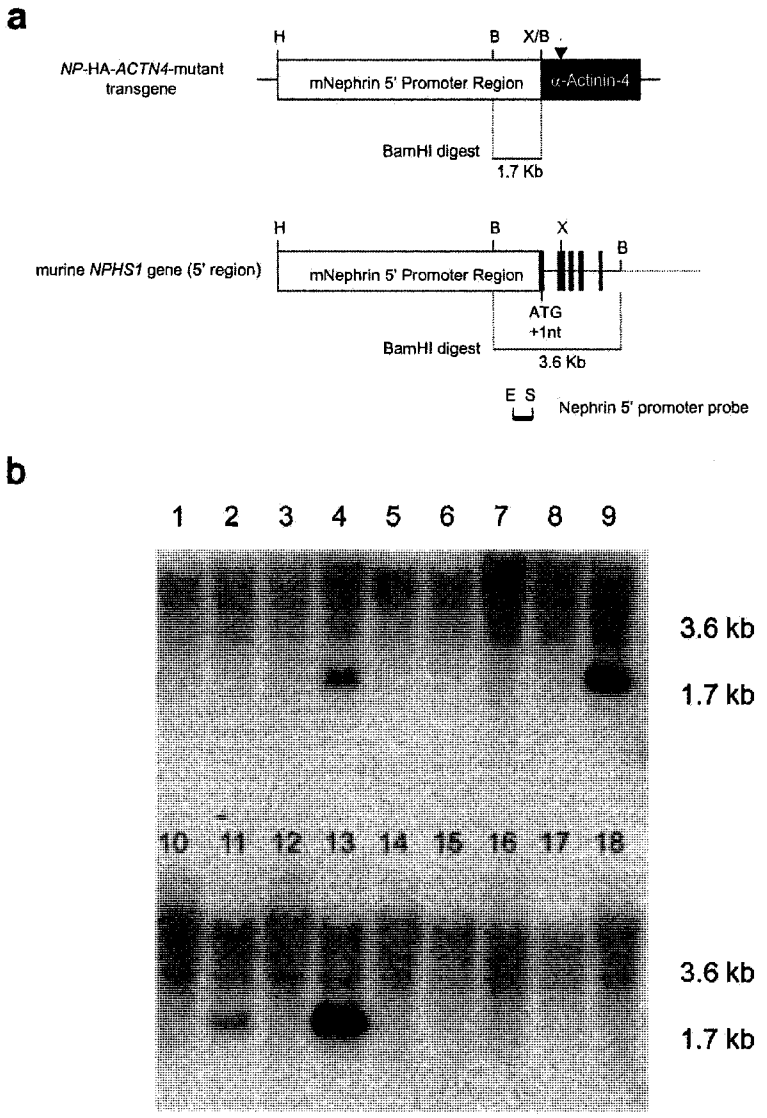

Figure 3.3 Nephrin promoter-*ACTN4*-mutant construct and genotypic analysis. **(a)** An 8.3 kb fragment of the murine nephrin promoter (*NP*) was cloned upstream of the HA-*ACTN4* mutant ORF. The resulting construct (*NP*-HA-*ACTN4*-mutant) was used to generate transgenic mice overexpressing mutant α-actinin-4 in a podocyte-specific manner. The A766G mutation is indicated by the arrow (⬇). An *EcoRI/StuI* (E/S) fragment was used as a probe in Southern blots. B:B*amHI*, X:*XhoI* and H:*HindIII*. **(b)** Genomic DNA was isolated from tail snips of resulting mice, digested with *BamHI* and analyzed by Southern blot to identify founders carrying the transgene. The endogenous nephrin gene yields a 3.6 kb fragment whereas the *ACTN4*-mutant transgene yields a 1.7 kb fragment. Shown is a representative blot with 18 of the 48 candidates. Four of the 12 mice with transgene incorporation are shown on this blot (lanes 4, 9, 11, 13).

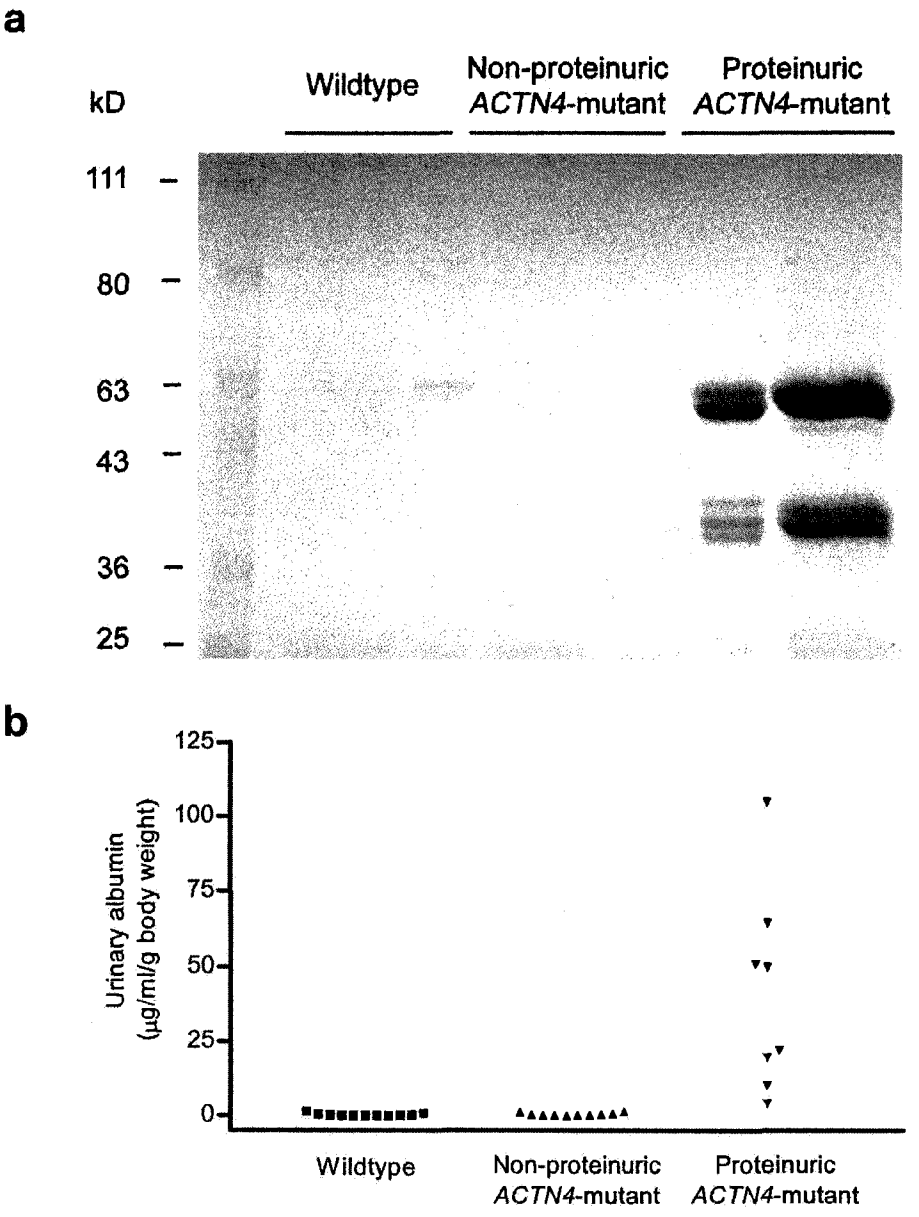

Figure 3.4 Urinalysis of wildtype and *ACTN4*-mutant mice.
(a) Urine samples (5 µL) of N_1 generation mice were resolved by SDS-PAGE and the gels stained with Coomassie Blue. A significant proportion of the *ACTN4*-mutant mice exhibit proteinuria. **(b)** Albumin levels determined by ELISA revealed significant albuminuria in 8 of 18 *ACTN4*-mutant animals (> 4 µg/day/g body weight). In the other 10 *ACTN4*-mutant mice, urinary albumin levels did not differ from wt littermates (a and b).

the age of weaning (data not shown) and was not gender-specific. This phenotype continues to be observed for both founder lines as backcrossed onto pure C57Bl/6 and C3H lines (each currently at generation N5). Urine of wt mice exhibited negligible albumin levels (0.3 ± 0.1 μg albumin/ml/g body weight, n=11). Three of the 8 proteinuric mice (1 derived from the 6445 founder line; 2 from the 7180 founder line) had microscopic hematuria, and exhibited elevated serum creatinine (36.7 ± 4.2 μmol/L) and urea levels (28.9 ± 3.7 mmol/L) compared to wt mice (14.8 ± 0.9 μmol/L and 9.6 ± 0.9 mmol/L, respectively, n=11). Creatinine and urea levels for the remaining proteinuric (12.8 ± 1.9 μmol/L and 8.4 ± 1.5 mmol/L, respectively, n=5) and non-proteinuric (12.4 ± 0.7 μmol/L and 7.8 ± 0.6 mmol/L, respectively, n=10) *ACTN4*-mutant mice were not significantly different from wt.

Podocyte-Specific Transgene Expression

Podocyte-specific expression of the mutant HA-α-actinin-4 protein was verified by immunofluoresence of cryostat sections. A representative panel of tissues from 2 N_1 mice (from both founder lines) was probed with a monoclonal anti-HA tag antibody (12CA5, 1:500). As shown in Figure 3.5 (left panels), the HA-tagged α-actinin-4 protein was detected in the glomeruli of transgenic animals exhibiting an expression pattern that is consistent with podocyte foot process localization. Expression in the podocytes was confirmed by immunofluorescence with nephrin using an anti-nephrin polyclonal antibody (1:1000) (Figure 3.5, middle panels). Transgene expression was not detected in liver, spleen, lung, skeletal muscle or heart (data not shown).

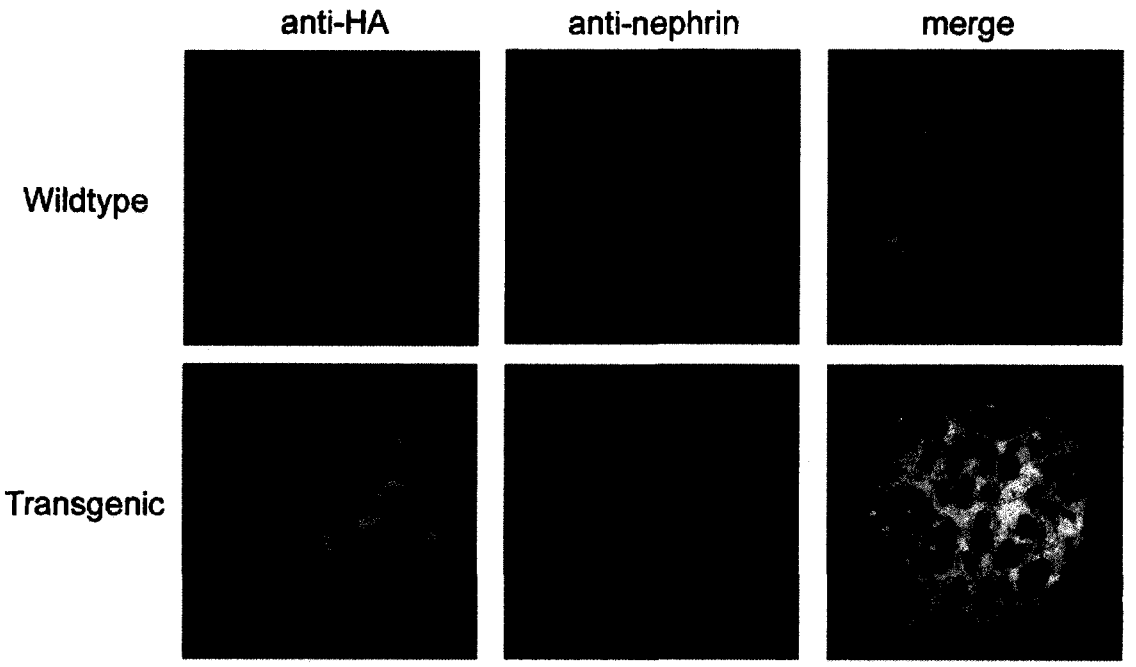

Figure 3.5 Podocyte-specific expression of mutant HA-α-actinin-4.
Left panels: immunofluorescence using a monoclonal anti-HA tag antibody (12CA5) and FITC-secondary antibody reveals glomerular-specific expression of mutant HA-α-actinin-4 in *ACTN4*-mutant mice but not in wt littermates. Middle and right panels: the pattern of expression overlapped with that of the podocyte-specific protein - nephrin (Cy3-conjugated secondary antibody).

Elevated Systolic Blood Pressure in ACTN4-Mutant Mice

The incidence of hypertension in human carriers of *ACTN4* mutations is significantly higher than for the general population, yet seems independent of proteinuria (21). We therefore assessed the systolic blood pressures of the *ACTN4*-mutant mice by tail cuff plethysmography. As shown in Figure 3.6, at 10 weeks of age the average systolic blood pressure of both proteinuric (110 ± 3 mmHg, n=8) and non-proteinuric *ACTN4*-mutant mice (112 ± 3 mmHg, n=10) was significantly elevated compared to that of the wt animals (101 ± 2 mmHg, n=11; $P < 0.05$).

Proteinuric ACTN4-Mutant Mice Exhibit Histological Features of FSGS

Renal biopsies from patients with autosomal dominant familial FSGS are characterized by areas of glomerulosclerosis with tuft collapse and solidification that are both focal and segmental (20-22, 28). PAS staining and light microscopy of kidney sections from proteinuric mice (n=6) revealed glomeruli that contained adhesions of the tuft (Figure 3.7a). Trichrome staining revealed many sections with segmental fibrosis - mainly in glomeruli of the outermost cortex (Figure 3.7b). Occasional formations reminiscent of "tip lesions" as seen in humans were also noted. Several mice had kidneys exhibiting extensive cortical scarring and prominent tubular dilatations containing proteinaceous material (Figure 3.7c). Silver stain frequently revealed recognizable increased mesangial matrix (Figure 3.7d). Proteinuric *ACTN4*-mutant mice exhibited modest, yet significant values of renal damage. For example, proteinuric *ACTN4*-mutant mice had 7.3 ± 2.8 % of their glomeruli globally sclerosed (wt, 0.4 ± 0.2 %; non-proteinuric *ACTN4*-mutant, 0%), and 10.4 ± 4.3 % with segmental sclerosis (wt, 0 %; non-proteinuric *ACTN4*-mutant, 0%). Furthermore, 5 of the 8 proteinuric *ACTN4*-mutant

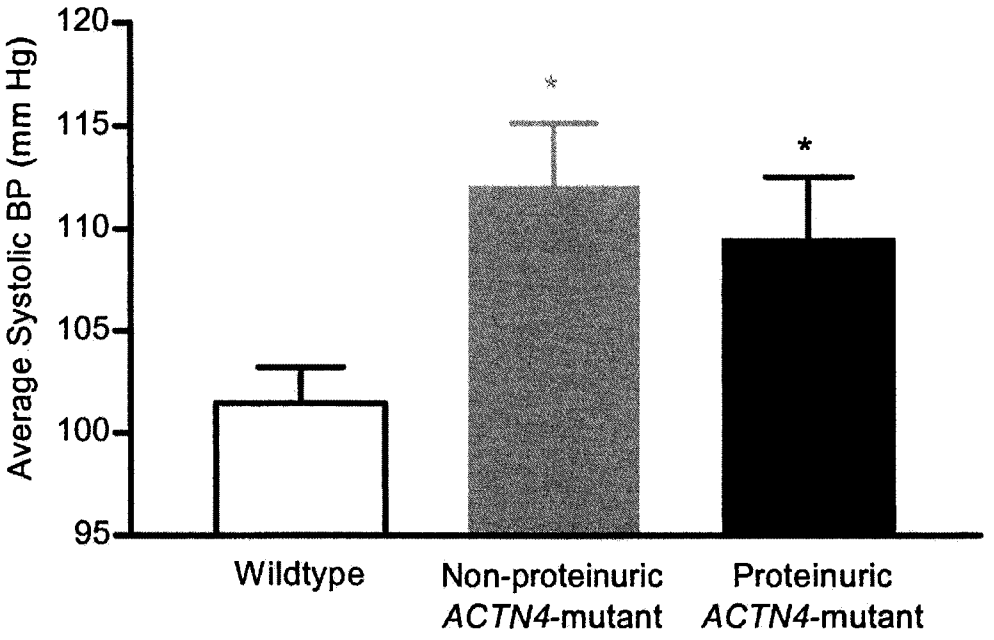

Figure 3.6 Elevated systolic blood pressure in *ACTN4*-mutant mice.
Systolic blood pressure was monitored in wt and *ACTN4*-mutant mice daily for 5 days by tail cuff plethysmography. Both proteinuric and non-proteinuric *ACTN4*-mutant mice exhibited elevated mean systolic blood pressure. * $P < 0.05$.

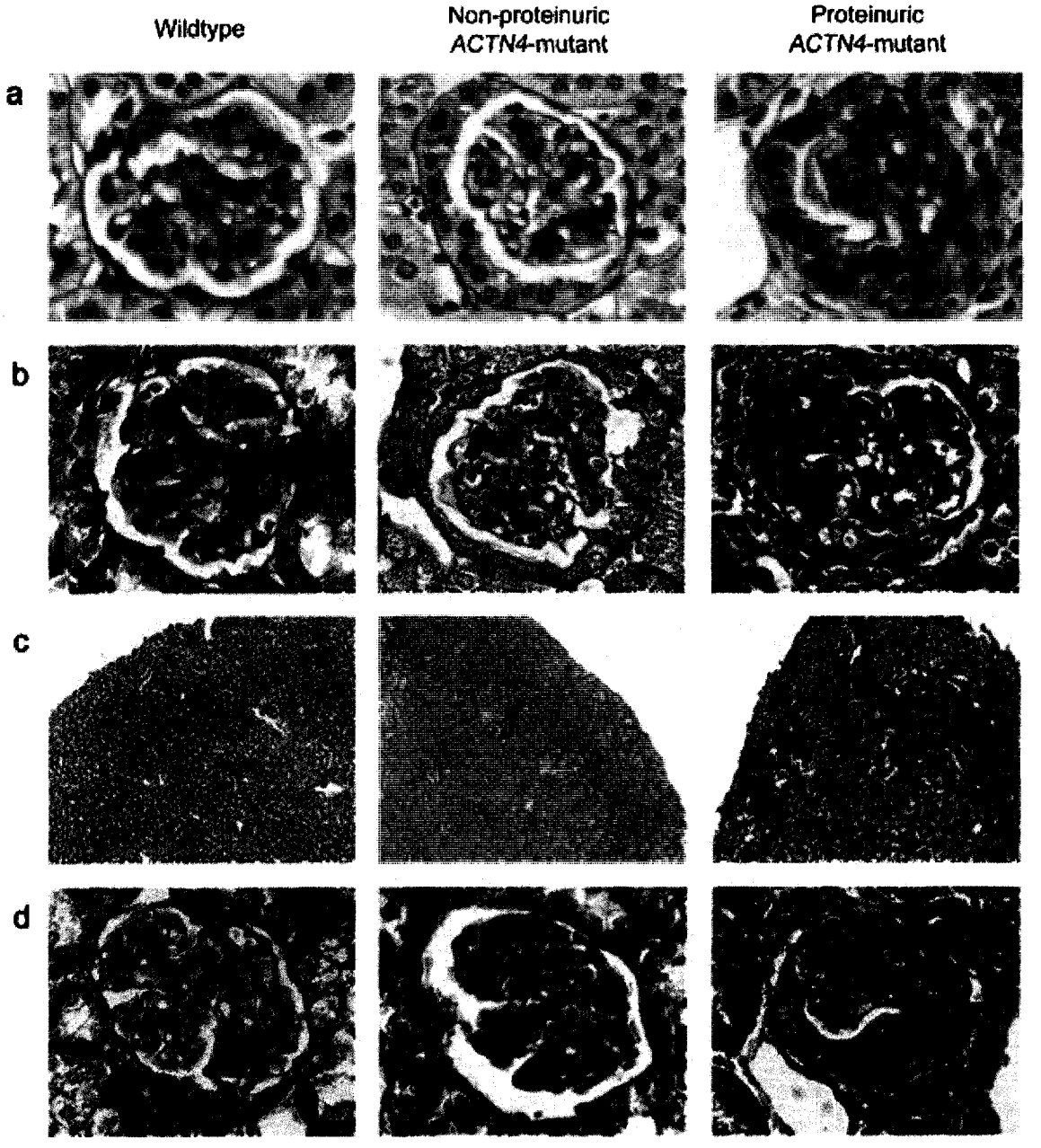

Figure 3.7 Proteinuric *ACTN4*-mutant mice exhibit histological features of FSGS. Kidney sections from 10 week-old wt, non-proteinuric *ACTN4*-mutant and proteinuric *ACTN4*-mutant mice were stained with PAS (a), Masson trichrome (b,c), or silver stain (d). (**a**) A representative glomerulus from a proteinuric *ACTN4*-mutant mouse showing tuft adhesion by PAS stain (400x); (**b**) Masson trichrome stain showing segmental sclerosis of a glomerulus (400x); (**c**) Extensive cortical scarring, tubular dilatation and accumulation of proteinaceous material in proteinuric *ACTN4*-mutant kidney (40x); (**d**) Silver stain showing substantial mesangial matrix (collagen) accumulation in proteinuric *ACTN4*-mutant glomerulus (400x).

mice had mild to moderate tubular dilatation accompanied by mild to moderate interstitial fibrosis, whereas this pathology was not observed for either wt or non-proteinuric *ACTN4*-mutant mice. An average of 129, 122, and 140 glomeruli per section were assessed in the wt, non-proteinuric *ACTN4* and proteinuric *ACTN4* mice, respectively.

In clinical cases of FSGS the GBM remains intact while the podocytes exhibit marked structural damage that includes: cell hypertrophy, foot process effacement, pseudocyst formation, cytoplasmic overload with reabsorption droplets and occasionally, detachment from the GBM. Electron microscopy (EM) analyses of proteinuric *ACTN4*-mutant mice revealed podocytes with patchy to more extensive foot process fusion although it was never diffuse (Figure 3.8, lower left panel). Numerous vacuoles in podocytes with some residual bodies were seen (Figure 3.8, lower right panel). GBM thickness did not appear to change, nor did we observe any notable differences between the podocytes of non-proteinuric *ACTN4*-mutant (n=6) and wt mice (n=6).

Elevated Expression Levels of Mutant HA-α-actinin-4 in Proteinuric ACTN4-Mutant Mice

To quantitate the relative expression of the HA-*ACTN4*-mutant gene product in *ACTN4*-mutant mice, we performed both immunofluorescence of kidney sections and real-time RT-PCR analysis of total kidney RNA derived from either proteinuric or non-proteinuric *ACTN4*-mutant mice. As shown in Figure 3.9a, an anti-HA antibody consistently detected more HA-α-actinin-4 in proteinuric vs non-proteinuric mice. This difference was observed for both founder lines. Furthermore, as shown in Figure 3.9b, relative HA-*ACTN4*-mutant mRNA expression levels, normalized to *GAPDH* mRNA, were 3.2-fold greater in proteinuric *ACTN4*-mutant mice compared to non-proteinuric

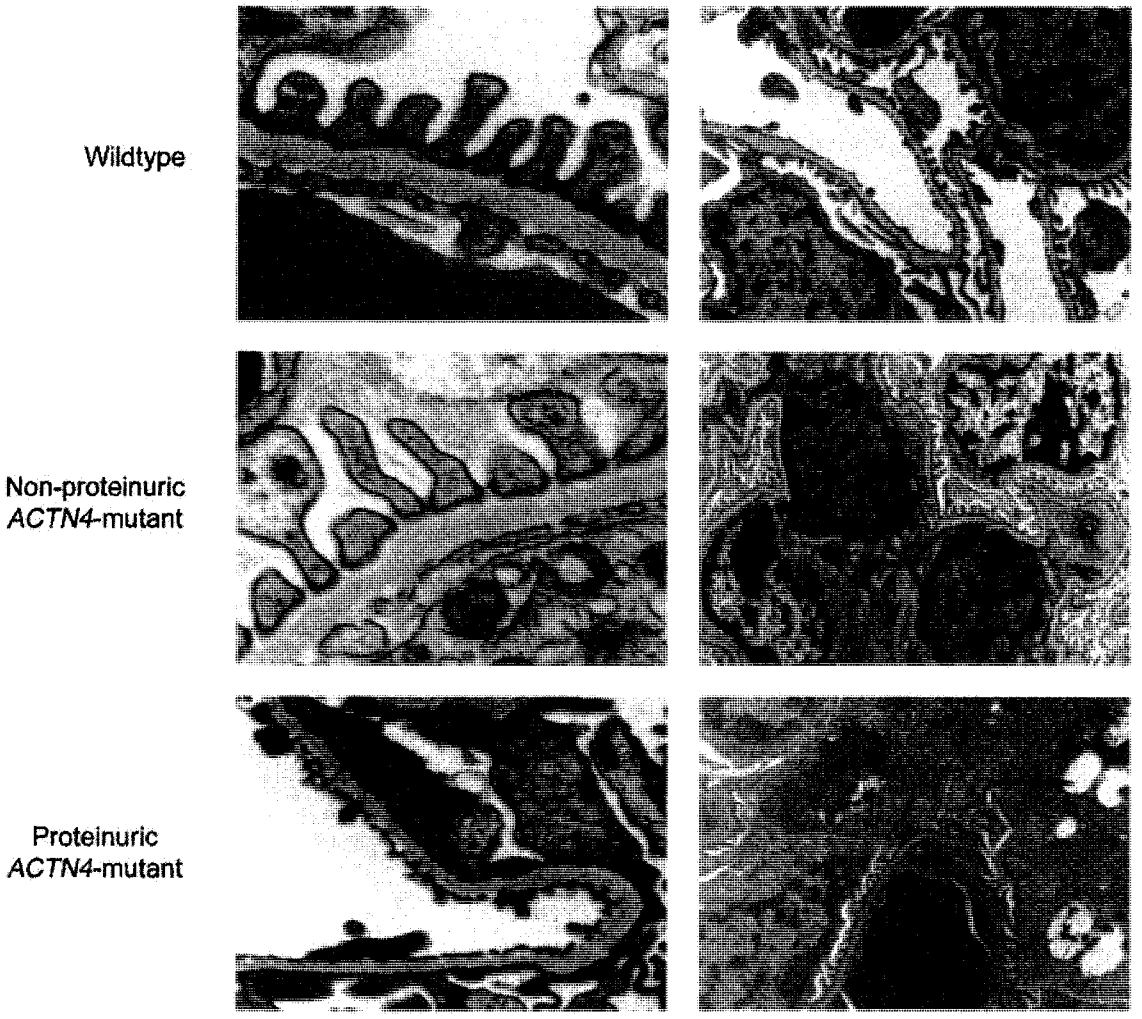

Figure 3.8 Podocyte foot process fusion in *ACTN4*-mutant mice.
Kidney sections from 10 week-old wt, non-proteinuric *ACTN4*-mutant and proteinuric *ACTN4*-mutant mice were assessed by electron microscopy. Representative podocyte foot process fusion in proteinuric *ACTN4*-mutant mice (lower left panel); Podocyte cell body vacuolization (arrow) in proteinuric *ACTN4*-mutant mice (lower right panel).

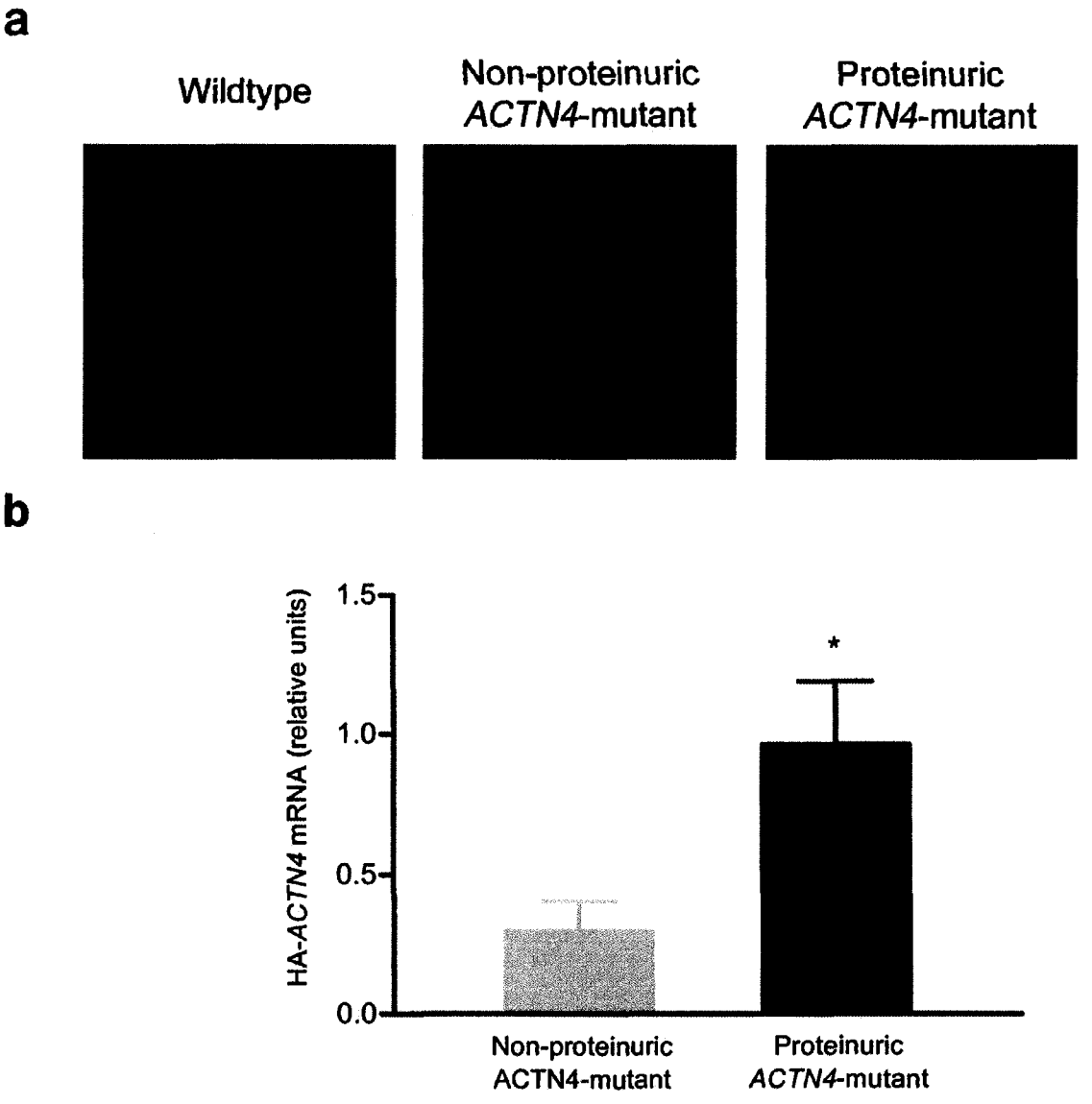

Figure 3.9 Expression of mutant HA-α-actinin-4 in proteinuric vs. non-proteinuric *ACTN4*-mutant mice.

(a) Immunofluorescence using an anti-HA tag antibody (rabbit polyclonal) and Cy3-conjugated secondary antibody reveals greater mutant HA-α-actinin-4 levels in proteinuric vs. non-proteinuric *ACTN4*-mutant mice. **(b)** Real-time RT-PCR of total kidney mRNA using a HA-*ACTN4*-specific MGB probe. Values were standardized to levels of *GAPDH* mRNA in each sample and have been normalized for the number of podocytes/glomerulus. HA-*ACTN4* mRNA levels are significantly higher in proteinuric *ACTN4*-mutant mice, n=8 (* $P < 0.01$ vs non-proteinuric *ACTN4*-mutant mice, n=10). No detectable product was amplified using RNA from wt mice (not shown).

ACTN4-mutant littermates (non-proteinuric *ACTN4*-mutant, 0.30 ± 0.11 relative units, n=10; proteinuric *ACTN4*-mutant, 0.96 ± 0.23 relative units, n=8; $P < 0.01$). Since the primer/probe combination is specific for the HA-tag, no signal could be detected for wt mice. We next compared endogenous renal *ACTN4* mRNA expression to that of the mutant transgene by real-time RT-PCR using a primer/probe combination that recognizes both forms. The total *ACTN4* mRNA expression (i.e., transgenic and endogenous *ACTN4*) as normalized to *GAPDH* was not different between groups of mice (wt, 1.16 ± 0.19 relative units; non-proteinuric *ACTN4*-mutant, 1.16 ± 0.15 relative units; proteinuric *ACTN4*-mutant, 1.20 ± 0.15 relative units; n=6) thereby demonstrating low-level expression of the transgene compared to endogenous *ACTN4*. Such expression is sufficient to generate a proteinuric phenotype in these mice and is consistent with the large gain in affinity for F-actin by the α-actinin-4 mutant protein, as predicted by in vitro results (Figure 3.2).

Decreased Nephrin mRNA and Protein Levels in Proteinuric ACTN4-Mutant Mice

The maintenance of the filtration barrier at the molecular level is accomplished in part by nephrin, a key component of the slit diaphragm (29). We examined nephrin protein expression by immunofluorescence using an anti-nephrin antibody. As shown in Figure 3.10a, nephrin expression levels were visibly reduced in proteinuric *ACTN4*-mutant mice compared to both wt littermate controls and non-proteinuric *ACTN4*-mutant littermates. We next evaluated the expression of the *NPHS1* gene product in *ACTN4*-mutant mice, by real-time RT-PCR analysis of total kidney RNA derived from either wt, proteinuric or non-proteinuric *ACTN4*-mutant mice. As shown in Figure 3.10b, relative nephrin mRNA expression levels, corrected for *GAPDH* mRNA, and normalized to the

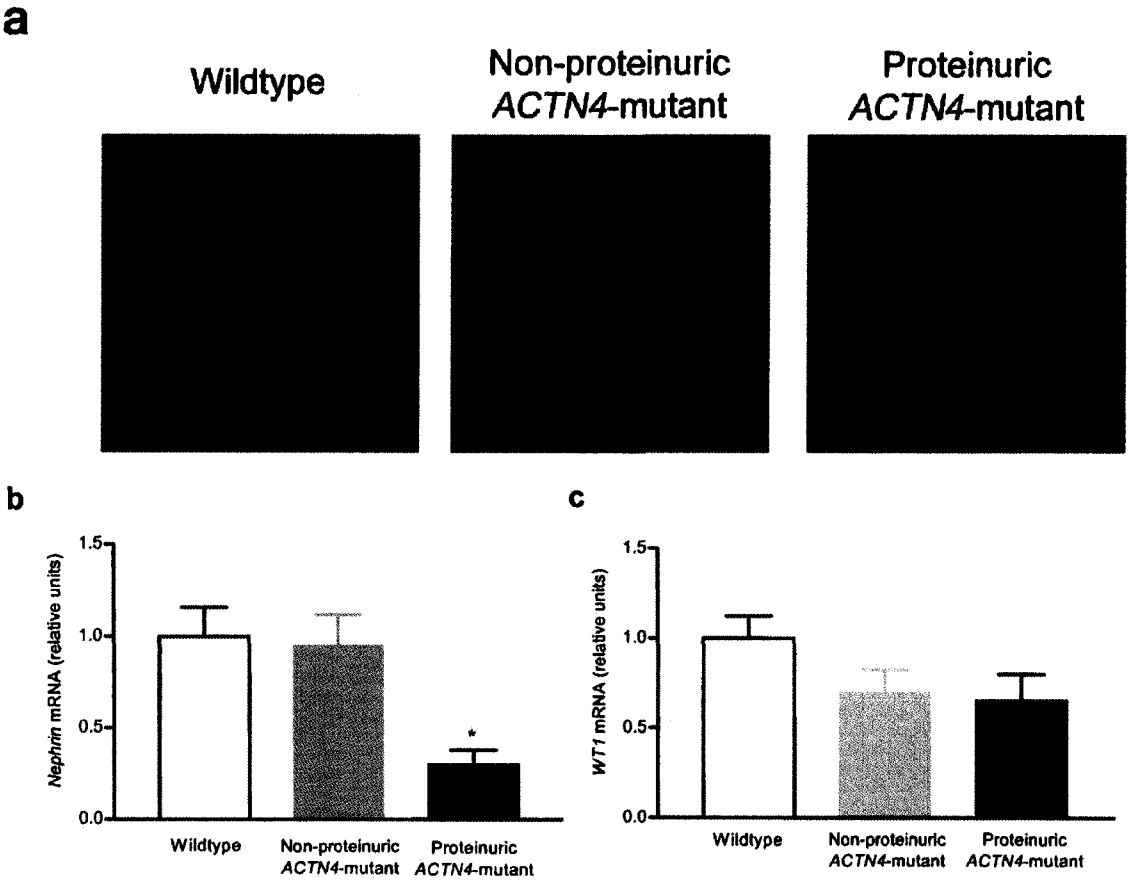

Figure 3.10 Reduced nephrin expression in proteinuric *ACTN4*-mutant mice. **(a)** Immunofluorescence using an anti-nephrin antibody and Cy3-secondary antibody reveals reduced expression of nephrin in proteinuric, but not in wt or non-proteinuric *ACTN4*-mutant littermates. **(b,c)** Total kidney RNA isolated from 10-week-old animals was analyzed by real-time RT-PCR using specific TaqMan probes. Values were standardized to levels of *GAPDH* mRNA and have been normalized for the number of podocytes/glomerulus. **(b)** Nephrin mRNA levels were significantly decreased in proteinuric *ACTN4*-mutant mice, n=6 (* $P < 0.01$ vs both wt, n=6, and non-proteinuric *ACTN4*-mutant mice, n=6). **(c)** Levels of *WT-1* mRNA were not significantly different between *ACTN4*-mutant mice and wt littermates.

number of podocytes/glomerulus were reduced by 70% in proteinuric *ACTN4*-mutant mice compared to both wt littermate controls and non-proteinuric *ACTN4*-mutant littermates (wt, 1.00 ± 0.16 relative units, n=6; non-proteinuric *ACTN4*-mutant, 0.95 ± 0.17 relative units, n=6; proteinuric *ACTN4*-mutant, 0.30 ± 0.08 relative units, n=6; $P < 0.01$). Furthermore, as shown in Figure 10c, mRNA levels of the podocyte-specific Wilm's Tumor-1 (*WT-1*) transcription factor were slightly lower yet not statistically significant in these transgenic mice as compared to wt controls (wt, 1.00 ± 0.12 relative units, n=6; non-proteinuric *ACTN4*-mutant, 0.70 ± 0.13 relative units, n=6; proteinuric *ACTN4*-mutant 0.66 ± 0.15 relative units, n=6; each not significant vs. wt).

Discussion

The renal syndrome known as focal segmental glomerulosclerosis now stands as the second leading cause of renal insufficiency, exceeded only by diabetes (7). The weight of recent evidence suggests that the cellular target in FSGS is the glomerular epithelial cell – or podocyte. Point mutations in *ACTN4,* a gene encoding for a key component of the podocyte's complex molecular architecture were recently identified in several families and show strong linkage with a steroid-resistant familial form of autosomal dominant FSGS (20). The sequelae of intermolecular events initiated by aberrant α-actinin-4/actin interactions resulting in the development of sclerosis are unknown. In the present study, we have generated a mouse model for this *ACTN4*-associated form of FSGS. To our knowledge, this is the first genetically-derived animal model based upon an inherited form of FSGS.

Clinical cases of *ACTN4*-associated FSGS are associated with a mild onset of proteinuria in the teenage years with slow but progressive loss of renal function and the development of ESRD in some individuals later in life (20-22). Furthermore, this syndrome is not fully penetrant since not all carriers of the *ACTN4* point mutations are proteinuric (22). Similarly in the present study, not all *ACTN4*-mutant mice were proteinuric, likely owing to lower expression of mutant α-actinin-4 compared to the proteinuric mice. This phenomenon was observed in both founder lines and could not be correlated with transgene copy number (data not shown). Furthermore, only 3 of the 8 proteinuric mice displayed reduced renal function by 10 weeks of age. Variable levels of *ACTN4*-mutant expression may therefore underlie the incomplete penetrance encountered

in human cases of *ACTN4*-associated FSGS. Hypertension is also a common finding associated with many forms of FSGS, including *ACTN4*-associated FSGS (21). The incidence of hypertension was reported to be abnormally high in the Oklahoma *ACTN4*-FSGS family and yet could not be correlated with proteinuria (21). Similarly, we found that the overall systolic blood pressure averages of both proteinuric and non-proteinuric *ACTN4*-mutant mice were elevated. Such phenotypic heterogeneity observed both in humans, and now in mice might be explained by genetic modifier effects.

Our results strongly support the notion that the lesions encountered with this form of familial FSGS find their root cause in the podocyte. We observed a number of renal pathological changes in mice expressing a high affinity variant of α-actinin-4 in a podocyte-specific manner that are consistent with an FSGS-like phenotype. These include – segmental glomerular fibrosis, occasional tip lesions, adhesion of the glomerular tuft, podocyte foot process fusion, podocyte vacuolization, and mesangial matrix accumulation. Additionally, we observed marked tubular dilatation, accompanied by the accumulation of a luminal proteinaceous material indicating severe damage due to the proteinuria. This combination of pathological and clinical data indicate that mice with podocyte-specific expression of mutant α-actinin-4 exhibit a phenotype similar to that seen in humans with this genetic mutation.

The *ACTN4*-associated form of FSGS was identified by Mathis et al., who employed a positional cloning approach to study an FSGS kindred from Oklahoma (21, 22). Subsequently, Kaplan and co-workers studied 3 families (including the Oklahoma family) with an autosomal dominant form of FSGS in order to identify the underlying genetic defect accounting for the observed FSGS (20). They identified an A-G

substitution in the *ACTN4* open-reading frame that resulted in a K to E mutation in a region immediately distal to the actin-binding site (20). Similar mutations in this region were identified in the 2 other families described in the study. Each of these mutations yield α-actinin-4 proteins that bind to F-actin with greater affinity than the wildtype form, implying that dysregulation of the actin cytoskeleton might underlie the ensuing podocyte damage and thereby render the glomerulus susceptible to sclerosis. Several reports have documented an abnormal distribution and disaggregation of podocyte actin microfilaments during the development of foot process effacement (30-32). Concomitant redistribution of actin and α-actinin in the podocytes of nephrotic rats has also been reported (30, 33), resulting in dysregulation of actin filament bundling in foot processes and finally yielding disruption of normal foot process function. Such alterations in cytoskeletal function might explain the characteristic changes to podocytes observed in FSGS.

Accordingly, point mutations in the *ACTN4* gene might have significant effects on key components of the podocyte cytoskeleton and slit diaphragm. Nephrin, a key player in the formation of the slit diaphragm, possesses extracellular IgG-like repeats, a single membrane-spanning region, and a cytoplasmic tail that interacts with intracellular molecules including CD2AP, which may anchor it to the actin cytoskeleton (17, 18). The importance of this molecule in maintaining the glomerular barrier to protein is illustrated by the fact that humans with mutations in the *NPHS1* gene, and *NPHS1*-null mice are nephrotic and fail to develop normal podocyte foot processes (19, 34). Many recent studies have associated reduced nephrin mRNA or protein expression with the onset of proteinuria in a number of nephropathies including diabetic nephropathy (35, 36), minimal change disease (37), passive heymann nephritis (38-40), and membranous

proliferative glomerulonephritis (37). Our results showing a 70% reduction in nephrin mRNA in proteinuric *ACTN4*-mutant mice, are consistent with the observed foot process fusion and are the first to demonstrate such a correlation in the context of FSGS. The reduced nephrin mRNA and protein levels were not a result of changes in podocyte numbers at this early stage of the disease's progression as both the number of podocytes per glomerulus and the number of glomeruli were no different between groups of mice. These changes in nephrin expression may reflect lower transcriptional activity at the *NPHS1* locus or perhaps enhanced nephrin mRNA or protein degradation. In either case, these results strongly suggest that a link exists between the regulation of cytoskeletal function and nephrin expression. Expression of the transcription factor *WT-1*, a podocyte-lineage marker, has been previously shown to be downregulated in some non-familial forms of collapsing and non-collapsing FSGS, thereby suggesting a reversion of the podocyte to a more immature / proliferating phenotype (4, 41, 42). However, we now show that *WT-1* mRNA levels and immunofluorescence of synaptopodin (data not shown), another podocyte-specific marker, were not significantly different between the 3 groups of mice, thereby suggesting that despite the reduced nephrin expression, these cells maintained a somewhat differentiated phenotype.

In summary, we have developed transgenic mice overexpressing a mutated variant of the α-actinin-4 protein in a podocyte-specific manner. As in clinical cases of familial FSGS, a significant proportion of these mice exhibit moderate proteinuria, marked podocyte foot process fusion as well as other classical FSGS hallmarks. Furthermore, nephrin mRNA levels are reduced in proteinuric animals, suggesting a cause and effect relationship between dysregulation of the actin cytoskeleton by mutant α-actinin-4 and the deterioration of the slit diaphragm complex. Future insights gained from such a model

uncovering the intra- and inter-cellular mechanisms by which mutations in the *ACTN4* gene yield an FSGS phenotype may provide novel therapeutic approaches for the prevention and treatment of this disease.

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Chapter 4

FSGS-associated α-actinin-4 (K256E) impairs cytoskeletal dynamics in podocytes

FSGS-associated α-actinin-4 (K256E) impairs cytoskeletal dynamics in podocytes

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Description and author contribution

This manuscript describes the effects of a disease-causing mutation on the function of α-actinin-4 in cultured podocytes. Mislocalization of the mutant protein severely impairs cytoskeleton-dependent processes such as cell spreading and migration. The results presented in this manuscript provide some insight into the mechanisms by which mutations in the *ACTN4* gene contribute to podocyte injury.

All the experiments presented in this manuscript were performed by myself, with guidance from Chris Kennedy. Koralee Chaisson and Robin Parks developed the adenoviruses used in this study. Writing of the manuscript was performed by myself, with subsequent editing by Chris Kennedy and Robin Parks. Both myself and Chris Kennedy contributed to the revision of the manuscript prior to publication.

Abstract

Mutations in the *ACTN4* gene, encoding the actin crosslinking protein α-actinin-4, are associated with a familial form of focal segmental glomerulosclerosis (FSGS). Mice with podocyte-specific expression of K256E α-actinin-4 develop foot process effacement and glomerulosclerosis, highlighting the importance of the cytoskeleton in podocyte structure and function. K256E α-actinin-4 exhibits increased affinity for F-actin. However, the downstream effects of this aberrant binding on podocyte dynamics remain unclear. Wild-type and K256E α-actinin-4 were expressed in cultured podocytes via adenoviral infection to determine the effect of the mutation on α-actinin-4 subcellular localization and on cytoskeletal-dependent processes such as adhesion, spreading, migration, and formation of foot process-like peripheral projections. Wild-type α-actinin-4 was detected primarily in the Triton-soluble fraction of podocyte lysates and localized to membrane-associated cortical actin and focal adhesions, with some expression along stress fibers. Conversely, K256E α-actinin-4 was detected predominantly in the Triton-insoluble fraction, was excluded from cortical actin, and localized almost exclusively along stress fibers. Both wild-type and K256E α-actinin-4-expressing podocytes adhered equally to an extracellular matrix (collagen-I). However, podocytes expressing K256E α-actinin-4 showed a reduced ability to spread and migrate on collagen-I. Lastly, K256E α-actinin-4 expression reduced the mean number of actin-rich peripheral projections. Our data suggest that aberrant sequestering of K256E α-actinin-4 impairs podocyte spreading, motility, and reduces the number of peripheral projections. Such intrinsic cytoskeletal

derangements may underlie initial podocyte damage and foot process effacement encountered in *ACTN4*-associated FSGS.

Introduction

Focal segmental glomerulosclerosis (FSGS) is a common glomerular lesion and a significant cause of end-stage renal disease (1, 2). Clinically, FSGS patients present with variable levels of proteinuria and a progressive loss of renal function. Pathologically, FSGS is characterized by segmental sclerosis in a proportion of glomeruli, the filtering units of the kidney (3). Accumulating evidence suggests that defects in podocytes initiate processes leading to the degeneration of filtration integrity and the development of sclerotic lesions (4-8).

Podocytes are terminally differentiated cells that line the outer aspects of the glomerular capillaries (9). The highly ordered podocyte architecture consists of a cell body from which emerge major processes, which branch into foot processes that interdigitate with those of neighboring podocytes to provide the structural platform upon which a molecular sieve is formed. The foot processes are endowed with a microfilament-based contractile apparatus composed of actin, myosin-II, α-actinin, talin, paxillin and vinculin (10), and are anchored to the glomerular basement membrane (GBM) via an $\alpha_3\beta_1$-integrin complex (11, 12). The intricate morphology of the podocyte, coupled to its exposure to distensile forces within the glomerular capillary render these cells susceptible to damage in many nephropathies, including FSGS.

The actin bundling α-actinins are members of the spectrin superfamily. Four isoforms have been described (α-actinin-1 to -4) (13). While α-actinins-2 and -3 are expressed at the Z-line of striated muscle, α-actinins-1 and -4 are more ubiquitously expressed. α-Actinin-4 is expressed in podocytes and is thought to play a key role in the

maintenance of this cell's architecture (14). The putative function of α-actinin-4, which exists as a head-to-tail homodimer, is to crosslink actin filaments through its N-terminal actin binding domain (ABD) comprised of 2 calponin homology domains. Increasing evidence suggests that α-actinin-4 may interact with a number of other proteins such as β_1-integrin (13, 15, 16), synaptodpodin (17), vinculin (18) and phosphatidylinositol 3-kinase (19). In addition to an ABD, a number of other functional domains are found in the α-actinin-4 sequence – including two calcium-binding EF hands, a phosphoinositide-binding domain, as well as a focal adhesion kinase (FAK) tyrosine phosphorylation consensus sequence. Accordingly, phosphorylation by FAK (20), binding of phosphoinositides (21, 22) and sensitivity to intracellular calcium (23) may modulate the actin binding properties and localization of α-actinin following various environmental stimuli.

Mutations in the *ACTN4* gene (K228E, T232I and S235P) are associated with an autosomal dominant form of FSGS (24, 25). We developed a mouse model of *ACTN4*-associated FSGS by expressing the murine correlate of the K228E mutation (K256E) in a podocyte-specific manner using the *mNPHS1* promoter (26). These mice exhibit significant proteinuria and develop FSGS-like lesions confirming that the disease originates in the podocyte. However, the mechanism by which mutations in α-actinin-4 dysregulate podocyte function is not fully understood. Abrogation of α-actinin-4 expression in mice yields severe glomerular disease (27). Furthermore, recent studies by Yao et al. suggest that the familial mutations promote α-actinin-4 aggregation and thereby target the protein for degradation via the proteosome pathway, resulting in a partial loss-of-function (28). In contrast, mutations in α-actinin-4 increase its affinity for filamentous actin (F-actin), suggesting a gain-of-function mechanism (24, 26). In support of the latter

mechanism, the severity of the FSGS-like phenotype correlates directly with K256E α-actinin-4 levels in transgenic mice (26). Thus, it remains unclear whether and how these two viewpoints can be reconciled.

To address this issue, we assessed the functional consequences of an FSGS-associated mutation (K256E) in α-actinin-4 at the cellular level. We now report the intracellular mislocalization of K256E α-actinin-4 in mouse podocytes, which undermines the processes of cell spreading and migration, and impairs the formation of actin-rich peripheral projections. Our data suggest that such defects in key cytoskeletal-associated processes may compromise the podocyte's ability to cope with the demands of the glomerular environment, maintain foot processes structure, and thereby initiate the progression towards sclerosis.

Materials and methods

Adenoviral Constructs

Adenoviral constructs containing wildtype or K256E murine HA-*ACTN4* sequences were generated by subcloning from the previously described pcDNA3-HA-*ACTN4* vectors (26). In these viruses, the HA-*ACTN4* expression cassette replaced the E1-region and transcription is directed rightward, relative to the conventional human adenovirus serotype 5 map. The E1-deleted, first-generation Ad vectors used in these studies were constructed using a combination of conventional cloning techniques and RecA-mediated recombination (39, 40), and were grown and titered on 293 cells, as previously described (41).

Cell Culture

Conditionally-immortalized mouse podocytes were cultured on collagen-I-coated dishes in RPMI-1640 supplemented with 10% FBS, 100 U/mL of penicillin, 100 μg/mL of streptomycin, 100 μg/mL of Normocin (InvivoGen, San Diego, CA) and 10 U/mL γ-interferon (Sigma, Oakville, ON) at 33°C (permissive conditions). For differentiation, cells were cultured at 38°C without γ-interferon (non-permissive conditions) for at least 10 days. Cells used for experiments were between passages 5 and 15 only. Differentiated podocytes were infected with a range of viral loads (multiplicity of infection (MOI) between 0 and 50 plaque forming units/cell) to determine optimal infection conditions. Lysates (10 μg total protein) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Immunoblots were incubated with a monoclonal

anti-HA antibody (HA-7, Sigma, 1:1000), followed by an HRP-conjugated secondary antibody and developed using Supersignal West Pico Chemiluminescence (Pierce). Immunoblots were stripped and reprobed with an anti-actin antibody (Sigma, 1:1000). For all subsequent experiments, differentiated podocytes were infected at an MOI of 25 plaque forming units/cell and incubated for 72 hours to achieve sufficient protein expression.

Fluorescence Microscopy

At 3 days post-infection, podocytes grown on collagen-coated coverslips were processed for immunofluorescence. Cells were fixed with 4% paraformaldehyde, washed with PBS, permeabilized with 0.2% Triton X-100, blocked with 2% BSA, and incubated with an anti-HA tag antibody (Inter Medico, Markham, ON, 1:200 or HA-7, Sigma, 1:1000). Cells were then double-labeled with appropriate Alexa Fluor-conjugated secondary antibodies and Alexa Fluor-conjugated phalloidin (both form Molecular Probes, Eugene, OR, 1:1000). To visualize focal adhesions, cells were labeled with an anti-vinculin antibody (Vin11.5, Sigma, 1:100) followed by appropriate Alexa Fluor-conjugated secondary antibodies and phalloidin. Images were captured using a Zeiss AxioCam and a Zeiss Axioskop 2 fluorescence microscope (Zeiss Axioskop 2 MOT, Zeiss, Germany).

Fractionation of Intracellular Actin Pools

Infected differentiated mouse podocytes were scraped into ice-cold lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.5% Nonidet P-40) supplemented with protease inhibitor cocktail (Sigma, 1:100) and

PMSF, and incubated at room temperature for 15 minutes. Cell lysates were then centrifuged at 15 000 *x g* to isolate the Triton X-100 insoluble (TI) fraction. The Triton X-100 soluble (TS) fraction was removed and further centrifuged at 100 000 *x g* to separate G-actin (supernatant, S) and F-actin (pellet, P). All pellets were resuspended in the original sample volume. A small aliquot of the total lysate was reserved for protein determination using a BCA Protein Assay Kit (Pierce, Rockford, IL). Samples of equal volume (10 µg total protein in lysate) were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with a polyclonal anti-HA tag antibody (Clontech, Palo Alto, CA, 1:1000) followed by an HRP-conjugated secondary antibody and developed using Supersignal West Pico Chemiluminescent Substrate (Pierce). The immunoblots were subsequently stripped and reprobed with an anti-actin antibody (Sigma, 1:1000). The membranes were exposed to film, images were scanned with a Hewlett Packard ScanJet 6100C and densitometry of the bands measured using Kodak 1D 3.5 software.

Cell Adhesion Assays

Differentiated mouse podocytes, having been infected with adenovirus 72 hrs earlier, were harvested by trypsin/EDTA treatment, centrifuged, and resuspended in RPMI containing 10% FBS and counted with a hemacytometer. Cells were seeded onto collagen-I-coated U-bottom 96-well plates ($5x10^4$ cells/well) and incubated at 38°C. At various times after seeding (3, 6, 12 and 24 hrs), the wells were washed with PBS and the cells fixed with 4% paraformaldehyde for 30 minutes at room temperature. Adherent cells were then stained with 1% crystal violet for 30 minutes, washed 5 times with PBS, and solubilized in 20% acetic acid. The absorbance (595nm) of each well was then

measured using a FLUOstar Galaxy plate reader. Data are from three separate experiments and are expressed as a function of control cells.

Cell Replating Assays

Differentiated mouse podocytes, having been infected with adenovirus 72 hrs earlier, were harvested by trypsin/EDTA treatment. Following trypsin inactivation with soybean trypsin inhibitor (Sigma, 0.5 mg/mL), the cells were collected by centrifugation, washed once, and held in suspension for 15 minutes at 38°C in RPMI containing 10% FBS. Suspended cells were counted with a hemacytometer and seeded onto collagen-I-coated glass coverslips ($5x10^4$ cells/well) and incubated at 38°C. At 3 and 6 hours following replating, the wells were washed with PBS, and adherent cells fixed with 4% paraformaldehyde and processed for immunofluorescence as above. The number of spreading cells was counted (three separate experiments) and is expressed as a percentage of infected cells.

Cell Migration Assays

Differentiated mouse podocytes, having been infected with adenovirus 72 hrs earlier, were harvested by trypsin/EDTA treatment. Following trypsin inactivation with trypsin inhibitor (0.5 mg/mL), cells were collected by centrifugation and resuspended in RPMI + 10% FBS. Suspended cells were counted by hemacytometer and seeded onto the upper chamber of transwell inserts (5×10^4 cells/insert) pre-coated on the lower surface with collagen-I. After a 24 hour incubation at 38°C, cells on the upper surface were removed with a cotton-tip applicator and the migrating cells on the lower surface of the insert were washed with PBS, fixed with paraformaldehyde and stained with 4',6-

diamidino-2-phenylindole (DAPI). Membranes were removed from the inserts, placed on slides, and migrating cells were quantified by counting DAPI-stained nuclei. Data are from five experiments expressed as the percentage of control.

Quantification of Peripheral Projections in Cultured Podocytes

Differentiated podocytes grown on collagen-I-coated coverslips were infected with adenoviruses for wildtype or K256E α-actinin-4 and processed for immunofluorescence as described above (*Fluorescence Microsocopy*). Peripheral projections emanating from the cell body of HA-positive cells were quantified in a minimum of 50 cells from four experiments. Data are the mean number of projections per cell. Uninfected cells served as controls.

Statistics

Values reported are the means ± S.E.M. from at least three experiments. Statistical comparisons were made using unpaired t-test or 1 way analysis of variance (ANOVA) followed by the Newman-Keuls multiple comparison test.

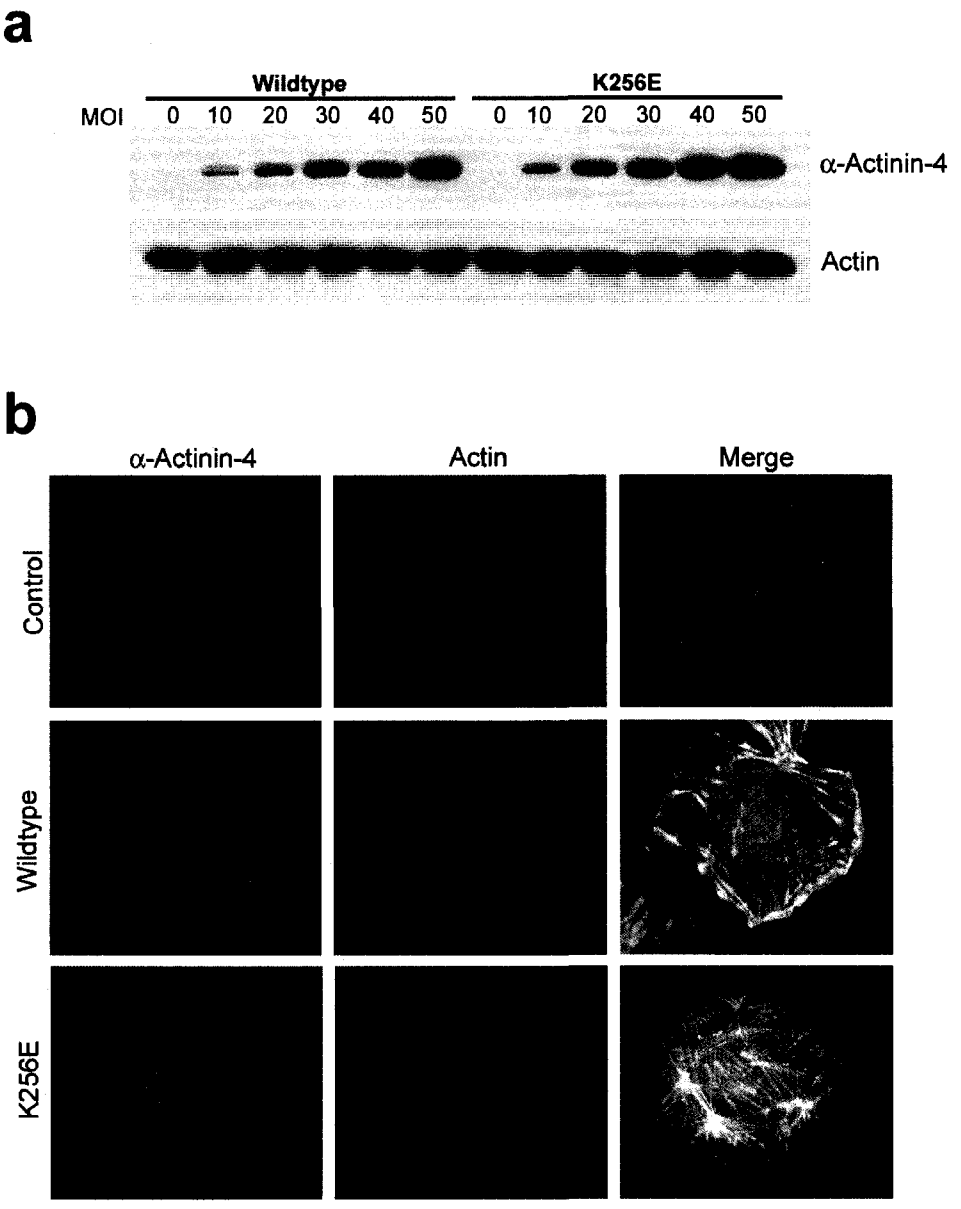
Results

Mislocalization of K256E α-Actinin-4 in Cultured Podocytes

Mutations in α-actinin-4 increase its affinity for F-actin *in vitro* (24, 26). However, the subcellular localization of mutant α-actinin-4 is not clearly defined. We therefore generated adenoviral constructs with HA-tagged wildtype or K256E α-actinin-4 and transduced a conditionally-immortalized mouse podocyte cell line. Podocytes were infected with a range of virus to determine a concentration yielding efficient expression (Figure 4.1a). For all subsequent experiments, infections were performed with a multiplicity of infection (MOI) of 25 and incubated for 72 hours. There was no apparent degradation of heterologously expressed K256E α-actinin-4 during this timeframe as its expression paralleled that of the wildtype protein (Figure 4.1a). As shown in Figure 4.1b, wildtype α-actinin-4 localized predominantly with cortical actin. The wildtype protein was also distributed along stress fibers and at focal adhesions, as identified by vinculin co-immunofluorescence (Figure 4.1c). Conversely, K256E α-actinin-4 was absent from the cell periphery, but was preferentially associated with stress fibers (Figure 4.1b) and focal adhesions (Figure 4.1c).

Differential Association of K256E α-Actinin-4 with Intracellular Actin Pools

We next performed cellular fractionation experiments to determine the association of wildtype and K256E α-actinin-4 with various intracellular actin pools. Podocytes expressing wildtype or K256E α-actinin-4 were lysed in Triton X-100-containing buffer and subject to differential centrifugation. As shown in Figures 4.2a and 4.2b, only 16.0 ±


a
Wildtype
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Wildtype
K256E

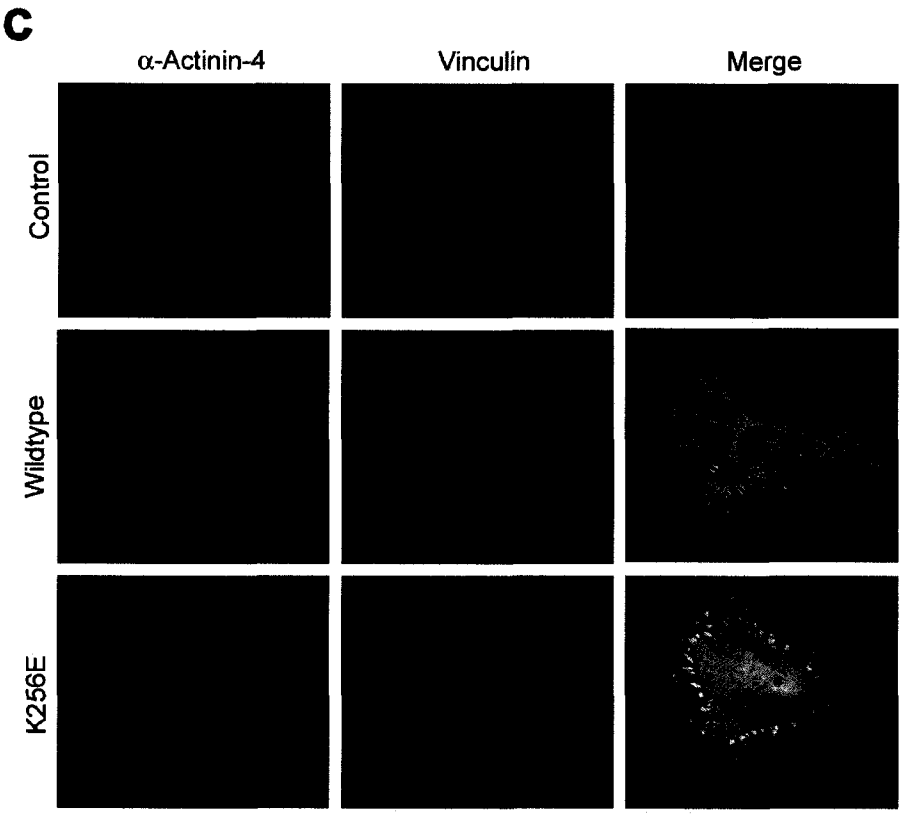

Figure 4.1 Subcellular localization of wildtype and K256E α-actinin-4 in mouse podocytes.
Differentiated podocytes were incubated with a range of adenoviral doses (MOI 0-50) for wildtype or K256E α-actinin-4 to determine optimal infection conditions. The expression achieved with both adenoviruses was similar for each condition (**a**). Three days following infection with viruses, differentiated podocytes were fixed and stained with an anti-HA tag antibody to detect α-actinin-4 and AlexaFluor 488-phalloidin to detect F-actin (**b**). Uninfected cells were treated in the same manner and serve as control. Wildtype α-actinin-4 is predominantly found with membrane-associated cortical actin and process-like projections with limited expression along stress fibers. Conversely, K256E α-actinin-4 is predominantly associated with stress fibers and is excluded from the cell periphery. Furthermore, both wildtype and K256E α-actinin-4 were colocalized with vinculin at focal adhesion complexes (**c**).

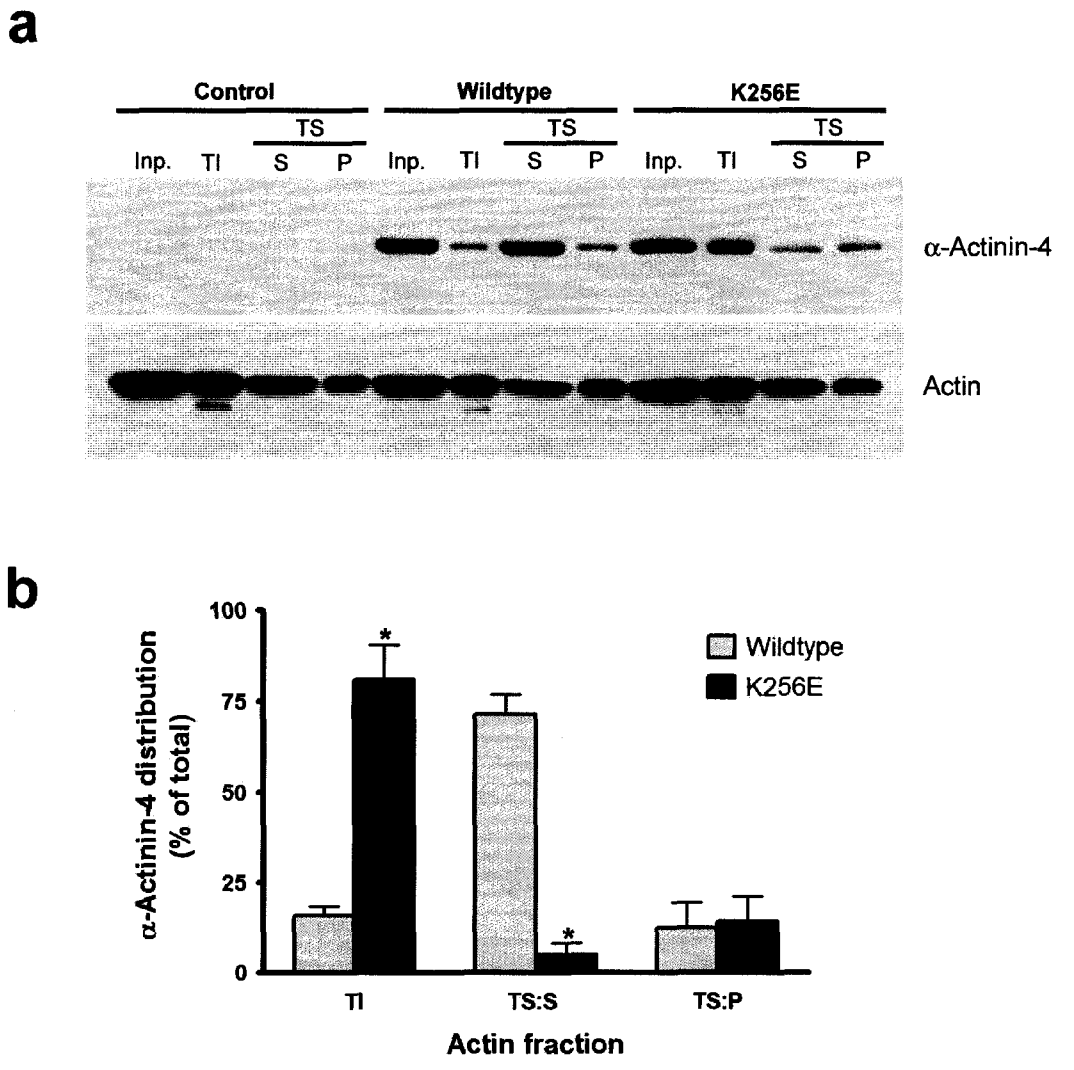

Figure 4.2 Distribution of wildtype and K256E α-actinin-4 among intracellular actin pools.

At three days post-infection, differentiated mouse podocytes were lysed in buffer containing 1 % Triton X-100 and centrifuged at 15,000 *x g* to pellet the Triton X-100 insoluble (TI) fraction containing large cytoskeletal structures such as actin bundles. The Triton X-100 soluble (TS) fraction was further centrifuged at 100,000 *x g* to separate G-actin (supernatant, S) from F-actin (pellet, P). Samples were then analyzed by Western blot using an anti-HA tag antibody and an anti-actin antibody. A representative blot **(a)** and the graphical representation **(b)** of three separate experiments illustrate the distribution of α-actinin-4 among the various actin pools. The majority (71.4 ± 5.5 %) of wildtype α-actinin-4 remained soluble (S, TS fraction) while the majority (81.0 ± 9.6 %) of K256E α-actinin-4 was associated with actin bundles (TI fraction). Uninfected cells were treated in the same manner and serve as control. Input lanes (Inp.) show similar expression levels of both wildtype and K256E α-actinin-4. *P<0.005 vs. wildtype, (n=3).

2.5 % of the wildtype α-actinin-4 associated with large cytoskeletal structures (Triton X-100 insoluble (TI) fraction), while 71.4 ± 5.5 % of the protein remained soluble (Triton X-100 soluble (TS):S fraction). Conversely, 81.0 ± 9.6 % of the K256E α-actinin-4 was associated with large cytoskeletal structures (TI fraction), and only 4.9 ± 3.4 % of the mutant protein remained soluble (TS:S fraction). Expression of both wildtype and K256E α-actinin-4 was similar, as evidenced by the input (Inp.). Furthermore, neither the expression of wildtype nor K256E α-actinin-4 altered total actin levels. These data reveal a differential association of wildtype versus K256E α-actinin-4, with K256E α-actinin-4 sequestered to large cytoskeletal structures such as actin bundles, while wildtype α-actinin-4 remains predominantly soluble.

Effect of K256E α-Actinin-4 Expression on Cell Adhesion, Spreading and Migration

The inappropriate association of K256E actinin-4 with the actin cytoskeleton suggested that it may negatively affect cytoskeletal dynamics. We therefore determined its effect on cytoskeletal-dependent processes such as cell adhesion, spreading and migration. Since α-actinin-4 is associated with focal adhesions, we hypothesized that the mutant protein may negatively affect the ability of cells to adhere to an extracellular matrix. Adhesion assays were performed using podocytes expressing GFP alone (control), wildtype α-actinin-4 or K256E α-actinin-4 and measured their ability to bind to collagen-I-coated wells (Figure 4.3). The number of adherent cells was quantified at various timepoints (3 to 24 hours). Irrespective of the time allowed for adhesion, there was no difference in adhesion between wildtype and K256E α-actinin-4 expressing podocytes, suggesting that cell-matrix interactions are not adversely affected by K256E α-actinin-4.

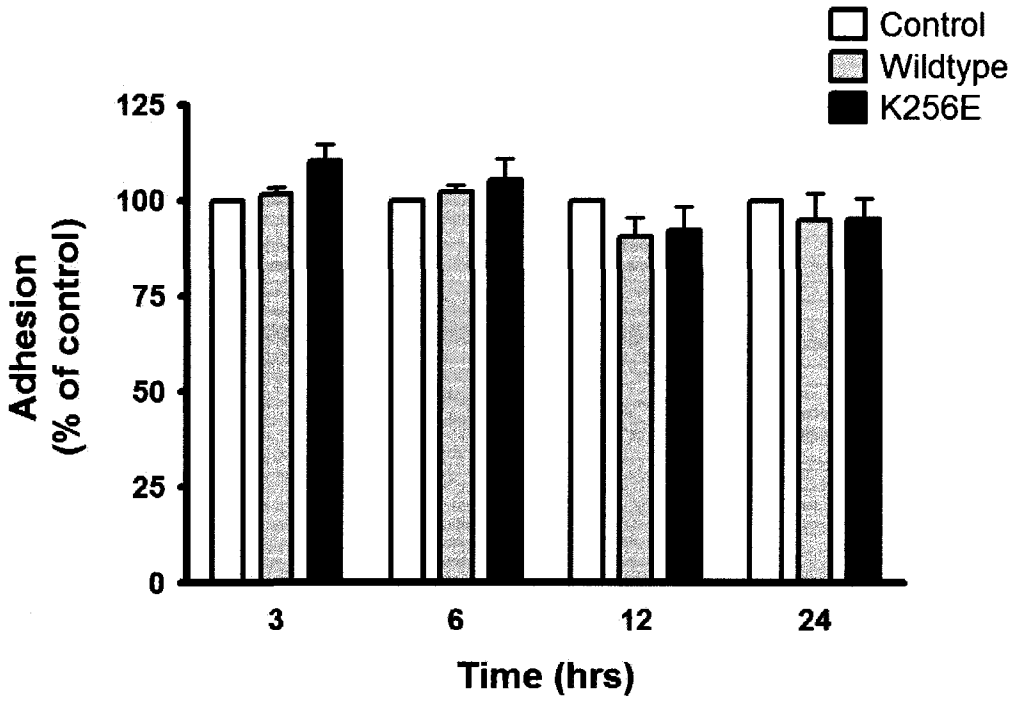

Figure 4.3 Effect of α-actinin-4 on podocyte adhesion.
Differentiated mouse podocytes expressing either GFP alone (control), wildtype or K256E α-actinin-4 were trypsinized and replated onto collagen-I-coated 96-well U-bottom plates at a density of $5x10^4$ cells/well. Cells were fixed with paraformaldehyde at various timepoints, stained with crystal violet, and the absorbance of each well measured at 595nm with a spectrophotometric plate reader. At every timepoint examined, there was no significant difference in the ability of wildtype or K256E α-actinin-4-expressing cells to adhere to an extracellular matrix. Data are from three separate experiments (n=3) and are expressed as a percent of control for each timepoint.

We next performed a replating assay to assess the ability of cells expressing either wildtype or K256E α-actinin-4 to efficiently spread on an extracellular matrix (collagen-I). Podocytes expressing GFP alone (control), wildtype or K256E α-actinin-4 were harvested and replated onto collagen-I-coated glass coverslips. Adherent cells were fixed after 3 or 6 hours and visualized by immunofluorescence. Within 3 hours of replating, a significant number of podocytes had adhered to the substratum and had begun to spread. For each condition, we observed no differences in the total number of podocytes adhering to the extracellular matrix. However, at both timepoints examined, the number of spreading podocytes was significantly lower in K256E actinin-4 expressing cells (13.0 ± 0.3 % at 3hrs; 17.3 ± 1.0 % at 6hrs) compared to control (29.2 ± 5.8 % at 3hrs; 37.7 ± 5.2 % at 6hrs) and wildtype (34.7 ± 4.4 % at 3hrs; 38.4 ± 5.8 % at 6hrs) (Figure 4.4a). Within 6 hours of replating, wildtype α-actinin-4 was localized with cortical actin at the cell periphery (Figure 4.4b). Conversely, K256E α-actinin-4 remained associated with F-actin and condensed in the cell body (Figure 4.4b), consistent with the observed impairment in cell spreading.

Cell migration relies upon a dynamic cytoskeleton. The increased affinity of K256E α-actinin-4 for F-actin could undermine this process. We therefore performed haptotactic transwell migration assays to determine the effect of K256E α-actinin-4 on cell migration. Podocytes expressing GFP alone (control), wildtype or K256E α-actinin-4 were plated onto the upper surface of transwell inserts. After 24 hours, the cells remaining in the upper chamber were removed and the cells that had migrated to the underside of the insert were quantified. Podocytes expressing wildtype α-actinin-4 migrated at a similar rate (90.8 ± 9.2 %) to control cells (Figure 4.5). Conversely, the migration of podocytes expressing K256E α-actinin-4 was significantly reduced (52.1 ±

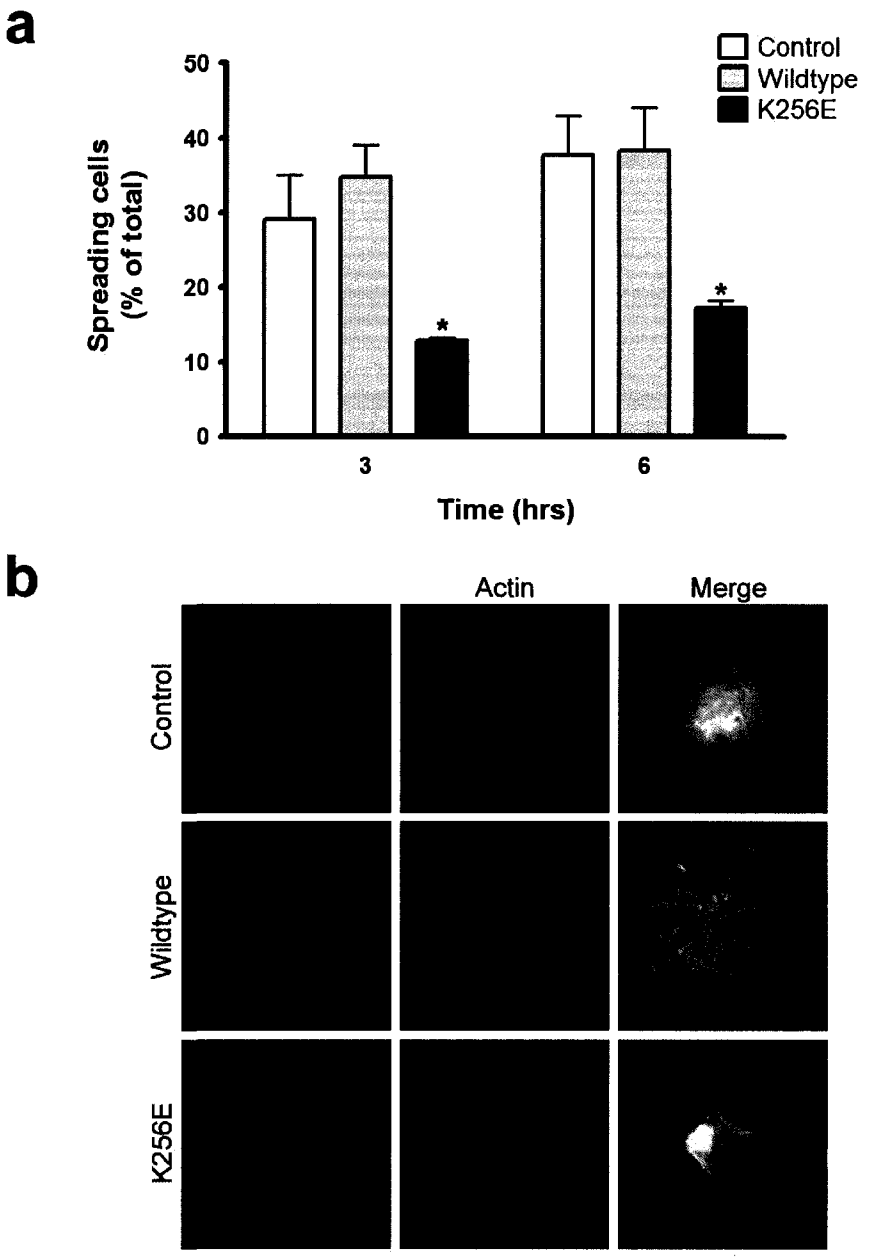

Figure 4.4 Effect of α-actinin-4 on podocyte spreading.
Differentiated mouse podocytes expressing GFP alone (control), wildtype or K256E α-actinin-4 were trypsinized, held in suspension for 15 minutes, then replated onto collagen-I-coated coverslips and analyzed by immunofluorescence at 3 and 6 hours. Expression of K256E α-actinin-4 significantly reduced the number of spreading cells at each timepoint examined (**a**). After 6 hours, wildtype α-actinin-4 was localized to cortical actin, while K256E α-actinin-4 remained condensed within the cell (**b**). *P<0.05 vs. control and wildtype of corresponding timepoint, (n=3).

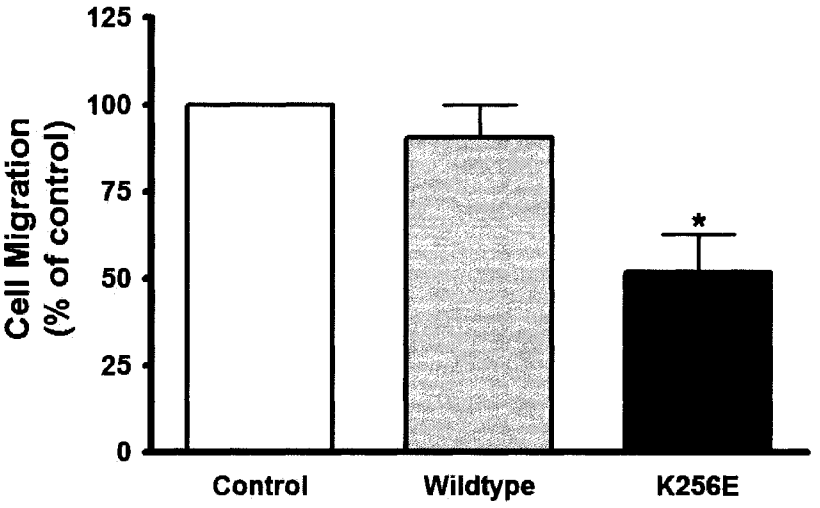

Figure 4.5 Effect of α-actinin-4 on podocyte migration.
Differentiated mouse podocytes expressing GFP alone (control), wildtype or K256E α-actinin-4 were trypsinized and replated onto the upper surface of transwell inserts. After 3 hours, the cells remaining in the upper chamber were removed and the cells on the lower surface were stained with DAPI and counted. K256E α-actinin-4 caused a 48% reduction in the number of migrating cells compared to control and wildtype. *P<0.01 vs. control and wildtype, (n=5).

10.7 %; P<0.01 vs control and wildtype). These data suggest that K256E α-actinin-4 causes defects in cytoskeletal dynamics and impairs cellular processes such as spreading and migration.

Quantification of Peripheral Projections in Cultured Podocytes

Podocyte-specific expression of K256E α-actinin-4 leads to podocyte damage and foot process effacement *in vivo* (26). The conditionally immortalized podocyte cell line used in the present studies have been shown to form foot process-like peripheral projections when cultured under non-permissive conditions (29). We therefore assessed the effect of K256E α-actinin-4 on peripheral projections in differentiated podocytes (Figure 4.6). Podocytes expressing wildtype α-actinin-4 displayed a slight increase in the mean number of projections (3.8 ± 0.8 projections/cell vs. 2.9 ± 0.6 for control), whereas podocytes expressing K256E α-actinin-4 exhibited a decrease in the mean number of projections (1.9 ± 0.4) (Figure 4.6b). Wildtype, but not K256E α-actinin-4, was readily detected in the peripheral projections, along with actin (Figure 4.6a). Furthermore, projections emerging from podocytes expressing wildtype α-actinin-4 appeared longer and thinner than those of control and K256E α-actinin-4-expressing podocytes. These data provide evidence that α-actinin-4 plays a key role in the formation and/or maintenance of peripheral projections in cultured podocytes.

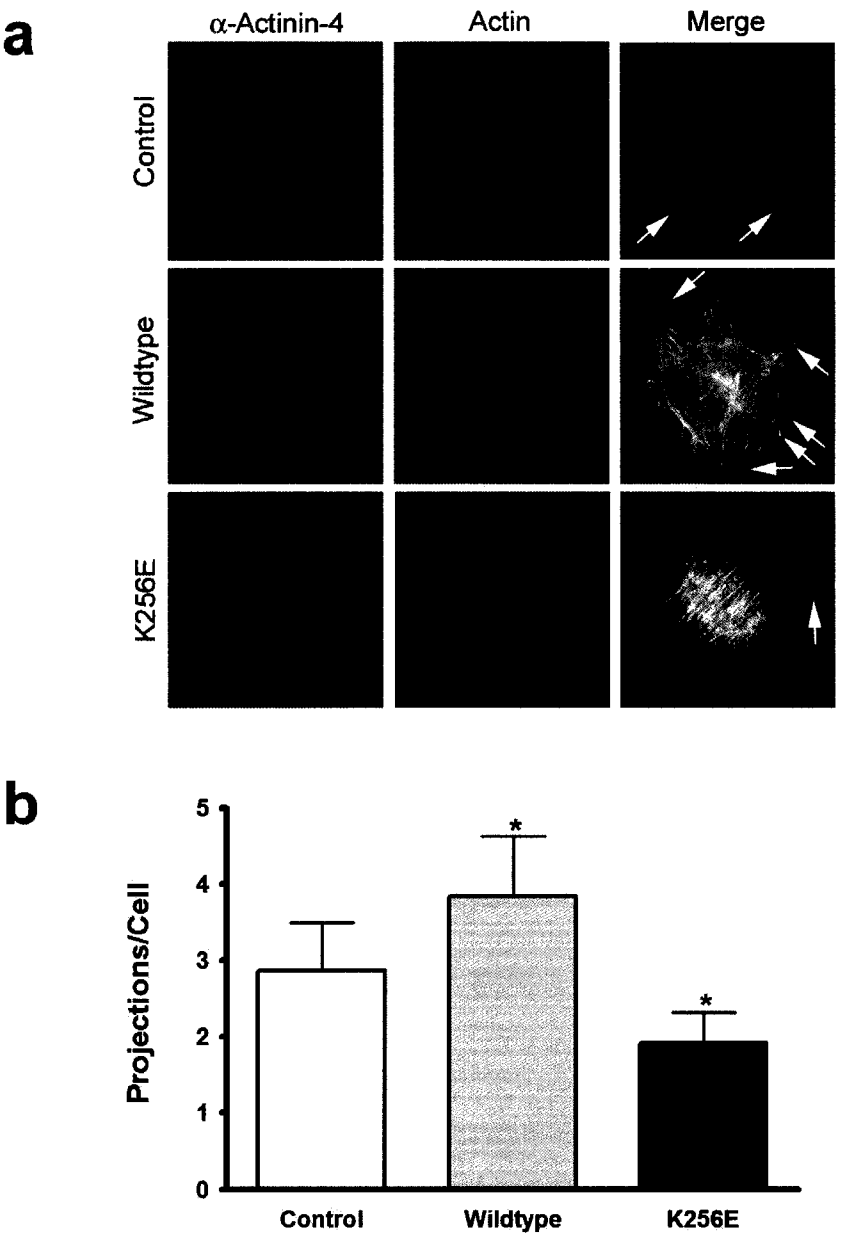

Figure 4.6 Effect of α-actinin-4 on peripheral projections of podocytes.
Three days following infection with viruses for wildtype or K256E α-actinin-4, differentiated podocytes were fixed and stained with an anti-HA tag antibody to detect α-actinin-4 and AlexaFluor 488-phalloidin to detect F-actin. Images in **(a)** are representative of cells for each condition and demonstrate actin-rich projections (arrows) emanating from the cell body. **(b)** Expression of wildtype α-actinin-4 caused an increase in the mean number of projections (3.8 ± 0.8 projections/cell vs. 2.9 ± 0.6 for control), while expression of K256E α-actinin-4 caused a decrease in number of projections (1.9 ± 0.4). *P<0.05 vs. control, (n=4).

Discussion

We previously developed a mouse model of an inherited form of FSGS by expressing a mutant variant of α-actinin-4 (K256E) under the control of a podocyte-specific promoter (*mNPHS1*) (26). In this model, approximately 50 % of mice develop FSGS-like lesions and display podocyte foot process effacement. However, the direct consequences of mutant α-actinin-4 on podocyte structure and function remained unclear. We therefore assessed the effects of K256E α-actinin-4 on the cytoskeletal dynamics of cultured podocytes.

The conditionally-immortalized podocyte cell line used in this study has previously been described in detail (29). These cells are relatively resistant to conventional transfection approaches for achieving heterologous expression of proteins. We therefore developed adenoviruses for both wildtype and K256E α-actinin-4 which yielded significant expression in a high proportion of cells (90%), rendering the cell population much more homogenous than previously attainable. The expression level of both wildtype and mutant K256E α-actinin-4 were similar (Figure 4.1a), suggesting that the mutant protein is not subject to degradation in these cells.

The most impressive feature upon expression of K256E α-actinin-4 is its distinct intracellular localization. Although the putative function of α-actinin-4 is that of an actin crosslinking protein, we found that the majority of wildtype α-actinin-4 colocalized with membrane-associated cortical actin (Figure 4.1b) or was detected in the Triton X-100 soluble pool (Figure 4.2). In contrast, K256E α-actinin-4 was consistently found along stress fibers and was retained in the Triton X-100 insoluble fraction (Figure 4.2). This sequestration is likely a direct effect of the increased affinity of K256E α-actinin-4 for F-

actin, and is consistent with a gain-of-function effect of such mutations. In support of this, an alternative splice variant of α-actinin-4 has been reported in small cell lung cancer (30). The splice variant contains 3 missense mutations in exon 8, the region containing FSGS-associated mutations. This mutant isoform also displays increased affinity for actin, and was found to colocalize mainly with actin stress fibers, unlike the wildtype protein which was concentrated along the periphery of the cell.

Several reports have identified an association of actinin isoforms with components of focal adhesion complexes, such as the β_1-integrin subunit and vinculin (13). Phosphoinositides may also bind to α-actinin and regulate its interaction with actin filaments or integrin receptors (21, 22). Furthermore, α-actinin is phosphorylated by focal adhesion kinase (FAK) in platelets (20), reducing its cosedimentation with actin filaments. These findings clearly indicate a role for α-actinin in mediating signals that could modulate the assembly of focal adhesions or the cytoskeleton. It is therefore interesting to speculate that mutations in α-actinin-4, which increase its affinity for actin, may render the protein insensitive to these factors and thereby perturb cytoskeletal dynamics.

The mislocalization of mutant α-actinin-4 led us to investigate its effects on cytoskeletal-dependent processes such as cell adhesion, spreading and migration. Although we did not observe a significant impact of K256E α-actinin-4 on podocyte adhesion (i.e., adhesion and replating assays), we found that it significantly affected spreading and migration. Indeed, podocytes expressing K256E α-actinin-4 remained rounded and showed severe defects in their ability to spread on collagen-I. This phenotype is reminiscent of that reported in FAK-deficient embryonic mesodermal cells

(31), which can adhere but fail to spread effectively on ECM, suggesting that FAK signaling may be impaired in cells expressing mutant α-actinin-4. Furthermore, as indicated by the present study, podocytes expressing K256E α-actinin-4 exhibit severe motility defects. These findings are in accordance with a role of actinin-4 in cell motility and cancer invasion (32-34), where cytoplasmic localization of α-actinin-4 is associated with an infiltrative phenotype in breast cancer cells, whereas cells with nuclear α-actinin-4 are non-invasive. Our results show that α-actinin-4 is significantly involved in cell spreading and migration. The motility deficit seen in cells expressing K256E α-actinin-4 is likely due to the sequestering of the protein to centrally located actin stress fibers, and away from motility based structures at the cell's periphery.

Expression of wildtype α-actinin-4 increased the mean number of actin filament-containing peripheral projections emanating from the cell body, reminiscent of podocyte foot processes *in vivo*. In contrast, K256E α-actinin-4 reduced the mean number of such projections (Figure 4.6). Although the arborized phenotype observed in cultured podocytes is not nearly as extensive as that seen in podocytes *in vivo* (35), our data suggests that due to mislocalization, K256E α-actinin-4 does not provide a suitable framework for the maintenance/formation of such projections. We previously showed that podocyte-specific expression of K256E α-actinin-4 leads to foot process effacement and glomerulosclerosis. Such damage may be explained by mislocalization of mutant α-actinin-4, rendering it unresponsive to appropriate signals (e.g., actin remodeling) or unable to provide structural support at the cell membrane, and thereby contribute to foot process effacement.

A recent study by Yao et al. attributes podocyte defects encountered in *ACTN4*-associated FSGS to a loss-of-function since mutations render α-actinin-4 more susceptible

to forming unstable aggregates which are rapidly degraded via the proteosome pathway (28). Conversely, in transgenic mice expressing an FSGS-associated mutant α-actinin-4, podocyte damage and sclerosis correlate directly with mutant transgene expression, consistent with a gain-of-function mechanism (26). We did not observe any degradation of exogenous K256E α-actinin-4 in cultured podocytes (Figure 4.1a) within the experimental timeframe. In light of these findings, we favor a synthesis of the two models to explain the dysregulated phenotype of podocytes expressing FSGS-associated mutant α-actinin-4. Gain-of-affinity mutations in α-actinin-4, coupled with some degradation of the mutant protein, could each contribute to a loss-of-function by effectively eliminating this protein from select intracellular locales (e.g., motility-based actin structures and process-like peripheral projections), thereby disturbing cytoskeletal-dependent processes such as cell spreading, migration and importantly, process maintenance/formation. In support of this hypothesis, dysregulation of the actin cytoskeleton and α-actinin-4 expression/localization has been reported in various glomerular disease models characterized by podocyte foot process effacement (6, 36-38), and is therefore likely to be a key event in the progression of podocyte injury.

In summary, we have further defined the functional consequences of an FSGS-associated form of the actin-crosslinking protein – K256E α-actinin-4. Whereas wildtype α-actinin-4 is predominantly localized to membrane-associated cortical actin in conditionally-immortalized podocytes, K256E α-actinin-4 remains tightly associated with stress fibers. Mislocalization of K256E α-actinin-4 causes severe defects in motility including cell spreading and migration. Furthermore, K256E α-actinin-4 caused a decrease in the number of foot process-like peripheral projections. Our data therefore

suggest that dysregulation of the podocyte cytoskeleton may play a key role in the progression of sclerotic lesions resulting from various podocyte injuries.

Acknowledgements

We are grateful to Peter Mundel (Albert Einstein, New York) for the conditionally-immortalized podocyte cell line. Robin J. Parks is supported by the Canadian Institutes of Health Research (CIHR), CIHR/Muscular Dystrophy Association of Canada/Amyotrophic Lateral Sclerosis Society of Canada Partnership Grant, Premier's Research Excellence Award, and the Jesse Davidson Foundation for Gene and Cell Therapy. C.R.J. Kennedy and R.J. Parks are CIHR New Investigator Awardees. Jean-Louis R. Michaud is a recipient of a CIHR Doctoral Award. This work is supported by an operating grant from the Kidney Foundation of Canada.

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Chapter 5

Modulating α-actinin-4 dynamics in podocytes

Modulating α-actinin-4 dynamics in podocytes

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Running title: Regulation of α-actinin-4 dynamics

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Description and author contribution

This manuscript explores the potential regulatory role of both calcium and phosphoinositides on α-actinin-4 function. FSGS-associated mutations (K256E) not only increase the affinity of α-actinin-4 for actin but also render the protein insensitive to such regulatory elements. These changes severly impair cytoskeletal remodeling following mechanically-induced stretch, a mimic of the intraglomerular forces exerted on podocytes in their natural environment.

All the experiments presented in this manuscript were performed by myself, with guidance from Chris Kennedy. Mona Hosseini-Abardeh performed preliminary studies on cytoskeletal changes induced by mechanical stimulation in the presence of wildtype and K256E α-actinin-4. Kevin Farah worked in the laboratory as a summer student and participated in the quantification of cytoskeletal changes following mechanical stretch. Writing of the manuscript was performed by myself, with subsequent editing by Chris Kennedy prior to submission.

Abstract

Podocytes are equipped with a dynamic cytoskeleton allowing them to contend with intraglomerular forces. FSGS-associated mutations in α-actinin-4, an actin bundling protein, increase its affinity for actin and hinder cytoskeletal dynamics. We hypothesized that this gain of affinity would override its sensitivity to regulatory factors such as calcium, acting via two EF hand motifs, and/or phosphoinositides (PI). We therefore generated phosphoinositide (mutPI)- and calcium (mutEF)-insensitive variants of α-actinin-4 and compared their properties to wildtype (wt) α-actinin-4 and a disease-associated mutant (K256E). α-Actinin-4^{mutPI} displayed increased affinity for actin, while the affinity of α-actinin-4^{mutEF} was unchanged. In actin sedimentation assays, calcium decreased, while PI increased α-actinin-4/actin association. Similar to α-actinin-4^{K256E}, α-actinin-4^{mutPI} was mislocalized in cultured podocytes, being sequestered mostly to actin stress fibers and focal adhesions. The turnover of α-actinin-4^{wt} and α-actinin-4^{mutEF} along stress fibers and focal adhesions was rapid, while the turnover of α-actinin-4^{K256E} and α-actinin-4^{mutPI} was reduced. Equibiaxial mechanical stretch, a mimic of intraglomerular forces, reduced podocyte surface area by 50%, but the decrease was more severe (~70%) in the presence of high-affinity mutants of α-actinin-4. These data suggest that dynamic regulation of α-actinin-4/actin interactions by calcium and/or PI may be necessary for maintaining podocyte structure in response to glomerular hydrostatic forces.

Key words:

FSGS, podocyte, cytoskeleton, α-actinin-4, mechanical stretch

Introduction

Podocytes are epithelial cells that line the outer aspect of the blood vessels within the kidney's filtering units (glomeruli). Podocytes are terminally-differentiated cells with a unique morphology; primary processes emanating from the cell body give rise to small interdigitating foot processes, upon which a molecular sieve is formed [1]. This molecular sieve, termed the slit diaphragm, is comprised of various proteins, including nephrin, podocin, CD2AP, Neph1, P-cadherin, FAT 1/2, TRPC-6 and others. Many slit diaphragm components are anchored to the podocyte cytoskeleton, a key player in maintaining the cell's peculiar shape [1]. Podocyte injury due to intrinsic or extrinsic factors disrupts podocyte architecture with ensuing loss of filtration barrier integrity, podocyte detachment and loss which lead invariably to progressive renal disease.

Mutations in the *ACTN4* gene are responsible for an autosomal dominant form of focal segmental glomerulosclerosis (FSGS), a renal disorder characterized by podocyte foot process effacement and proteinuria [2]. The product of the *ACTN4* gene, α-actinin-4, is highly expressed in healthy podocytes. Loss of α-actinin-4 or overexpression of a high-affinity mutant (K256E) both lead to FSGS in mice [3, 4]. Furthermore, we previously showed that murine α-actinin-4^{K256E} is sequestered solely to actin stress fibers and focal adhesion in cultured podocytes [5]. This localization differs substantially from that of wildtype α-actinin-4 which is found predominantly at the cell periphery. The mislocalization of α-actinin-4^{K256E} significantly impedes the podocyte's ability to spread and migrate on an extra-cellular matrix, highlighting the importance of the podocyte cytoskeleton.

α-Actinin-4, a member of the spectrin superfamily, exists as a head-to-tail homodimer and is thought to crosslink and bundle actin filaments via its N-terminal actin binding domain (Figure 1). The central spectrin repeats are involved in dimer formation and serve as a docking platform for numerous molecular partners [6]. α-Actinin-4 also contains two calcium-sensing EF hand motifs at its C-terminal end. Binding of calcium to the EF-hand motifs of α-actinin-1, another non-muscle isoform of α-actinin, caused a decrease in its association with filamentous actin (F-actin) [7]. The actin binding domain of α-actinin-4 consists of two calponin homology (CH) domains, the first (CH1) being critical for binding to actin, whereas the second (CH2) may play a modulatory role. In support of this, a phosphoinositide-binding site has been mapped to the CH2 domain of α-actinin-4 [8]. Binding of phosphoinositides (PI(4,5)-P_2 and PI(3,4,5)-P_3) to α-actinin-1 inhibit bundling by blocking the binding of α-actinin to F-actin, thereby modulating cytoskeletal remodeling [9, 10].

In the present study, we examined the role of α-actinin-4's regulatory domains both *in vitro* and in cultured podocytes. We report that both calcium and phosphoinositides modulate α-actinin-4 / F-actin interactions. High-affinity variants of α-actinin-4 lead to a dysregulated cytoskeleton, rendering podocytes unable to cope with hydrodynamic pulsatile pressures exerted within the glomerulus.

Materials and methods

Subcloning, protein purification and adenovirus production

Constructs containing hemagglutinin (HA)-tagged wildtype (wt) and K256E α-actinin-4 in pcDNA3.1 were described previously [5]. A calcium-insensitive variant (α-actinin-4^{mutEF}) was obtained by mutating key residues within both EF-hand motifs (EF1: D779A/D781A/G783A, EF2: D820A/N822A/S824A) using PCR-based mutagenesis (Figure 1). These mutations have been shown to abolish calcium sensitivity in the dictyostelium 34kDa protein [11, 12]. A phosphoinositide-insensitive variant (α-actinin-4^{mutPI}) was also obtained by mutating key residues (K182I/H190L/K194I) within the phosphoinositide binding site (Figure 1), as previously described for α-actinin-1 [9]. All mutations were confirmed by nucleotide sequencing.

α-Actinin-4 constructs were then subcloned into pGEX-4T-3 (Amersham Biosciences) to obtain glutathione S-transferase (GST) fusion constructs. The GST-fusion proteins were expressed in BL21 bacteria (Invitrogen), purified using Glutathione-sepharose 4B (Amersham Biosciences) and eluted with reduced glutathione, according to the manufacturer's instructions.

Variants of α-actinin-4 were also subcloned into the pEGFP-N2 plasmid (Clontech) to obtain green fluorescent protein (GFP) fusion constructs. α-Actinin-4 in these constructs contains an N-terminal HA-tag and a C-terminal GFP-tag. For adenovirus production, GFP-tagged variants of α-actinin-4 were subcloned into pShuttle-CMV (Stratagene). This shuttle vector undergoes homologous recombination in bacteria with pAdEasy-1 to constitute the recombinant adenoviral plasmid. Linearized

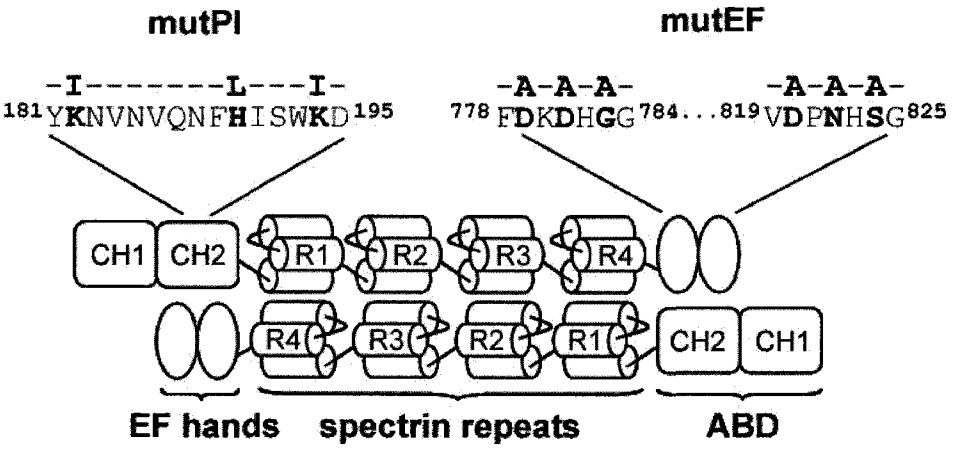


Figure 5.1 Diagram of α-actinin-4 structure.
α-Actinin-4 exists as a head-to-tail homodimer and crosslinks actin filaments via its N-terminal actin binding domain (ABD), which consists of two calponin homology (CH) domains. The presence of a phosphoinositide binding site (within CH2) and two EF-hand motifs suggest that α-actinin-4/actin interactions may be modulated by phosphoinositides and/or calcium. Phosphoinositide- and calcium-insensitive variants (mutPI and mutEF, respectively) were generated by introducing point mutations within the regulatory domains as indicated.

recombinant adenoviral plasmid was transfected into AD-293 cells to obtain a primary viral stock, which was amplified, purified and titered according to the manufacturer's recommendations.

In vitro actin sedimentation assay

The binding of GST-α-actinin-4 variants to filamentous actin (F-actin) was assessed by actin sedimentation assay as previously described [9]. Briefly, GST-α-actinin-4 was added to freshly polymerized actin (Cytoskeleton), incubated at room temperature for 30 minutes and centrifuged at 100 000 x *g* for 30 minutes at 18°C. The supernatant and pellet were separated and analyzed by SDS-PAGE. Proteins in the gel were stained with Coomassie brilliant blue for 1 hour and the gels destained overnight. Imaging was performed using an AlphaImager gel documentation workstation and densitometry analyzed with the accompanying AlphaEase FC Software Version 4.1.0 (Alpha Innotech Corporation). To determine the effect of calcium on α-actinin-4/actin interactions, actin sedimentation assays were performed in the presence of increasing levels of $CaCl_2$ (final concentration, 0, 0.1, 1.0 and 10 mM). To determine the effect of phosphoinositides on α-actinin-4/actin interactions, actin sedimentation assays were performed in the absence or presence of either 50 μM $PI(4,5)P_2$ or $PI(3,4,5)P_3$ (diC16, Echelon Biosciences Inc.).

Cell culture and fluorescence microscopy

Conditionally-immortalized mouse podocytes were cultured as previously described [13]. Podocytes were differentiated for a minimum of 10 days, infected with

appropriate adenoviruses, and cultured for another 3 days. Cells used for fluorescence microscopy were fixed in 4% paraformaldehyde, permeabilized with PBS containing 0.2% Triton X-100 and incubated with Alexa fluor 594-phalloidin (Molecular Probes/Invitrogen) to visualize the actin cytoskeleton. Cells were observed with a Zeiss Axioskop 2 MOT microscope and images captured with a Zeiss AxioCam (Zeiss, Germany). Image cropping and processing was performed using Adobe Photoshop 5.5.

Subcellular fractionation

Differentiated podocytes previously infected with appropriate adenoviruses were scraped into ice-cold lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.5% Nonidet P-40) supplemented with protease inhibitor cocktail (Sigma) and PMSF. Cell lysates were centrifuged at 10 000 x *g* for 15 minutes to isolate actin bundles (Triton X-100 insoluble (TI) fraction). The Triton X-100 soluble (TS) fraction was removed and further centrifuged at 100 000 x *g* for 30 minutes to separate unbundled F-actin (pellet, P) from soluble globular actin (supernatant, S). Equal volumes (5 μg total protein in lysate) were separated by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with anti-HA (clone HA-7, Sigma) or anti-actin (Sigma) antibodies. The membranes were developed for chemiluminescence (Pierce) and exposed to film. Imaging and densitometric analysis was performed using an AlphaImager workstation as described above.

Fluorescence recovery after photobleaching (FRAP)

A7r5 cells were cultured on glass-bottom Delta T dishes (Bioptechs Inc.) and infected with appropriate adenoviruses. Cells were visualized with a Zeiss LSM 5

PASCAL microscope (Zeiss, Germany). Three images were taken prior to photobleaching to calculate the initial fluorescence intensity. Fluorescence in the marked area was bleached by 5 rapid iterations of a 488-nm argon laser at full intensity. Images were subsequently captured at 1% laser intensity at 2 second intervals. Recovery of fluorescence within the bleached area was calculated as previously described following $I_{rel} = (I_{(t)}/I_{(0)}) * (T_{(0)}/T_{(t)})$, where $I_{(t)}$ is the intensity in the bleached area at time t, $I_{(0)}$ is the intensity of the bleached area before bleaching, $T_{(t)}$ is the total cellular intensity at time t, and $T_{(0)}$ is the total cellular intensity before bleaching [14]. Average pixel intensities were determined using Image J software (National Institutes of Health, Bethesda, Maryland, USA) and normalized for background fluorescence. A minimum of 5 separate cells were used for quantification.

Mechanical stretch

Podocytes were cultured on collagen-I-coated BioFlex plates (Flexcell, International) and subjected to mechanical stretch using a Flexercell FX-4000T instrument. Parameters of the stretch experiments were as follows: 10 % biaxial strain (sinusoidal) at a frequency of 0,5 Hz for 24 hours. Control cells were cultured under identical conditions but were not exposed to stretch. Following stretch, cells were fixed with 4% paraformaldehyde and processed for immunofluorescence as described above. Cell surface area was determined by tracing the perimeter of the cells using Image J software and expressed as μm^2.

Statistical analysis

Values reported are the means ± S.E.M. from at least three experiments. Statistical comparisons were made using t test or one way analysis of variance (ANOVA) followed by the Newman-Keuls multiple comparison test, where appropriate.

Results

Calcium and phosphoinositides modulate binding of α-actinin-4 to actin in vitro

In order to characterize the functional role of α-actinin-4's regulatory domains, we generated calcium- and phosphoinositide-insensitive variants (Figure 1). Mutations within the EF-hand motifs of the dictyostelium 34kDa protein, an actin-bundling protein, abolish its calcium sensitivity [11, 12]. The calcium-insensitive variant (α-actinin-4^{mutEF}) was obtained by introducing analogous mutations in full-length α-actinin-4 by PCR-based mutagenesis. A phosphoinositide-insensitive variant of α-actinin-1, a non-muscle isoform of α-actinin with approximately 90% homology to α-actinin-4, has previously been described [9]. The phosphoinositide-insensitive variant (α-actinin-4^{mutPI}) was obtained by introducing analogous mutations in a similar fashion. The ability of α-actinin-4 variants to bind to F-actin was assessed by *in vitro* actin sedimentation assays using purified GST-α-actinin-4-fusion proteins. As shown in Figure 2, α-actinin-4^{wt} is predominantly soluble, while the high-affinity variant α-actinin-4^{K256E} displays increased binding to actin. Mutations in the EF hand motifs of α-actinin-4 did not modify its affinity for actin, as the distribution of α-actinin-4^{mutEF} mimics that of α-actinin-4^{wt}. Conversely, mutations in the phosphoinositide binding site of α-actinin-4 caused a significant increase in its affinity for actin. Indeed, the distribution of α-actinin-4^{mutPI} is very similar to that of α-actinin-4^{K256E}. GST-alone served as control, and did not associate with F-actin.

To examine the extent to which α-actinin-4/actin interactions can be modulated, we performed *in vitro* actin sedimentation assays in the presence of calcium and phosphoinositides. Under baseline conditions, just over 40% of α-actinin-4^{wt} is

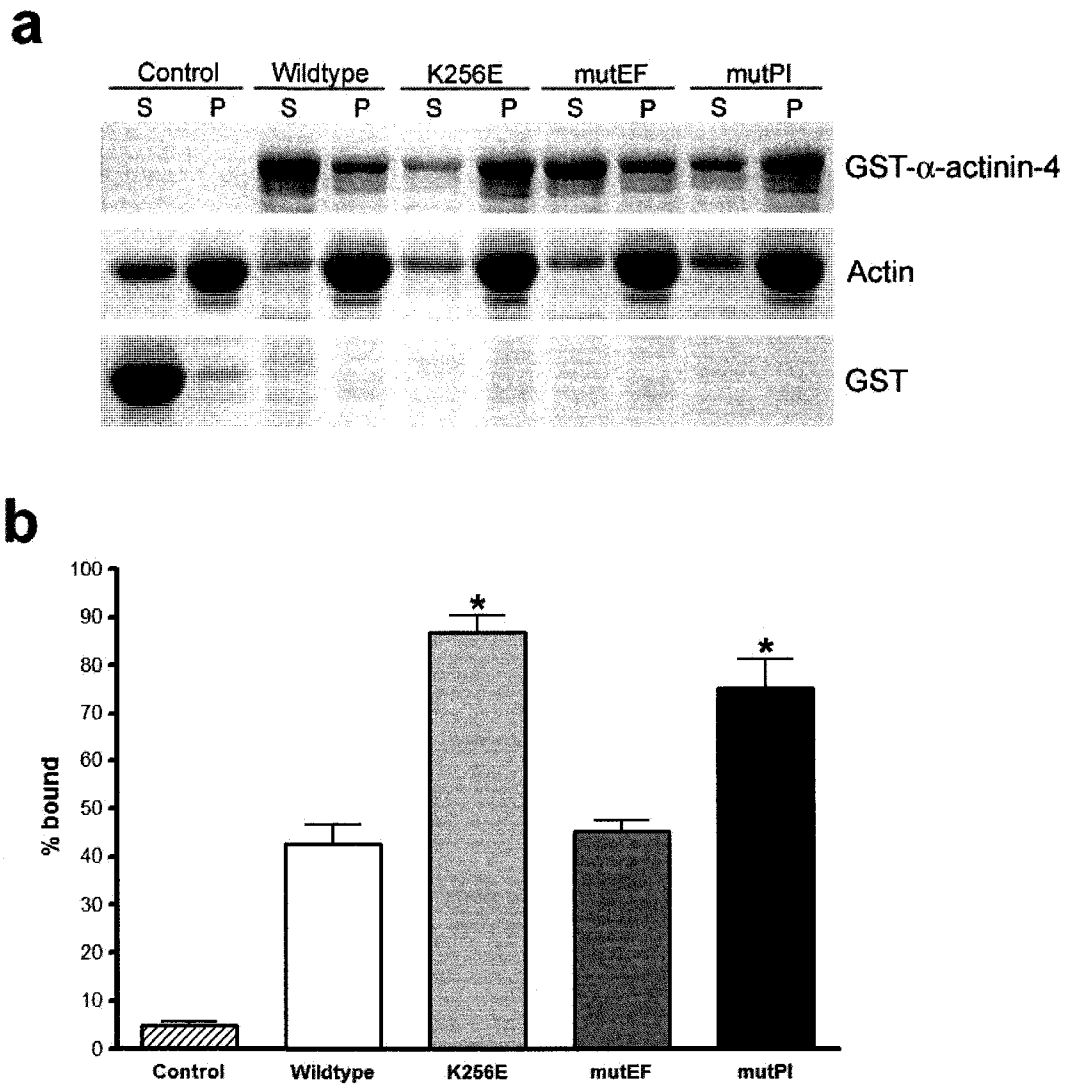


Figure 5.2 Mutations in α-actinin-4 alter its binding to actin.
(a) GST-α-actinin-4 variants were purified, incubated with polymerized actin and centrifuged at 100 000 x *g* for 30 minutes to isolate F-actin and bound α-actinin-4. Supernatants (S) and resuspended pellets (P) were analyzed by SDS-PAGE and the gels stained with Coomassie Blue. **(b)** The percentage of α-actinin-4 detected in the pellet (% bound) was determined by densitometry. GST alone did not associate with F-actin and served as control. * $P < 0.001$, $n = 3\text{-}7$.

associated with F-actin. As shown in Figure 3a, increasing calcium concentrations displaced α-actinin-4^{wt} from the pellet fraction (F-actin) to the soluble fraction. α-Actinin-4^{K256E} was insensitive to such regulation, as it remained exclusively associated with F-actin. As expected, α-actinin-4^{mutEF} was insensitive to regulation by calcium. Although not statistically significant, α-actinin-4^{mutPI} retained minimal calcium sensitivity *in vitro*, despite its increased affinity for F-actin. In contrast to the effect of calcium, phosphoinositides unexpectedly caused a general increase in the association of α-actinin-4 with F-actin (Figure 3b and c). $PI(3,4,5)P_3$ displayed a greater modulatory effect compared to $PI(4,5)P_2$, especially for α-actinin-4^{wt}. α-Actinin-4^{K256E} was insensitive to phosphoinositides, possibly owing to its increased baseline affinity for actin. Taken together, these results demonstrate that both calcium and phosphoinositides can modulate α-actinin-4/actin interactions. Such modulation may allow actin bundle assembly/disassembly and likely plays an important role in cytoskeletal remodeling.

Localization of α-actinin-4 variants in cultured podocytes

We previously reported that a gain-of-affinity mutation in murine α-actinin-4 (K256E) sequesters the protein to actin stress fibers and focal adhesions in cultured podocytes [5]. Although α-actinin-4 is described as an actin-bundling protein, we observed the majority of α-actinin-4^{wt} at the cell periphery and within membrane ruffles in cultured podocytes. We hypothesized that the increased affinity of α-actinin-4^{mutPI} would alter its subcellular localization, similar to α-actinin-4^{K256E}. Since cultured podocytes are relatively resistant to common transfection methods, we developed adenoviruses encoding GFP-tagged α-actinin-4 variants. Adenoviral infection allows for controlled expression in a high proportion of cultured podocytes. As shown in Figure 4,

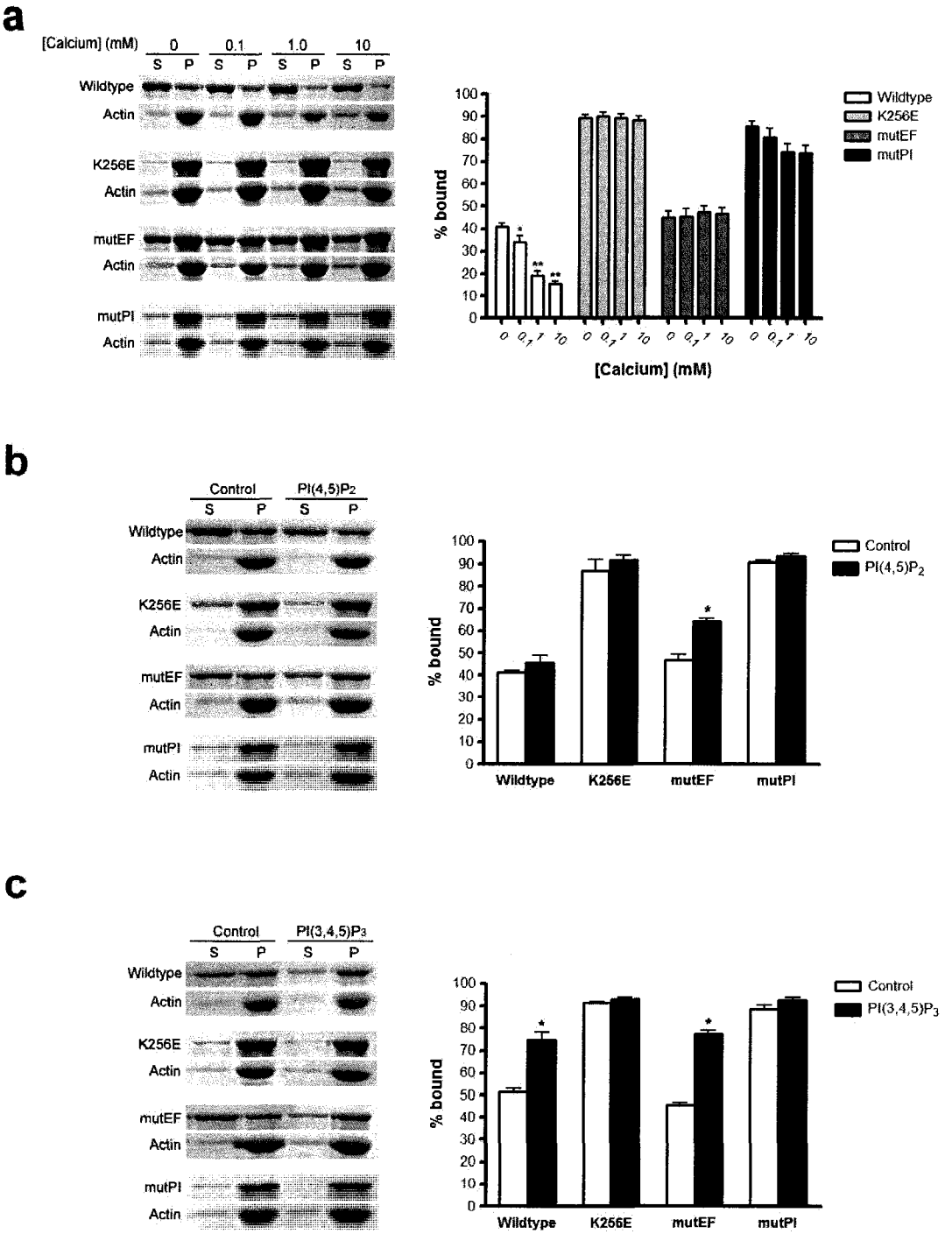


Figure 5.3 Calcium and phosphoinositides modulate α-actinin-4/actin interactions. *In vitro* actin sedimentation assays were performed in the presence of calcium **(a)**, PIP_2 **(b)**, or PIP_3 **(c)**. Levels of α-actinin-4 variants and actin in the supernatant (S) and pellet (P) of a representative experiment are shown. The percentage of α-actinin-4 bound to actin was determined by densitometry and is shown in graphical form. * $P < 0.05$ and ** $P < 0.001$ vs. control, n = 3-7.

α-actinin-4^{wt} was detected along stress fibers (small arrows), at focal adhesions, and within peripheral membrane ruffles (arrowheads). The expression pattern of α-actinin-4^{mutEF} was mostly indistinguishable from that of α-actinin-4^{wt}. Conversely, the localization of α-actinin-4^{mutPI} was very similar to that of α-actinin-4^{K256E}; both variants were preferentially localized to actin stress fibers and away from cortical membrane structures. Furthermore, high-affinity variants (K256E and mutPI) also caused the formation of abnormally-bundled actin (large arrows). These abnormal bundles were observed in numerous cells expressing α-actinin-4^{K256E}, and in a smaller proportion of cells expressing α-actinin-4^{mutPI}. The subcellular localization of α-actinin-4 variants was confirmed in various cell lines (mouse embryonic fibroblasts (MEFs), A7r5, and COS-7), both with and without the C-terminal GFP-tag (unpublished observations and Figure S1).

In order to quantify this aberrant localization, we performed subcellular fractionation experiments by differential centrifugation. In this assay, large cytoskeletal structures such as actin bundles are isolated by low-speed centrifugation (Triton X-100 insoluble fraction; TI). The Triton X-100 soluble (TS) fraction is removed and centrifuged at high speed to separate non-bundled actin filaments (pellet, TS:P) from soluble components (supernatant, TS:S). As shown in Figure 5, the majority of α-actinin-4^{wt} (81%) and α-actinin-4^{mutEF} (77%) was detected in the supernatant of the TS fraction. Conversely, α-actinin-4^{K256E} and α-actinin-4^{mutPI} are predominantly detected in the TI fraction (67% and 62%, respectively). In order to rule out the possibility that the shift observed for α-actinin-4 was due to changes in actin levels, we also quantified the amount of actin in each fraction by densitometry. Actin levels were similar in each fraction, regardless of the α-actinin-4 variant (Figure 5c). These results demonstrate that variants of α-actinin-4 with increased affinity for actin (K256E and mutPI) are sequestered to

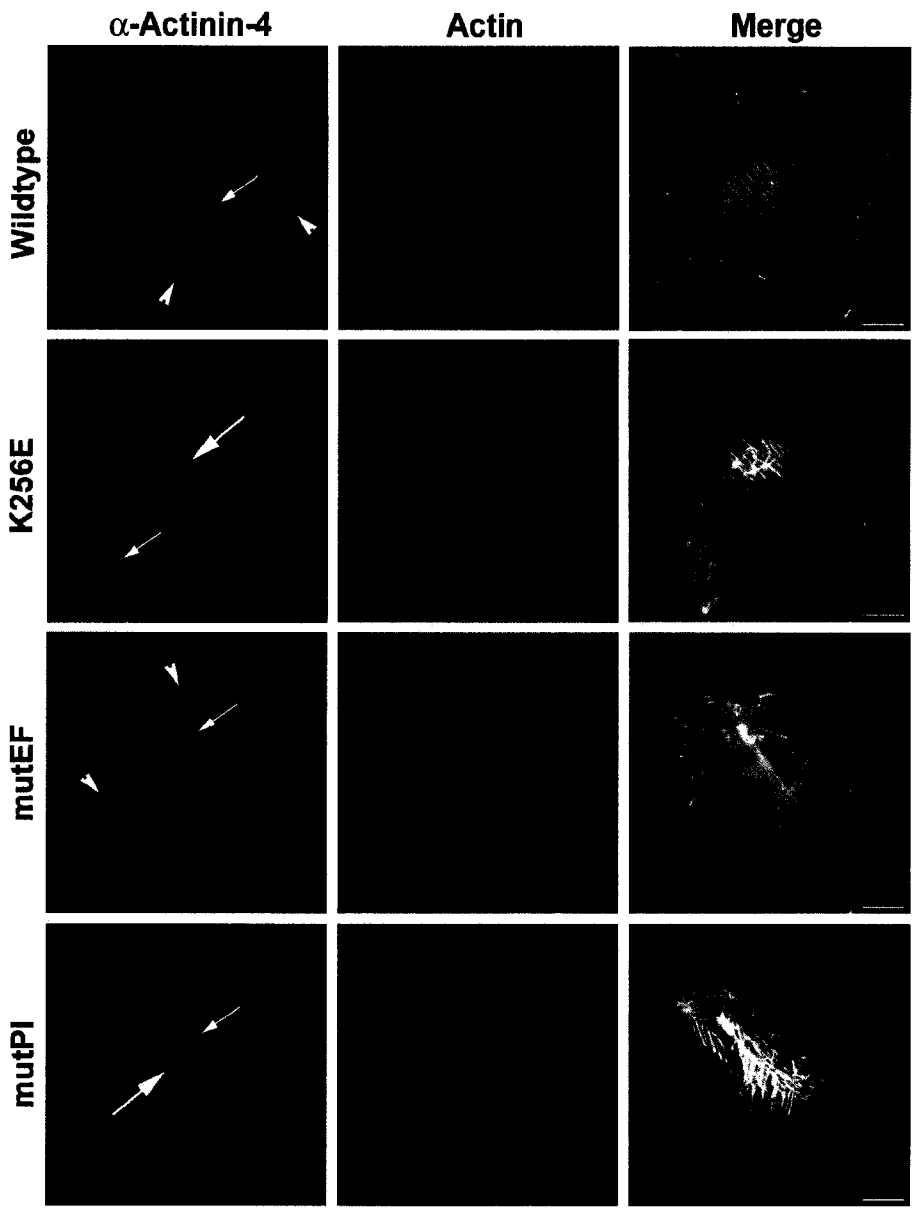


Figure 5.4 Localization of α-actinin-4 variants in cultured podocytes.
Differentiated podocytes were infected with adenoviral constructs encoding GFP-α-actinin-4 variants and processed for immunofluorescence after 72 hours. F-actin was stained with Alexa Fluor 594-conjugated phalloidin. Arrowheads indicate peripheral membrane ruffles, small arrows indicate actin stress fibers, and large arrows indicate abnormally bundled actin. Bar = 20 μm.

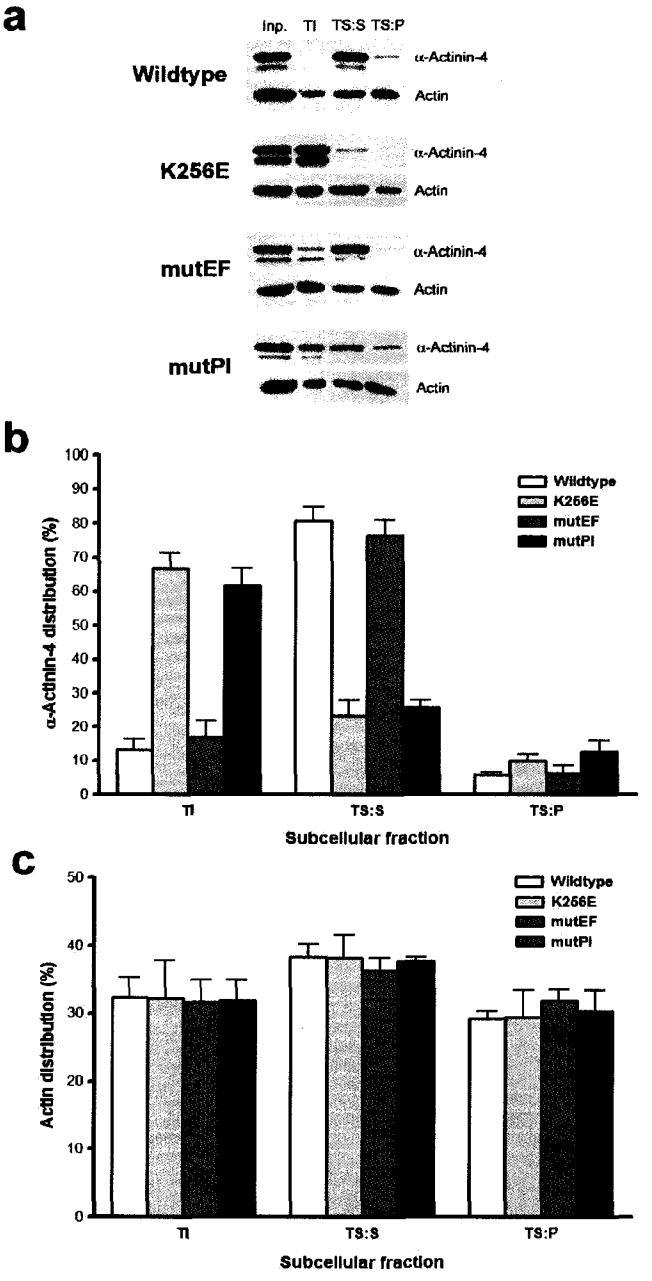
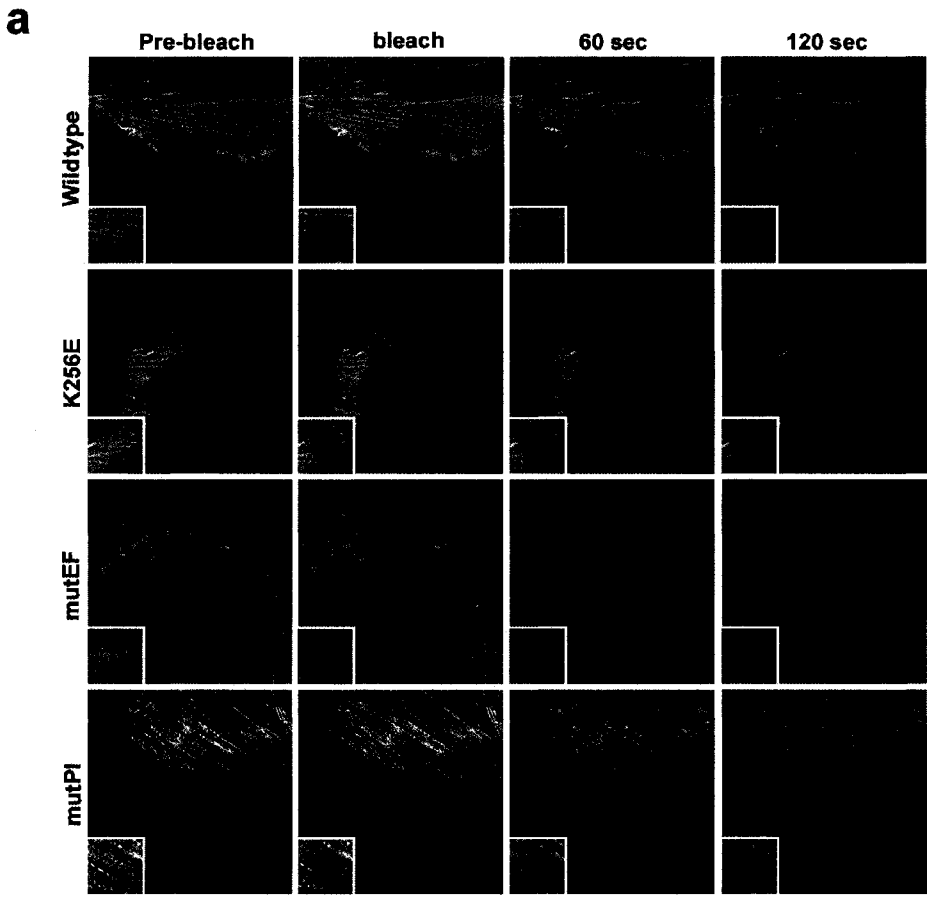


Figure 5.5 Subcellular distribution of α-actinin-4 variants in cultured podocytes. **(a)** Differentiated podocytes expressing GFP-α-actinin-4 variants were lysed in Triton X-100-containing buffer and subjected to low-speed centrifugation to isolate actin bundles (Triton insoluble fraction: TI). The Triton-soluble fraction (TS) was then centrifuged at high speed to isolate unbundled actin filaments (P) from soluble components (S). Equal volumes of each fraction were analyzed by SDS-PAGE and immunoblotted with anti-HA or anti-actin antibodies. The amount of α-actinin-4 **(b)** and actin **(c)** in each fraction was determined by densitometry and are expressed as a percentage. Input (Inp.) lanes indicate similar expression levels for each α-actinin-4 variant.

stable actin structures in cultured podocytes. These results were also confirmed in various cell lines (MEFs, A7r5, COS-7), both with and without the C-terminal GFP-tag (unpublished observations and Figure S2).

Affinity for actin dictates α-actinin-4 dynamics

We performed fluorescence recovery after photobleaching (FRAP) to examine the dynamics of α-actinin-4 variants in cultured cells. A7r5 cells (rat smooth muscle) were chosen since they display a robust cytoskeleton containing prominent parallel actin bundles and large, stable focal adhesions, making them an ideal cell line for FRAP experiments. FRAP was performed along stress fibers, and at focal adhesions, two areas of prominent α-actinin-4 expression. α-Actinin-4^{wt} is very dynamic, as illustrated by the rapid recovery of fluorescence along actin stress fibers (Figure 6a and 6b). Conversely, the behavior of α-actinin-4^{K256E} is very static, with very little fluorescence recovery after 2 minutes, or at later time points (not shown). Mutations within the calcium-sensing EF hands had little effect on α-actinin-4 dynamics, while mutations within the phosphoinositide binding site significantly impaired α-actinin-4 turnover along stress fibers. The recovery curves obtained following photobleaching of individual focal adhesions (Figure 6c and 6d) were very similar to those obtained from stress fibers. Interestingly, the turnover of α-actinin-4^{mutPI} was more severely reduced at focal adhesions than along stress fibers, suggesting that phosphoinositides play an important role in modulating α-actinin-4 dynamics within focal adhesions.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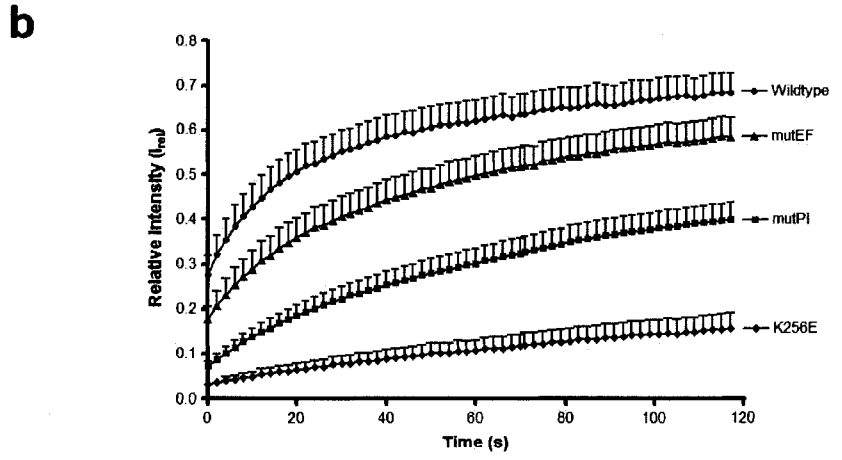
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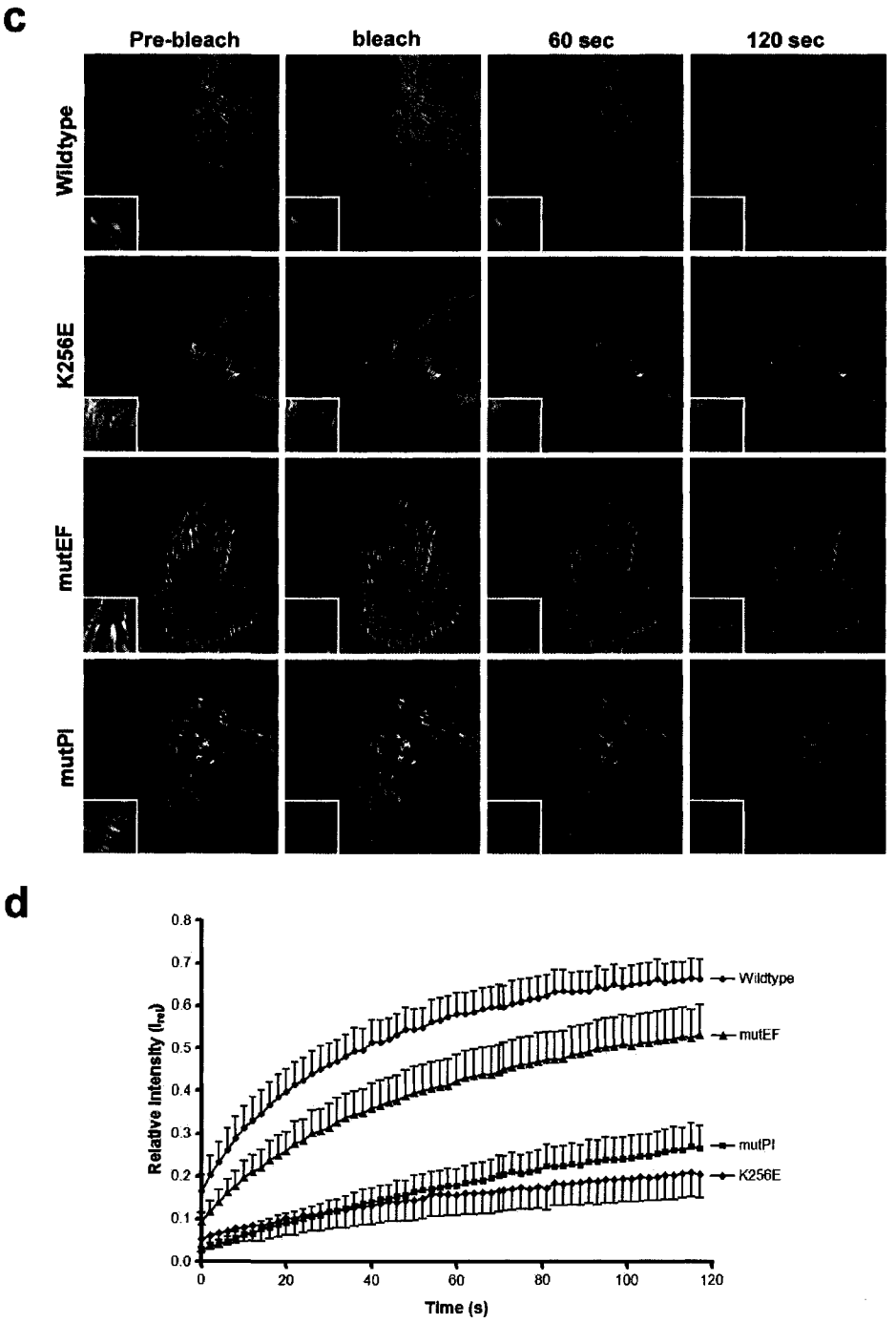

Figure 5.6 Assessment of α-actinin-4 dynamics by FRAP.
A7r5 cells were infected with adenoviruses encoding GFP-α-actinin-4 variants and processed for FRAP. FRAP was performed along actin stress fibers **(a,b)** and at focal adhesions **(c,d)**, areas of prominent α-actinin-4 expression. Representative images **(a,c)** are shown, with emphasis on the bleached areas (inset). Recovery curves **(b,d)** were constructed by calculating the relative intensity of the bleached area over time. n = 5-14 separate cells.

Dynamic α-actinin-4/actin interactions are required for cytoskeletal remodeling

The results detailed above demonstrate that increasing the affinity of α-actinin-4 for actin results in a static protein that is sequestered to heavily bundled actin structures. We sought to determine the effect of such changes in α-actinin-4/actin dynamics on the ability of podocytes to withstand physiological levels of mechanical stretch, as podocytes *in vivo* contend with such hydrodynamic forces within glomeruli. Podocytes were cultured on flexible silicone membranes and subjected to 10% equibiaxial stretch. Mechanical stimulation caused cytoskeletal remodeling, indicated by the disappearance of stress fibers, and a 50% decrease in cell surface area after 24 hours of stretch (Figure 7). Interestingly, expression of α-actinin-4^{wt} had a slightly protective effect, with the cells suffering only a 43% decrease in surface area. Cells expressing α-actinin-4^{K256E} displayed a reduced ability to withstand mechanical stretch as illustrated by a significant loss in surface area (↓72%). Expression of α-actinin-4^{mutEF} did not significantly affect the decrease in surface area following stretch (↓47%). α-Actinin-4^{mutPI} had an effect similar to that of α-actinin-4^{K256E}, causing a significant loss in surface area (↓69%). Finally, it is interesting to note that the abnormal actin bundles observed in cells expressing α-actinin-4^{K256E} and α-actinin-4^{mutPI} appeared to be very stable as they were not disassembled during the period of mechanical stimulation.

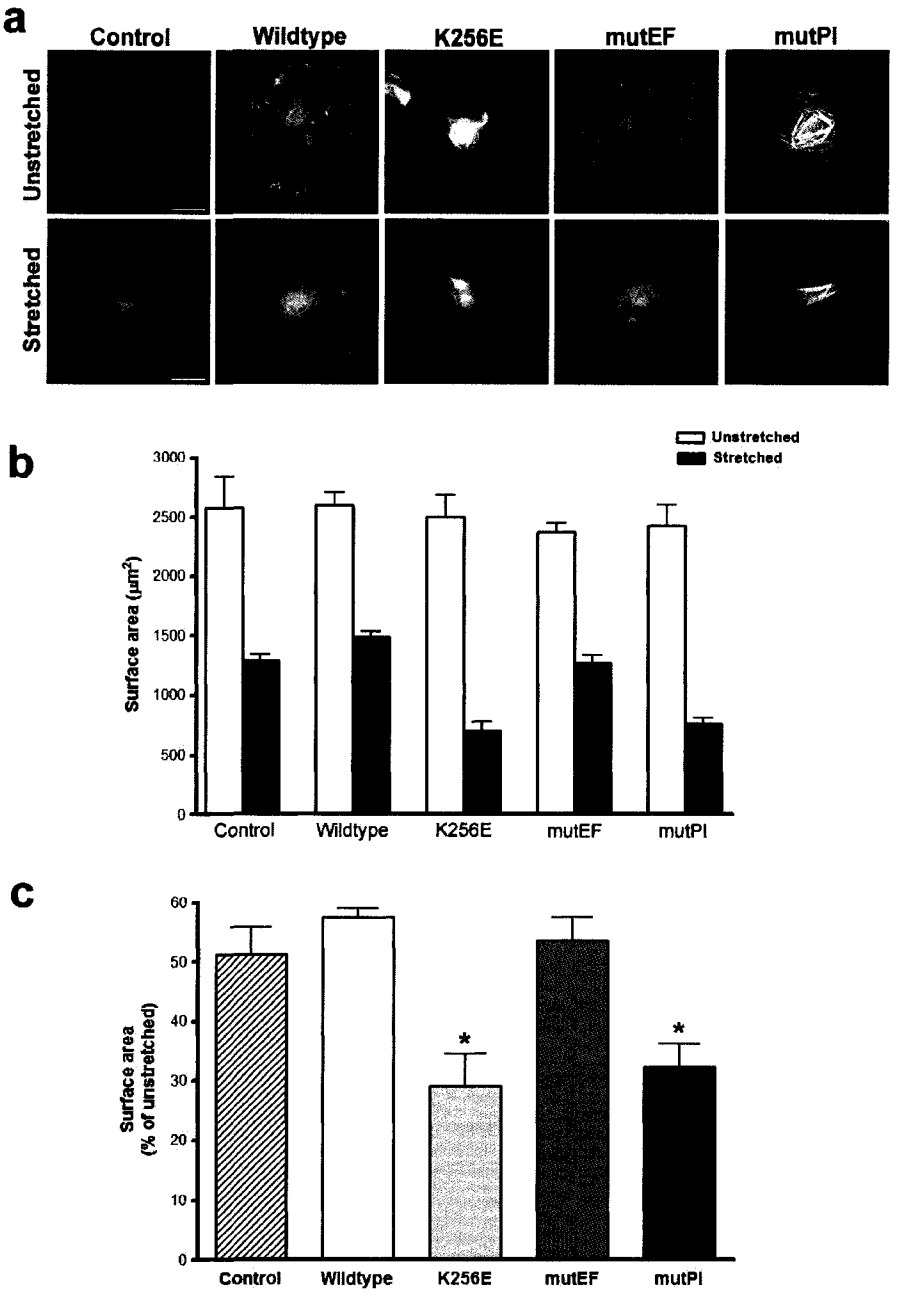

Figure 5.7 Mechanical stretch induces cytoskeletal remodeling in podocytes. **a)** Podocytes were differentiated on flexible silicone membranes and infected with adenoviruses encoding GFP-α-actinin-4 variants. Following 24 hours of mechanical stretch at an amplitude of 10%, cells were processed for immunofluorescence. F-actin was visualized with Alexa Fluor 594-conjugated phalloidin and nuclei were stained with DAPI. Uninfected cells served as control. **b)** Cell surface area (μm^2) was measured in stretched and unstretched cells. **c)** The cell surface area following stretch was expressed as a percentage of the respective unstretched cells. * $P < 0.01$ vs. control, $n = 4$ separate experiments.

Supplemental Figures

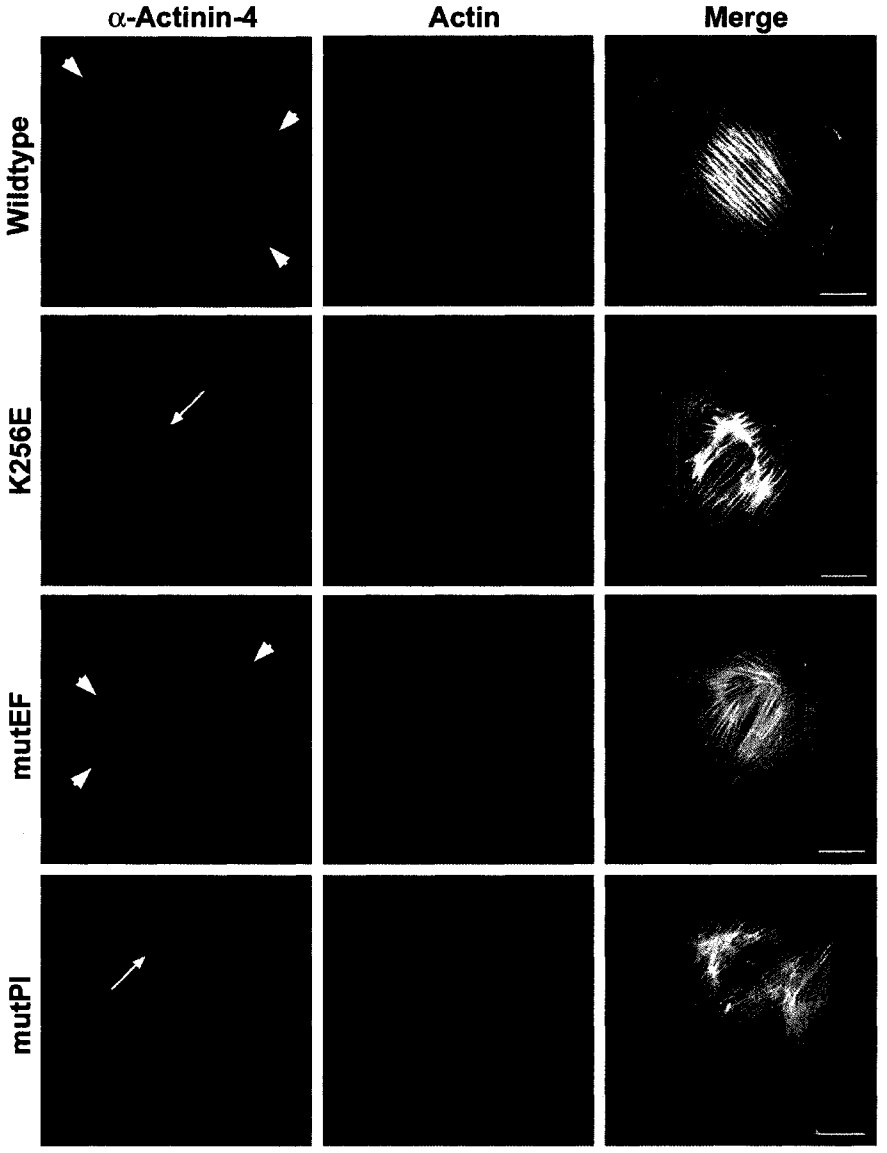

Figure S1 Localization of α-actinin-4 variants in A7r5 cells.
Cells were transfected with constructs encoding HA-tagged α-actinin-4 variants and processed for immunofluorescence after 72 hours. α-Actinin-4 was detected with an anti-HA antibody while F-actin was stained with Alexa Fluor 488-conjugated phalloidin. Arrowheads indicate peripheral membrane ruffles; arrows indicate abnormal actin bundles. Bar = 20 μm.

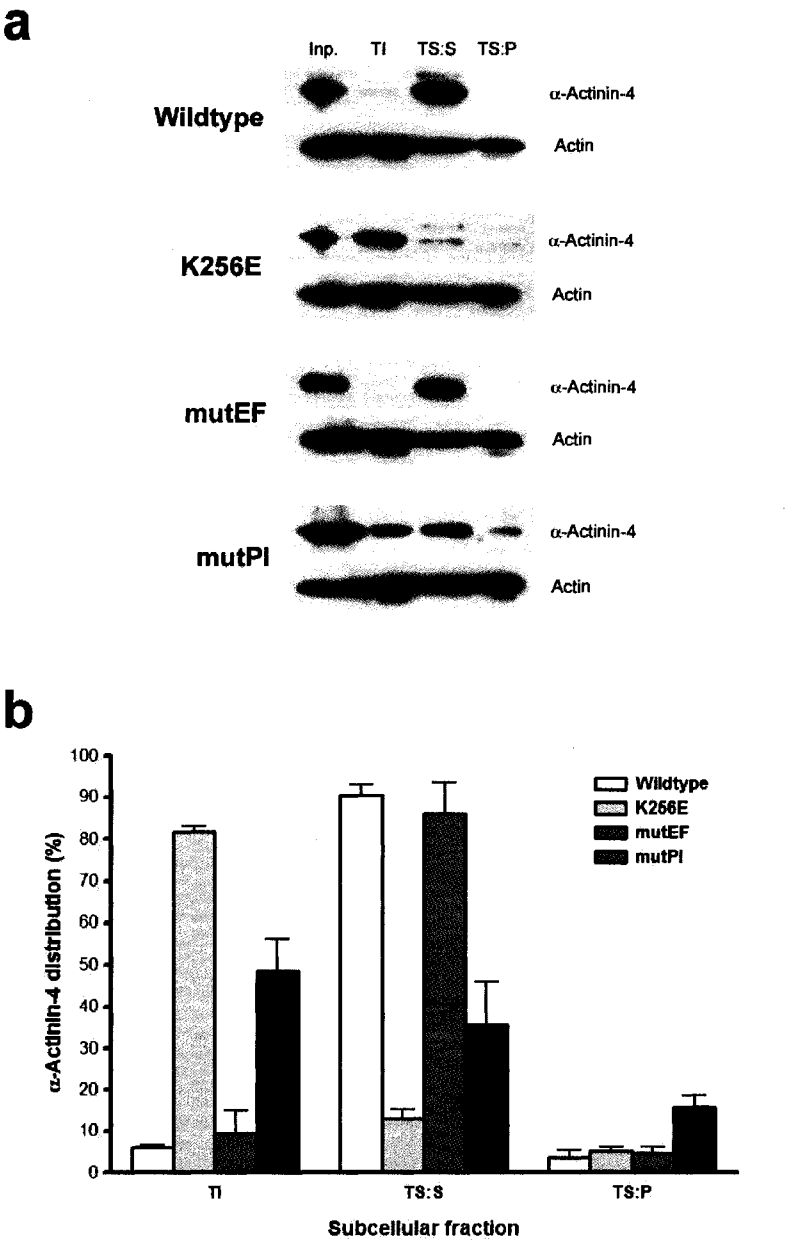

Figure S2 Subcellular distribution of α-actinin-4 variants in MEFs.
a) MEFs transfected with constructs encoding HA-tagged α-actinin-4 variants were lysed in Triton X-100-containing buffer and subjected to low-speed centrifugation to isolate actin bundles (Triton insoluble fraction: TI). The Triton-soluble fraction (TS) was then centrifuged at high speed to isolate unbundled actin filaments (P) from soluble components (S). Equal volumes of each fraction were analyzed by SDS-PAGE and immunoblotted with anti-HA or anti-actin antibodies. **(b)** The amount of α-actinin-4 in each fraction was determined by densitometry and expressed as a percentage of the total signal. Input (Inp.) lanes indicate similar expression levels for each α-actinin-4 variant. n = 2.

Discussion

Mutations in the actin bundling protein α-actinin-4 are associated with a familial form of focal segmental glomerulosclerosis [3]. We have previously shown that introduction of a point mutation (K256E) within the open reading frame of murine α-actinin-4 results in mislocalization of the protein and impairs podocyte motility [5]. The factors involved in the regulation of α-actinin-4 dynamics have not been fully defined. Based on previous studies involving α-actinin-1 and its putative regulatory domains, we hypothesized that both calcium and phosphoinositides play a key role in the modulation of α-actinin-4/actin interactions, and that the K256E mutation would disrupt such modulation. Our findings show that inactivation of the calcium-sensing EF hand motifs does not significantly alter the baseline binding of α-actinin-4 to actin. Conversely, mutation of the phosphoinositide binding site significantly increases the binding of α-actinin-4 to actin, similar to the K256E mutation. Evidence from a recent study suggests that mutations within the CH2 region of the actin binding domain of α-actinin-4 cause the protein to adopt an "open" conformation, exposing a previously buried actin binding site [15]. Since the phosphoinositide binding site is also found within the CH2 domain, it is possible that the mutations in α-actinin-4^{mutPI} that prevent phosphoinositide binding, may in fact induce a conformational change exposing the "third" actin binding site, resulting in the increased affinity for actin.

Despite the changes in affinity observed for the various α-actinin-4 variants, we were able to demonstrate that *in vitro*, both calcium and phosphoinositides can modulate the binding of wildtype α-actinin-4 to actin. Increasing levels of calcium decreased the affinity of α-actinin-4^{wt} for actin. Conversely, $PI(4,5)P_2$, and to a greater extent

$PI(3,4,5)P_3$, increased the association of recombinant α-actinin-4 with actin filaments. This contrasts with recent work that suggests that phosphoinositides generally promote dissociation of α-actinin-1 from actin [9, 10]. The reason for this difference is presently unclear. An important factor may be that full-length recombinant α-actinin-4 was used for actin-binding assays in the present study, as opposed to the truncated α-actinin-1 (i.e. only the ABD) used in previous studies. Interestingly however, earlier studies indicate that $PI(4,5)P_2$ dramatically increases the gelating activity of smooth muscle α-actinin [16]. These confounding results may suggest that α-actinin variants have retained or developed different modes of regulation. In any case, FSGS-associated α-actinin-4^{K256E} was insensitive to both Ca^{2+} and phosphoinositide regulation, suggesting that the gain of affinity may "override" its sensitivity to regulatory factors. Other than gain-of-affinity mutations, factors that increase α-actinin-4's affinity for actin have not been described. It is plausible that phosphoinositides may perform such regulation by causing the protein to adopt a temporary, high-affinity "open" conformation, thereby modulating α-actinin-4 dynamics. The dissociation of phosphoinositides from α-actinin-4 would then return the crosslinking protein to a low-affinity state. In contrast, the K256E mutant would be incapable of such dynamics. Furthermore, we speculate that the mutations in α-actinin-4^{mutPI} might, ironically, mimic the effect of phosphoinositide binding resulting in a chronic high affinity "open" conformation to thereby yield a phenotype not unlike that of the K256E mutant.

We have previously reported the mislocalization of HA-tagged α-actinin-4^{K256E} in cultured podocytes. In that study, HA-α-actinin-4^{wt} was detected along stress fibers, at focal adhesions, and associated with cortical actin structures. Conversely, HA-α-actinin-4^{K256E} was restricted to actin stress fibers and focal adhesions, with no detectable

expression at the cell periphery. In the present study, GFP-tagged α-actinin-4 was used for all experiments. We observed that addition of the GFP-tag caused some aggregation of α-actinin-4^{K256E}. Although GFP-α-actinin-4^{K256E} was again clearly detected along actin stress fibers and at focal adhesions, we observed prominent abnormal actin bundles in a high proportion of cells. These abnormal actin bundles were also observed in MEFs, A7r5 cells, and COS-7 cells expressing GFP-α-actinin-4^{K256E} (unpublished observations). The localization of α-actinin-4^{mutEF} was indistinguishable from that of α-actinin-4^{wt}; this result was anticipated since inactivation of the calcium-sensing EF hands had no effect on the binding of α-actinin-4^{mutEF} in actin sedimentation assays *in vitro*. Similar to α-actinin-4^{K256E}, we observed a significant change in the localization of α-actinin-4^{mutPI}; the protein was again sequestered to actin stress fibers and focal adhesions, and also caused the formation of abnormal actin bundles. This result is consistent with the reported localization of phosphoinositide-insensitive α-actinin-1 in cultured fibroblasts [9].

In the current study, FRAP experiments revealed that high-affinity variants of α-actinin-4 are very static, both along stress fibers and at focal adhesions. Since the time required for recovery was so great for α-actinin-4^{K256E} and α-actinin-4^{mutPI}, we were unable to determine if the mobile fractions were equivalent for each variant. In general, the turnover rates of α-actinin-4 variants were slightly slower at focal adhesions compared to stress fibers. This finding is in contrast with a previous study that reported faster turnover of α-actinin-1 at focal adhesions vs. stress fibers [10]. Interestingly, the turnover rate of α-actinin-4^{mutPI} was much slower at focal adhesions compared to stress fibers, possibly due to local differences in levels and availability of phosphoinositides.

In their natural environment, podocytes are constantly exposed to pulsatile forces exerted by the capillary wall. It is hypothesized that a dynamic cytoskeleton is required

for the podocytes to withstand such forces. In mice, podocyte-specific expression of α-actinin-4^{K256E} leads to podocyte foot process effacement and FSGS, highlighting the importance of the podocyte cytoskeleton. In the present study, forces exerted on podocytes *in vivo* were mimicked using a Flexcell system. Podocytes expressing high-affinity variants of α-actinin-4 (K256E and mutPI) displayed a significant loss in their surface area after 24 hours of stretch. Interestingly, we observed that expression of α-actinin-4^{wt} had a slightly protective effect, as evidenced by a milder surface area reduction. These results support the notion that a dynamic cytoskeleton is required for the maintenance of podocyte architecture. According to our *in vitro* data, we propose that calcium acts as a negative regulator of α-actinin-4/actin interactions. Conversely, phosphoinositides may act as positive regulators and increase the association of α-actinin-4 with actin. Dynamic regulation by these two opposing pathways may play an important role in podocyte plasticity. Our data also indicate that α-actinin-4^{K256E} is insensitive to both of these regulatory factors, and tips the balance towards a static cytoskeleton. Unable to remodel their actin cytoskeleton, the podocytes therefore suffer a substantial loss of surface area when exposed to significant mechanical strain. These findings provide a likely explanation for the phenotype observed in transgenic mice expressing α-actinin-4^{K256E}; unable to withstand the glomerular pulsatile pressure, podocytes suffer a loss of surface area (foot process effacement), which leads to FSGS.

Taken together, the results presented in this study suggest that dynamic modulation of α-actinin-4/actin interactions is required for podocyte function. We suggest that gain-of-affinity mutations, coupled with the ensuing insensitivity to regulatory factors, both contribute to the podocyte damage observed in α-actinin-4-associated FSGS.

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Chapter 6

General Discussion

6.1 Mouse model of FSGS : an invaluable tool

During the last decade, our knowledge of the biology of the glomerular podocyte has increased dramatically (Pavenstadt, Kriz et al. 2003). The discovery of a podocyte-specific gene – *NPHS1*, encoding the protein nephrin – mutated in families affected with congenital nephrotic syndrome of the Finnish type triggered an intense period of research that continues to this day. Indeed, the podocyte is now regarded as the most important cell type in glomerular filtration.

Various studies have unequivocally established that damage to the podocyte leads to proteinuria and is a key event initiating the progression to glomerular scarring. However, many of these studies have relied on the use of chemical agents toxic to the podocyte, such as puromycin aminonucleoside or protamine sulfate. The use of such toxic compounds has provided some insights into the mechanisms of podocyte injury, but does not allow long-term studies on the mechanisms involved in disease progression.

Based on the original description of FSGS-associated mutations in the *ACTN4* gene, we developed a novel mouse model of familial FSGS (Chapter 3) by expressing α-actinin-4^{K256E} in a podocyte-specific manner. The K256E mutation clearly and markedly increased the binding of α-actinin-4 to actin (Kaplan, Kim et al. 2000) and was chosen simply for this reason. Mice with podocyte-specific expression of α-actinin-4^{K256E} develop proteinuria and display foot process effacement and glomerular lesions similar to that observed in human patients. This mouse model has proved useful on multiple fronts. Firstly, it serves as proof-of-principle that damage originating within the podocyte leads to FSGS lesions, and that the structure of the podocyte is crucial for its function within the glomerular filtration barrier. Secondly, unlike nephrotoxic models, it has allowed a

more detailed study of the mechanisms involved in the progression of FSGS lesions. In humans, α-actinin-4-associated FSGS lesions appear later in life (third or fourth decade), and progress slowly to ESRD (Kaplan, Kim et al. 2000). Similarly, mice with podocyte-specific expression of α-actinin-4^{K256E} show a gradual decline in kidney function, but can live beyond 12 months of age, allowing studies at various stages of disease progression. For example, we have determined by microarray analysis that many genes involved in the complement pathway are dysregulated following α-actinin-4-dependent podocyte injury (unpublished observations). These studies therefore suggest that inappropriate activation of the complement pathway contributes to the renal damage observed in transgenic mice. Lastly, this mouse model of FSGS has also fostered international collaborations with various researchers in the field of podocyte biology.

The development of such a mouse model has therefore not only furthered our understanding of podocyte biology, but also provides an avenue for future studies on the progression of FSGS lesions. Such studies will hopefully lead to novel strategies for treatment and the prevention of FSGS.

6.2 The podocyte cytoskeleton in health and disease

The structural integrity of the podocyte is highly dependent on the hierarchical organization of its cytoskeletal components. The cytoskeleton of the primary processes (microtubule-based) provides a connection between the metabolic machinery of the cell body and the foot processes. The function of the foot process cytoskeleton (actin-based) is coupling the slit diaphragm complex (cell-cell interactions) and the podocyte-GBM

contact points (cell-matrix interactions). Since the glomerular capillary wall undergoes cyclic distension with every beat of the heart, the podocyte cytoskeleton must provide mechanical strength while retaining a certain degree of flexibility.

Structural alterations at the level of the cytoskeleton are a common finding in podocyte damage. Indeed, it is generally accepted that foot process effacement is initiated at the cytoskeleton and results in altered cell-cell (slit diaphragm) and/or cell-matrix (adhesion) interactions (Pavenstadt, Kriz et al. 2003). A dramatic reorganization of the actin cytoskeleton is often observed during foot processes effacement. Longitudinal actin bundles are replaced by a dense network of microfilaments at the base of effaced foot processes, which adopt a sheet-like morphology as opposed to their characteristic pear-like shape. In addition to these changes in actin organization, many actin-associated molecules – such as synaptopodin, α-actinin-4 and heat shock protein 27 (*hsp27*) – are also dysregulated in podocyte damage (Pavenstadt, Kriz et al. 2003). Monomeric GTPases involved in cytoskeletal organization have recently emerged as likely mediators of the signaling mechanisms leading to podocyte damage (Asanuma, Kim et al. 2005; Faul, Asanuma et al. 2007). Taken together, these changes illustrate that cytoskeletal proteins are critically relevant for podocyte morphology and support the hypothesis of the cytoskeleton as the common link in podocyte failure.

It is well known that the cytoskeleton is not only implicated in mediating cell shape, but also numerous other processes such as cell adhesion and migration. The effects of a gain-of-affinity mutant α-actinin-4 (K256E) on such processes were therefore assessed in cultured podocytes (Chapter 4). Though slight differences were observed in the recruitment of α-actinin-4^{K256E} to focal contacts, we could not detect any changes in the overall ability of the cells to adhere to an extracellular matrix. However, we found

that expression of α-actinin-4^{K256E} significantly impaired podocyte spreading and migration.

What is the significance of these results, considering the fact that podocytes are usually not viewed as a migratory cell type? Although the adult podocyte is likely incapable of migrating in whole to another location, structural changes at the level of the foot processes can be seen as migratory events (Reiser, Oh et al. 2004), involving cytoskeletal-driven forces on the plasma membrane. In support of this, podocytes display the ability to recover their characteristic architecture following chemically-induced foot process effacement (Whiteside, Prutis et al. 1989). Urinary loss of podocytes (via detachment from the GBM or apoptosis) is also commonly observed in glomerular disorders. In such cases, the surrounding podocytes could migrate in an attempt to cover the denuded GBM and prevent adhesions between the glomerular tuft and the parietal epithelium. Whether such migratory events are primarily relevant following injury, or for the normal function of the podocyte, requires further characterization. It is highly likely that intact cytoskeletal dynamics are indispensable for both maintenance of the podocyte's arborized morphology under normal conditions and for mediating repair following podocyte injury.

6.3 Understanding α-actinin-4 dynamics

While both non-muscle isoforms of α-actinin are highly homologous, our data suggests significant differences in their function and modes of regulation. It was originally thought that α-actinin-1 may be a linker between actin filaments and focal

adhesions, and that α-actinin-4 was primarily involved in bundling actin filaments. However, we have clearly shown that α-actinin-4 is readily incorporated into stable focal adhesions in cultured podocytes. A role for α-actinin-4 in focal adhesions is further supported by studies demonstrating decreased strength of integrin-mediated linkages to the cytoskeleton in α-actinin-4-deficient podocytes (Dandapani, Sugimoto et al. 2007). α-Actinin-4 in also readily detected in both peripheral membrane ruffles and circular dorsal ruffles (unpublished observations), suggesting a key role in actin-based cellular dynamics.

Most of what is known about non-muscle α-actinin dynamics has come from experiments with α-actinin-1. It is attractive to assume that the non-muscle isoforms share common regulatory mechanisms, and that findings obtained with actinin-1 can be applied to actinin-4. Similarly to α-actinin-1, our work indeed demonstrates that α-actinin-4 is sensitive to increasing levels of calcium, which decrease the binding of α-actinin-4 to actin (Chapter 5). However, in contrast to α-actinin-1, we have demonstrated that α-actinin-4 is positively regulated by phosphoinositides; PIP_3, and to a lesser extent PIP_2, both increased the binding of wildtype α-actinin-4 to actin. These results are in stark contrast with previous studies suggesting that phosphoinositides are negative regulators of α-actinin-1/actin interactions (Fraley, Tran et al. 2003). Although the experiments were performed in a similar fashion, the use of a truncated version of α-actinin-1 (i.e. only the actin binding domain) may explain these opposing results. The results presented in this thesis therefore suggest that non-muscle α-actinin isoforms display similar (calcium) and divergent (phosphoinositides) modes of regulation.

Another important finding of these studies is that α-actinin-4^{K256E}, a high-affinity variant (K_d of 0.05 μM vs. 0.27 μM for wildtype) associated with FSGS, remains insensitive to regulation by both calcium and phosphoinositides. It was recently

discovered that a subset of FSGS-causing mutations (including K256E) map to the CH1-CH2 interface of α-actinin-4's ABD and cause the protein to adopt an "open" conformation, thereby exposing ABS1 (Weins, Schlondorff et al. 2007). Exposure of all three ABSs, rather than two in a "closed" conformation, would result in an increased affinity for F-actin and may override α-actinin-4's sensitivity to calcium. These findings also provide a logical mechanism for the positive regulation observed in the presence of phosphoinositides. Since the phosphoinositide-binding site is also near the CH1-CH2 interface, binding of PIP_2/PIP_3 may cause a temporary conformational change exposing ABS1, thereby increasing the binding of α-actinin-4 to F-actin. Taken together, these results suggest that both negative and positive regulators are important for α-actinin-4 dynamics.

A consistent theme throughout these studies is that variants of α-actinin-4 with increased affinity for actin are very static compared to the wildtype protein. In light of the dynamic nature of the podocyte cytoskeleton, α-actinin-4 emerges as a key protein involved in the maintenance of foot process architecture. Negative regulation of α-actinin-4/actin interactions by calcium may provide the podocyte with the ability to disassemble actin bundles in order to remain flexible following extracellular cues such as distension or stretch. Conversely, positive regulation by phosphoinositides may provide actin filament and/or focal adhesion stability when required. Dysregulation of the F-actin-binding properties of α-actinin-4 clearly hinders cytoskeletal dynamics, and likely contributes to the morphological changes observed in podocyte injury.

6.4 Future studies

The results described in Chapter 5 provide *in vitro* evidence that both calcium and phosphoinositides modulate the F-actin-binding properties of α-actinin-4. However, extrapolation of *in vitro* findings to an *in vivo* situation is not always straightforward. The next logical step is therefore to confirm the *in vitro* findings in cultured cells. Increasing the levels of intracellular calcium using a calcium ionophore should cause the redistribution of α-actinin-4 from a detergent insoluble fraction (i.e. F-actin associated) to a detergent soluble fraction in actin co-sedimentation assays. To confirm the effect of phosphoinositides on α-actinin-4 function, actin co-sedimentation assays could be performed in the presence of constitutively active PI3-kinase. According to the *in vitro* data, increased levels of phosphoinositides should increase the binding of α-actinin-4 to F-actin.

If α-actinin-4 is indeed sensitive to regulatory factors such as calcium and phosphoinositides *in vivo*, what signaling mechanisms would be involved in triggering these intracellular effects? Since podocytes are constantly exposed to cyclical stretch, it is plausible that stretch receptors may be involved in mediating calcium influx into the podocyte to relieve cytoskeletal tension. Conversely, integrin binding to the ECM may provide outside-in signaling in order to stabilize podocyte-matrix contacts. Such signals may be mediated by FAK and subsequent activation of PI3-kinase, leading to increased binding of α-actinin-4 to F-actin along stress fibers and at focal adhesions. A balance between positive and negative signals likely provides appropriate regulation of α-actinin-4 dynamics under physiological conditions. Deviations from this equilibrium likely contribute to podocyte dysfunction and subsequent glomerular injury.

6.5 Conclusion and outlook

Despite significant advances in the field of podocyte biology, injury to this fragile epithelial cell and the ensuing glomerular lesions remain a leading cause of ESRD. The work presented in this thesis, and that of numerous others, have provided a better understanding of the mechanisms involved in podocyte injury. It has become clear that the cytoskeleton is not only important for determining podocyte architecture, but also to stabilize the cell-cell and cell-matrix contacts of the podocyte. α-Actinin-4 has emerged as a key player in podocyte dynamics – the ability to negatively and positively control its association with actin filaments may allow dynamic changes to the podocyte cytoskeleton in response to changes in its environment (Figure 6.1). Many factors that contribute to podocyte injury in glomerular diseases have now been identified. However, the precise signaling mechanisms involved remain poorly understood. Elucidation of these pathways in normal podocyte function and in pathological conditions will hopefully serve as stepping stones towards the development of novel therapeutic tools for preserving podocyte function.

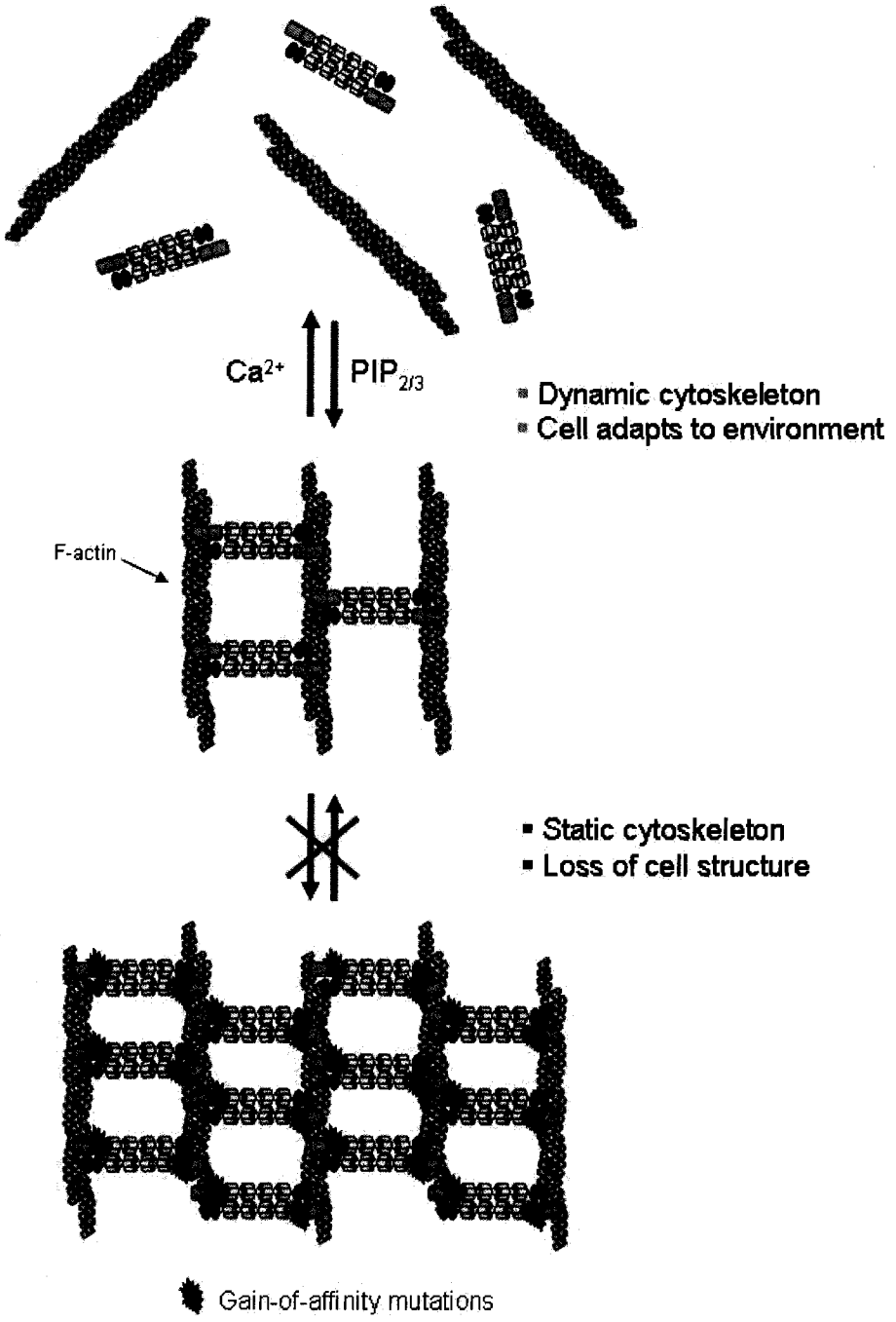

Figure 6.1 Regulation of α-actinin-4 dynamics in podocytes.
The binding of α-actinin-4 to F-actin is modulated by calcium and phosphoinositides, allowing for a dynamic cytoskeleton. The regulation of α-actinin-4 would therefore allow the podocyte cytoskeleton to adapt to various physiological stimuli, such as capillary distension. Gain-of-affinity mutations not only increase the binding of α-actinin-4 to F-actin, but also render it insensitive to regulatory factors. Such dysregulation leads to a static cytoskeleton and renders podocytes unable to cope with the demands of their environment.

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