Investigation of Dok1 and Dok2 as novel modulators of focal adhesion dynamics in kidney podocytes

by
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ABSTRACT

INVESTIGATION OF DOK1 AND DOK2 AS NOVEL MODULATORS OF FOCAL ADHESION DYNAMICS IN KIDNEY PODOCYTES

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Podocyte adhesion to the underlying glomerular basement membrane is required for proper filtration and is mediated by dynamic signalling events. Integrins are a major component of focal adhesions, and their activity is negatively regulated by Dok1. Dok is a member of the Dok family of adaptor proteins and though its expression has been detected in podocytes, its function therein has yet to be investigated. Here, we show that expression of Dok1 and the highly related Dok2 increases in human kidney disease. Using Dok1/2 knockout mice, we demonstrate an attenuated response to podocyte injury. We further show in glomerular protein lysates that Dok1/2 knockout animals display higher surface levels of integrin β1 relative to WT controls, and that phosphorylation of focal adhesion proteins are significantly increased. Based on these findings, we conclude that Dok1/2 regulate integrin signaling in podocytes, which may have implications in adhesion and maintenance of glomerular filtration.

ACKNOWLEDGEMENTS

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To my family, my continuous supporters and best friends, I truly could not have done this without you. To my mum and dad, thank you always being there when I needed you the most. I've grown to be the person I am today because of you two. Thank you to my siblings, Matthew and Zoe for making me laugh and always being more mature than me.

Although this journey comes to an end, another will begin. I hope we will always continue to support each other in whatever times necessary.

DECLRATION OF WORK PERFORMED

The experiments presented in this thesis were performed by Nikkita Trisha Dutta with a few exceptions. Peihua Lu performed all of the ACRs. Dr. Claire Martin and Peihua Lu helped with glomeruli isolation. All mice used in this project were organized and handled by Dr. Laura New and the University of Guelph Central Animal Facility and Isolation unit.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
C3	Complement Component 3
CD8	Cluster of Differentiation 8
CKD	Chronic Kidney Disease
DMEM	Dulbecco's Modified Eagle Media
DOK	Downstream of Kinase
ECL	Enhanced chemiluminescence
ELISA	Enzyme Linked Immunosorbent Assay
ERK	Extracellular Signal-Regulated Kinase
FAK	Focal Adhesion Kinase
GAP	GTPase-Activating Protein
GBM	Glomerular Basement Membrane
GTP	Guanosine Triphosphate
HEK	Human Embryonic Kidney
HPA	Human Protein Atlas
IFNy	Interferon Gamma
Ig	Immunoglobulin
IgA	Immunoglobulin A
ILK	Integrin-Linked Protein Kinase
IRS	Insulin Receptor Substrate
KO	Knockout
MEF	Mouse Embryonic Fibroblast
NIH	National Institutes of Health
NTS	Nephrotoxic Serum
PI3K	Phosphoinositide 3-kinase
PAGE	Polyacrylamide Gel Electrophoresis
PAS	Periodic Acid-Schiff
PH	Pleckstrin Homology
PINCH	Particularly Interesting New Cys-His Protein
PLC	Phospholipase C
PS	Protamine Sulfate
PTB	Phosphotyrosine Binding
PVDF	Polyvinylidene Difluoride
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of the Mean
SH2	Src Homology 2
SH3	Src Homology 3
SI	Sclerosis Index
TBST	Tris Buffered Saline with Tween 20
TEM	Transmission Electron Microscopy
WT	Wildtype

CHAPTER 1: INTRODUCTION

1.1 Chronic Kidney Disease and Treatment Options

Chronic kidney disease (CKD) is a condition defined by damaged kidneys that are unable to filter blood. Up to three million Canadians are estimated to have CKD and internationally, 10% of the population is affected by CKD(*1*). Kidney disease can range from mild to severe, and in some cases, leads to kidney failure, also known as end-stage kidney disease. Often times, kidney disease starts slowly and develops without obvious symptoms over a number of years. This leads to a late diagnosis and poor prognosis, where kidney function capabilities are low. Currently, there is no way to reverse the damage, and the only treatment options for end stage kidney failure are limited to dialysis or kidney transplantation(*2*). Almost 50% of individuals with severely reduced kidney function that are not on dialysis are unaware of having CKD (NIH, 2016). Therefore, screening and early detection of kidney disease through regular examinations are vital to prevent its progression and to reduce the risk of cardiovascular morbidity. Globally, CKD causes nearly 2.4 million deaths per year, making it the 6th fastest growing cause of death (Kidney Foundation, 2019). Understanding the molecular changes that affect kidney function and give rise to kidney disease is imperative, as it will give insight into discovering new ways to understand and avert the progression of kidney disease.

1.2 Kidney Anatomy

The kidneys are two bean shaped organs located below the ribcage, one on either side of the spine. The primary function of the kidney is to filter blood in order to prevent toxic buildup of waste and extra fluid. Both kidneys function to filter about 120 – 150 litres of blood daily, producing 2 litres of urine(*3*). Through filtering blood, the kidney is also responsible for regulating fluids, pH and blood pressure balance. The kidney itself is made up of millions of

functioning units called nephrons. A single nephron consists of a glomerulus, tubules and the loop of Henle. Each nephron works through a two-step process, where filtration occurs at the glomerulus and transport of waste occurs at the tubules.

1.2.1 The Glomerulus

The glomerulus is the site of filtration as it functions to retain large proteins in the blood, while allowing excretion of waste products, a process that is essential for the maintenance of homeostasis. A damaged glomerulus leads to significant loss of vital proteins from the bloodstream, disrupting the blood protein balance, and resulting in proteinuria, a classic hallmark of kidney damage where high levels of proteins are detected in urine. The filtration barrier of the kidney glomerulus is composed of three separate layers: the fenestrated endothelium, the basement membrane and the podocytes (Figure 1)(*4*). The first layer, the endothelial cells, have numerous openings. These openings range between 70 to 100 nm in diameter and are termed fenestrae(*5*). Glycocalyx, a glycoprotein-polysaccharide, lines the luminal surface of the endothelium to form the permeability barrier. Glycocalyx is a negatively charged mesh of cell surface anchored proteoglycans(*5*). The endothelium is maintained by vascular endothelial growth factors produced by podocytes(*5*). The second layer, the glomerular basement membrane (GBM) is an acellular matrix with a thickness of 300 to 350 nm(*4*). The GBM is rich in type IV collagen, proteoglycans, laminin and nidogen, which together provides structural support for the capillary wall(*4*). However, the main contribution of the GBM to the macromolecular filtration barrier is not clearly understood. Despite this lack of knowledge, it is believed that the GBM is imperative for glomerular development, morphology and function[6]. The final layer, made up of podocytes, is essential for creating the slit diaphragm.

The slit diaphragm is the most important component of the kidney's ultrafiltration barrier(*6*). It serves as the primary mechanism to filter blood proteins to keep albumin and other plasma proteins from entering the urinary space.

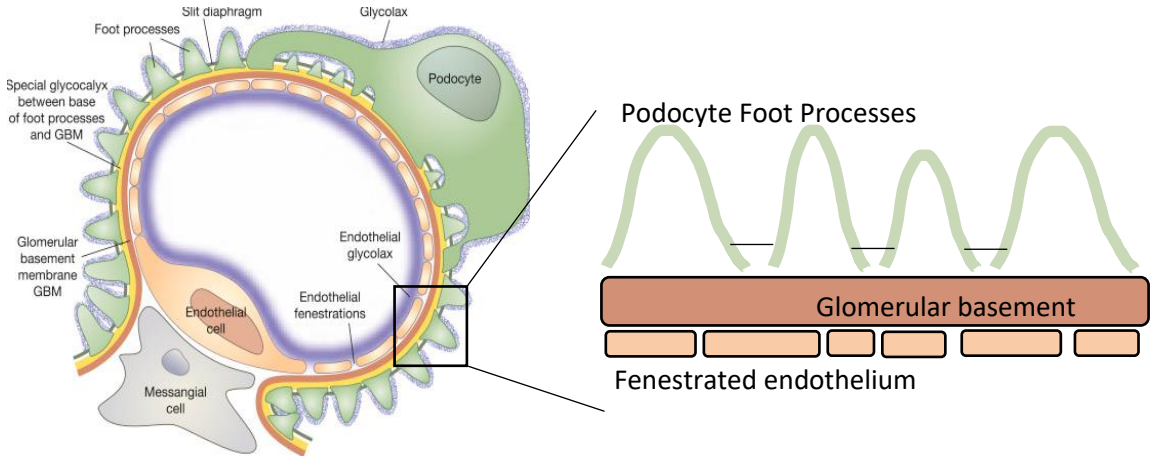


Figure 1: Glomerular filtration system. Cross section of the glomerular filtration barrier. It is composed of three layers: the fenestrated endothelium (orange), the glomerular basement membrane GBM (brown) and the podocytes (green) which project foot processes (FP). Blood is filtered from the endothelium towards the foot processes. (Adapted from Pollock, 2018).

1.2.2 The Unique Podocyte Cytoarchitecture

Podocytes are terminally differentiated cells lining the outer surface of the glomerular capillaries(*7*). Podocytes have a complex cellular architecture consisting of a cell body with major processes that extend outward from their cell body, which in turn have interdigitating foot processes that enwrap the glomerular capillaries. Major processes are tethered by microtubules and intermediate filaments, while foot processes contain actin-based cytoskeleton(*8*). Podocyte foot processes create a functioning slit diaphragm in between a meshwork of proteins actively participating in podocyte signaling(*9*). Foot processes have a thick, negatively charged coat (glycocalyx) facing the urinary space, creating an electrostatic

repel between neighbouring foot processes(*10*). The cytoskeletal dynamics and structural plasticity of podocytes as well as the signaling between each of these distinct layers are essential for an efficient glomerular filtration barrier. The genetic or acquired impairment of podocytes may lead to foot process effacement (also known as retraction), a morphological hallmark of proteinuric renal diseases(*11*).

1.3 Signaling Complexes

Podocyte development occurs in four steps; vesicle stage, S-shaped body stage, the capillary loop stage and the final maturation stage within the glomerulus(*12*). Podocytes develop from columnar epithelial cells, then form capillary loops, where they lose their lateral cell-cell contacts and begin to migrate(*12*). As podocytes mature, they lose their mitotic activity and begin to establish their complex cell architecture, including the reorganizations of cell-cell junctions, or signaling platforms. During normal podocyte development, the cell junction between the apical and basolateral surfaces appears to migrate downward to where foot processes adhere to the GBM. This modified adhesive junction, called the slit diaphragm, connects adjacent foot processes to form the filtration barrier. Another key interaction centre is the interface of podocytes with the GBM, where attachments are made through a series of focal adhesion complexes and cell surface receptors.

1.3.1 Lateral Signaling Complex

Nephrin is a specialized glomerular adhesion protein that is expressed as the podocyte matures and begins to differentiate and form extensions and foot processes. Nephrin is a 189 kDa type I transmembrane protein of the immunoglobulin (Ig) superfamily(*4*). It consists of a short intracellular domain, a transmembrane domain, and a large extracellular domain(*13*). The

extracellular domain consists of eight IgG motifs which interact in a zipper-like intercellular junction associating two foot processes together and creating the slit diaphragm. Clustering of the nephrin ectodomains leads to the scaffolding of various proteins to form a large stable signaling platform (Figure 2)(*14*). In the kidney, only podocytes express nephrin. When nephrin is not present, massive proteinuria, absence of a slit diaphragm and neonatal death are observed(*6*). Nephrin has a cytoplasmic domain with several tyrosine phosphorylation sites that can be phosphorylated via Src-family kinases such as Fyn(*14*). These phosphorylation sites are important for cell signaling pathways at the membrane. Nephrin signaling can be defined and differentiated by the dependency on different groups of tyrosine residues. Nephrin phosphorylation of both groups leads to the recruitment of p85/PI3K and Nck(*15*, *16*). Signaling downstream of p85/PI3K leads to the activation of Akt, while Nck signaling impacts actin remodeling(*15*, *16*). Mice lacking nephrin phosphorylation develop progressive proteinuria accompanied by structural changes in the filtration barrier, including foot process effacement, irregular thickening of the GBM and dilated capillary loops.

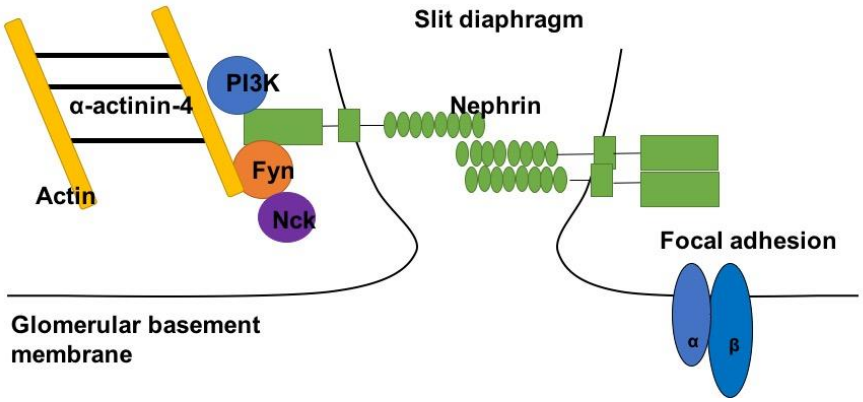


Figure 2: The lateral signaling complex. Nephrin is the key protein that creates the slit diaphragm. Nephrin interacts with PI3K, Fyn, and Nck. α-actinin-4 is an actin binding protein that regulates actin turnover to maintain normal morphology of podocytes.

1.3.2 Basal Signaling Complex

The interface between podocytes and the GBM is another critical signaling complex. Podocytes express cell surface receptors including integrins which recognize and bind to the extracellular matrix (*17*). The GBM is a dense meshwork of a number of extracellular components including collagen IV and laminin. It provides a scaffold for endothelial cells and podocytes to form an effective glomerular filtration barrier. Integrity and function depend on both cell-to-cell and cell-to-matrix adhesion(*18*). Active integrins recruit adaptor and effector proteins to sites known as focal adhesions. Focal adhesion complexes interact with the GBM to mediate podocyte adhesion(*12*). Phosphorylation of kinases, like focal adhesion kinase (FAK), and the interaction with downstream effector proteins like p130cas, regulate focal adhesion turnover(*18*).

1.4 Integrin Receptors

Integrins are heterodimeric transmembrane receptors composed of an α and β subunit (Figure 3)(*19*). The integrin family contains 18 α- and 8 β-subunits that bind noncovalently to form 24 distinct αβ integrin heterodimers in all cell types(*20*). Integrin β1 subunit is the most abundantly expressed and it heterodimerizes with at least 12 different α subunits. Together, they form dimers that are critical for interactions with the extracellular matrix components.

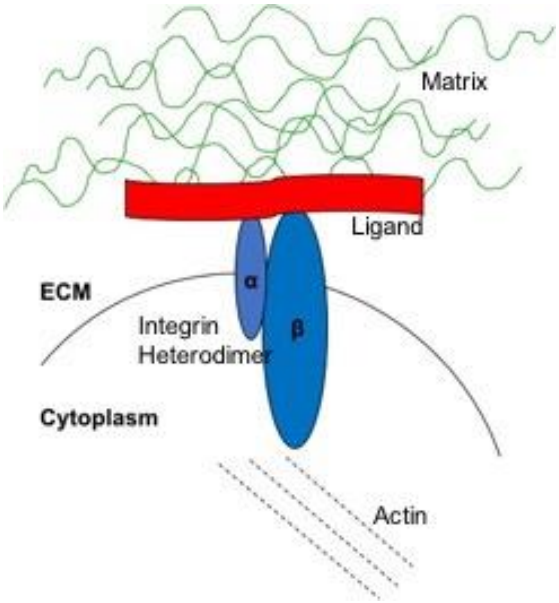


Figure 3: Integrin receptors integrating the extracellular matrix (ECM) and actin cytoskeleton. Integrin is a transmembrane protein composed of an alpha and beta subunit. Intracellular proteins can bind to integrin and through an inside-out signaling pathway affect extracellular matrices. The extracellular matrix (ECM) is an intricate network composed of multidomain macromolecules and is responsible for controlling proliferation, adhesion and migration through collagen, proteoglycans and other ECM components.

Integrins work in three conformations: inactive closed, primed closed, and active open (Figure 4)(*21*). When integrin engagement of the extracellular matrix occurs, there is integrin clustering and activation. This activation leads to bidirectional signaling that is responsible for

cellular events such as adhesion, spreading, migration and organization of the extracellular matrix leading to physiological changes of the barrier. Integrin activation and signal transduction can occur in an “inside-out” and “outside-in” manner. Inside-out signaling occurs by the induction and binding of many adaptor and effector proteins to the cytoplasmic domains of integrin B subunits that activate ligand binding in the ECM. Conversely, integrins can be activated when various ligands, like collagen, in the ECM bind and stimulate the cell to sense extracellular environments and react accordingly. Inactive integrins have a bent shape and are unable to transmit molecular signals(*21*). Primed but closed integrins are elongated but have yet to make either intracellular or extracellular interactions to promote signaling at focal adhesions(*21*). The switch between active and inactive integrin conformations is further enabled by endocytic or exocytic shuttling(*22*).

To bypass the lack of intrinsic enzymatic activity, integrins can transmit signals to the cell through recruitment of various adaptor and effector proteins to the focal adhesions(*23*). At least 232 protein components are recruited to adhesion complexes in a cell type and context dependent manner, highlighting the potential for adhesion signaling to bring about different cellular outcomes(*24*). The groups of proteins recruited to sites of active integrins include adaptors, actin remodeling proteins, signaling proteins, GTPase regulators, GTPase activating proteins, and numerous serine, threonine and tyrosine kinases and phosphatases(*25*).

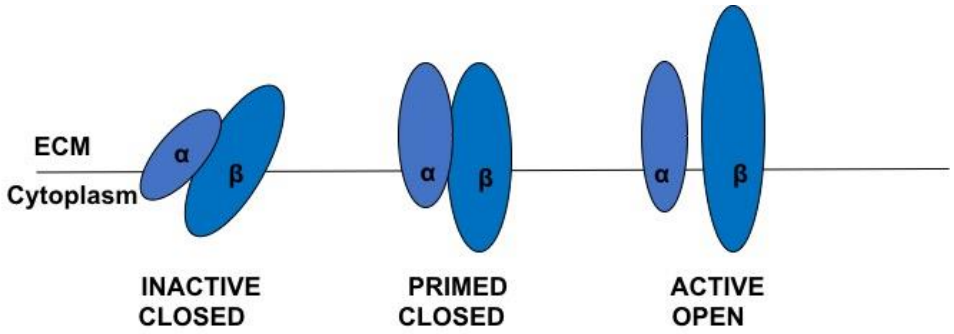


Figure 4: Three conformational states of integrin heterodimers. Integrin is conformationally bent when it is inactive, leading to closed bi-directional signaling. Integrin can be primed through protein interaction but still unable to transduce signaling. In the active conformation, integrin heterodimer undergoes a conformational change to allow signaling.

The α3β1 heterodimer is the most highly expressed integrin on the podocyte cell surface(*26*). Homozygous mutations in *ITGA3,* the gene encoding integrin α3, in humans leads to congenital nephrotic syndrome highlighted by defects in the GBM. Mice lacking α3 subunit die in the neonatal period and have aberrations in glomerular capillary loops, a disorganized GBM and podocyte foot process abnormalities(*27*). More specifically, podocyte specific α3 deletion caused nephrotic syndrome by 5 weeks of age and complete foot process effacement, and the inability to form mature foot processes(*28*). No human mutations affecting the β1 subunit have been described to date. Podocyte specific β1 deletion resulted in mice that developed severe end stage renal failure by three weeks of age characterized by proteinuria, podocyte loss and glomerulosclerosis(*29*).

1.5 Integrin Signaling in the Kidney

Matrix adhesion controls integrin activation and in turns recruits a number of focal adhesion proteins such as talin, integrin linked kinase (ILK), focal adhesion kinase (FAK), PINCH, α-actinin-4, p130Cas and Dok (Figure 5). Talin is a 270 kDa protein comprised of an N-terminal round head and a malleable rod domain. The talin head domain binds directly to integrin β

cytoplasmic tails and participates in inside-out signaling, where integrins transform from their resting state to an active state (integrin activation)(*30*, *31*). Phosphorylation of tyrosine resides on the cytoplasmic tails of integrin inhibits talin binding, therefore inhibiting integrin activation. Talin dependent recruitment of further proteins to active integrins causes the subsequent formation of focal adhesions(*32*). Talin-1 expression in podocytes is essential for the unique actin morphology of foot processes. Podocyte-specific *Tln1*-knockout mice develop proteinuria and succumb to death within ten weeks. *Tln1*-knockout mice exhibited a disturbed actin cytoskeleton and podocyte foot process effacement(*31*). Therefore, talin is an integral component involved in signal transduction between integrins and the actin cytoskeleton in podocytes.

Vinculin is another key adaptor protein involved with integrin-mediated adhesion complex formation. Vinculin is a 123kDa protein recruited by talin to focal adhesions. Vinculin comprises of an N-terminal head, proline rich neck, and a C-terminal tail domain. Upon talin recruitment, vinculin can undergo a conformational change to become an open active state(*33*). Contractile force pressure from extracellular matrix ligands promotes recruitment and release of focal adhesion proteins, like α-actinin, actin and paxillin(*34*). The N-terminal head of vinculin modulates integrin clustering, while the C-terminal tail domain links to actin(*33*). Mice with podocyte specific knockout of vinculin had increased albuminuria and foot process effacement following injury *in vivo*, suggesting that vinculin is necessary to maintain the integrity of the glomerular filtration barrier by modulating foot processes and in turn, stabilizing intercellular junctions.

Paxillin, a scaffolding protein, is another integral component of focal adhesions. It contains a C-terminal LIM domain(*35*). Localization of paxillin to focal adhesions is regulated through tyrosine and serine phosphorylation. The N-terminus of paxillin provides a docking site for vinculin, FAK, and Crk. Paxillin, like vinculin, is recruited to focal adhesions by talin. This results in downstream recruitment of Rho family small GTPases(*36*).

FAK is a non-receptor tyrosine kinase that is recruited to focal adhesions via talin and paxillin. It is responsible for recruiting p130Cas, Crk1/2 and Src family kinases. Total knockout of FAK in mice resulted in embryonic lethality characterized by migration defects*(38)*. Podocyte specific deletion of FAK in mice resulted in a normal phenotype with no proteinuria, however, uniquely, these mice were resistant to podocyte injury(*38*). Furthermore, podocyte injury was reduced when a FAK inhibitor was administered in a mouse model of glomerular injury.

p130Cas localizes to the focal adhesions with accumulation at the ends of F-actin stress fibers in foot processes, and is able to interact with the GBM and the slit diaphragm though its interactions with other adaptor proteins(*39*). Systemic deletion of p130Cas leads to embryonic lethality(*40*) and a podocyte-specific model is not yet characterized.

In conclusion, cell adhesion to the GBM occurs through a complex cell-matrix interface. Scaffolding proteins localize to focal adhesions propagating recruitment of effectors to transmit information between extracellular and intracellular environments.

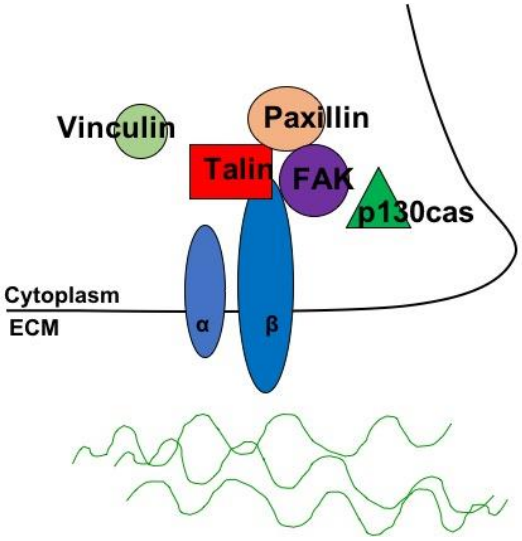


Figure 5: Integrin signaling through various proteins at the glomerular basement membrane to control focal adhesion dynamics. In the active open confirmation, integrins interact with Talin, FAK, paxillin, p130cas and vinculin to form focal adhesions.

1.6 Dok Family of Adaptor Proteins

Integrin signaling is further regulated by Dok (downstream of tyrosine kinase) proteins, particularly Dok1 and Dok2. The Dok family of adaptor proteins contains 7 homologues: Dok1-7. Dok1, the first Dok protein to be discovered, was found to be readily tyrosine phosphorylated downstream of a wide range of tyrosine kinases as well as upon cell adhesion and it can associate with p120rasGAP and Nck(*41–44*). Dok1-3 are preferentially expressed in immune cells and regulate several signaling pathways therein(*45*). However, studies suggest that these Dok proteins are also detectable in non-hematopoietic cells, suggesting a non-immunological function(*45*). Dok4-7 are preferentially expressed in non-hematopoietic cells(*46*).

All members of the Dok family have NH_2-terminal pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains, followed by SH2 target motifs in the COOH-terminal

moiety (Figure 6)(*41*). PTB domains can be separated into three unique classes according to their structures and binding preferences. Dok PTB domains fit into the insulin receptor substrate (IRS)-1 like group because they have a similar basic PH domain-like core structure(*45*). PTB domains show preferential binding to phosphotyrosine or tyrosine residues embedded within an NPxY motif, though domains of the IRS-1 like groups tend to prefer phosphorylated ligands(*47*).

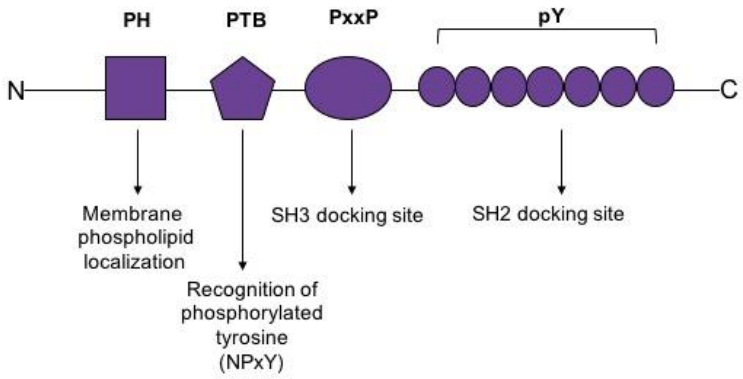


Figure 6: Schematic of Dok protein. Dok1 contains a pleckstrin homology (PH) domain, a phosphotyrosine binding domain (PTB), and lastly a carboxyl-terminal tail rich in tyrosine and proline residues. Dok1 shares high homology sequence with Dok2 and Dok3.

The Dok1 PTB domain has been shown to compete with the talin PTB domain for binding to the NPxY sequence on the integrin cytoplasmic tail. Binding of talin to the non-phosphorylated NPxY sequence on integrin β3 induces inside-out activation of integrin. However, when integrin β3 is phosphorylated on this sequence by Src, talin is unable to bind to phosphorylated integrin β3 (Figure 7)(*48*). Under these circumstances, Dok1 is able to outcompete talin, as the PTB domain of Dok1 can now bind the phosphorylated NPxY sequence on the cytoplasmic tail of integrin β3(*48*). Altogether this results in a conformational change and reduces inside-out activation/matrix adhesion of integrins. Notably, this NPxY sequence is also present on integrin β1(*48*). Consistent with this effect on integrins, Dok1 localizes to focal

adhesions. Overexpression of Dok1 enhances cell motility, while Dok1 loss in mouse embryonic fibroblasts (MEFs) impairs cell spreading, similar to that observed in Nck1/2-null MEFs(*49*).

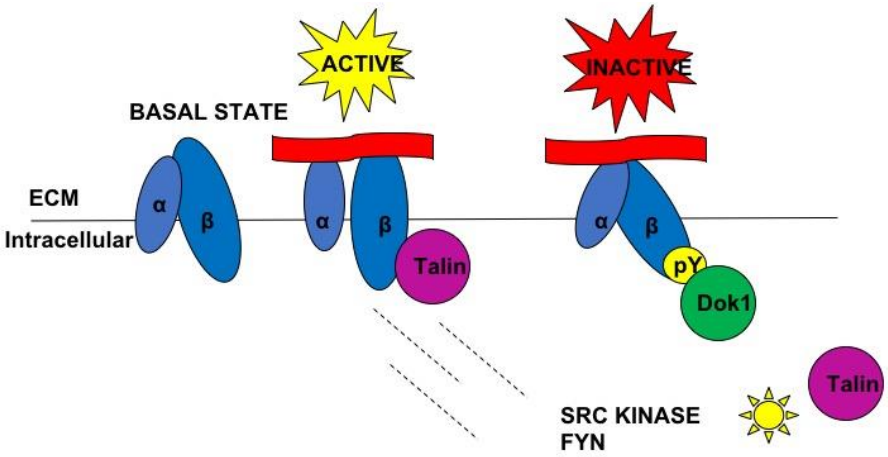


Figure 7: Competition of integrin binding between talin and Dok1. Talin PTB binds and activated integrin β1 to promote adhesion. Dok1 PTB binds Src-phosphorylated integrin β1, inactivating it and disrupting matrix binding.

Dok2 was identified as one of the several tyrosine phosphorylated proteins derived from bone marrow taken from patients with chronic myeloid leukemia(*50*). Dok2 is the closest related family member of Dok1, and shares similar functions. Dok2 undergoes tyrosine phosphorylation in platelets in response to stimulation by thrombin and integrin receptors. It has been shown to establish docking sites for downstream SH2 domain-containing signaling proteins including Nck(*44*). Overexpression of Dok2 negatively influences cell growth and Ras activation in response to various stimuli while loss of Dok2 enhances platelet adhesion due to increased integrin activation(*51*, *52*).

A podocyte-specific survey of focal adhesion proteins using genetic reporter models in combination with iTRAQ-based mass spectrometry led to the *in vivo* description of almost 3,500 proteins, including Dok1(*53*). Furthermore, Dok1 was identified as a putative binding partner of

Nck in an unpublished mass spectrometry study conducted in cultured human podocytes by our lab. The role of Dok proteins in podocytes has yet to be investigated.

1.6.2 Characterization of Dok Knockout Mice

Knockout mouse models have been created for Dok1, Dok2, Dok3 and Dok7(*50*, *54*). No single KO mouse models have been established for Dok4-6. Along with single KOs, a double knockout of Dok1/2 has been created, as well as a triple Dok1/2/3 KO. Dok1 KO mice have increased lung adenocarcinoma incidence, abnormal T cell and fibroblast proliferation, and abnormal bone marrow cell morphology/development(*54*). Dok2 KO mice have increased lung adenocarcinoma incidence and abnormal granulocyte physiology. Dok3 KO mice only have increased lung adenocarcinoma incidence. Single, double or triple compound loss of Dok in mice results in lung cancer, with penetrance and latency dependent on the number of lost Dok alleles(*54*).

Two independent Dok1/2 KO mouse lines were generated, on two separate backgrounds: C57BL/6 and 129/SV(*46*, *50*). Dok1 and Dok2 were shown to negatively regulate responses downstream of several immune receptors in lymphoid and myeloid cells. Dok1/2 KO mice develop myeloproliferative disease and exhibit diminished CD8+ T cell response against Herpes Simplex Virus-1(*55*). Dok1/2 KO mice are highly susceptible to induced colitis(*56*, *57*). C57BL/6 Dok1/2 KO animals develop lupus-like glomerulonephritis by 12 months of age(*57*). This phenotype was not reported in 129/SV Dok1/2 KO animals, nor in this line once migrated to a C57BL/6 background.

1.7 Rationale for Thesis and Objectives

Podocyte adhesion is evidently important for glomerular barrier integrity. Adhesion is dependent on adhesion complexes located at the GBM. Podocyte effacement is dynamic, phosphorylation driven and dependent on signaling proteins that regulate the actin cytoskeleton. By preventing or reducing some of the significant events that increase podocyte effacement, like altered focal adhesion turnover, it may be possible to prevent this pathogenic change. However, to truly understand this process, there needs to be more research completed in order to understand what regulates focal adhesion dynamics in podocytes. Dok1 was identified in an adhesion complex screen and is a known regulator of integrin activation. However, there has yet to be any research that investigates Dok1 and Dok2 proteins and their implications within the kidney. In this thesis, I will test the hypothesis that Dok1 and Dok2 proteins are novel candidates to regulate focal adhesion complexes in podocytes and may play an important role in signaling complexes within the podocyte adhesome.

The objectives of this thesis are to:

1. Profile Dok expression in glomeruli and podocytes in normal and disease models
2. Investigate kidney related phenotypes in Dok1/2 double knockout mice
3. Observe phenotype of Dok1/2 double knockout mice when subjected to a podocyte injury model
4. Investigate the signaling changes in Dok1/2 double knockout animals

CHAPTER 2: MATERIALS AND METHODS

Antibodies

Primary antibodies used for these experiments were as follows: mouse anti-B-actin (Sigma Aldrich, A41978), rabbit anti-Integrin β1 (Santa Cruz, 8978), mouse anti-p130cas (BD Transductions, 6102), rabbit anti-phospho-p130cas (Cell Signalling, 4011), rabbit anti-FAK (Cell Signalling, 3285), rabbit anti phospho-FAK (Cell Signalling, 3283), mouse anti-Paxillin (Upsate, 05-417), rabbit anti phospho-Paxillin (Cell Signalling 2541), rabbit anti-Flag (Sigma Aldrich, F7425), mouse anti-Dok1 (Santa Cruz, 6929), rabbit anti-Dok2(*58*), rabbit anti-phospho-Dok (Abcam, ab75741). Secondary antibodies (all Invitrogen) included horseradish peroxidase (HRP)-conjugated goat anti-rabbit (#A11008) and HRP-conjugated goat anti-mouse (#A11001).

Cell Culture

Human embryonic kidney (HEK) 293T cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich) supplemented with 10% Fetal Bovine Serum, 200 Units/mL penicillin and 200 μg/mL streptomycin (Invitrogen) and maintained at 37°C with 5% CO_2. Transient transfection was performed using 1-5 μg of DNA with polyethyleneimine (PEI) for 24-48 hours.

Cell Lysis and Western Blotting

Cells were lysed in Phospholipase C (PLC)+ lysis buffer (50 mM Hepes [pH 7.5], 150 mM NaCl, 10% glycerol, 1% Triton X-100, 15 mM MgCl2, 1 mM EGTA, 10 mM NaPPi, and 100 mM NaF) supplemented with fresh protease and phosphatase inhibitors (1 mM PMSF, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) by vortexing and sonicating on ice. Proteins from cleared lysates were resolved via SDS-PAGE and transferred to PVDF membrane

(Millipore). Membranes were blocked in TBST containing 5% nonfat milk powder or bovine serum albumin, and incubated overnight at 4°C with primary antibody. After washing, membranes were incubated with HRP-conjugated secondary antibody diluted at 1:10,000 in TBST for 1 hour at room temperature. Signals were detected using ECL Western blotting substrate (Pierce) or Luminata Crescendo (Millipore), and membranes were exposed to film (Pierce). Values used for densitometry were obtained using ImageLab v2.0 analysis software (BioRad).

Glomerular Isolation

Whole kidneys were collected and digested with collagenase A at 37°C for 10 minutes with gentle agitation. The digested tissue was gently pressed through a 100um cell strainer, followed by ice-cold sterile PBS flushing. The cell suspension was centrifuged at 1,000 rpm. The supernatant was discarded and red blood cells in the pellet were lysed in 10ml of Ack lysis buffer. After centrifugation, the supernatant was collected and remaining cells were lysed in PLC+, sonicated, and centrifuged for 10 minutes at 12,000rpm. The supernatant was collected and saved for future assays. Alternately, glomeruli were subject to biotinylation.

Cell Surface Biotinylation

Intact glomeruli were incubated with 5 ml of 1 mg/ml Sulfo-NHS-SS dissolved in borate buffer (10 mM boric acid, 150 mM NaCl [pH 8.0]) for 45 minutes under gentle agitation at 4°C. Coupling was terminated by washing two times (2 minutes each) with 15 mM glycine in PBS at 4°C. After a final wash in PBS, glomeruli were lysed in PLC+ buffer, and sonicated for 10 seconds. Lysates were then subjected to streptavidin agarose precipitation overnight at 4°C or resuspended in 2xSDS loading buffer to act as the input loading control. After overnight

precipitation, beads were washed three times successively in PLC+, and spun at 3000 × *g* for 1 minute between washes before finally being resuspended and boiled in 50 µl of 2xSDS loading buffer. Surface Integrin β1 in each sample was determined by dividing the density of band observed in immunoblotting of the precipitated sample by the density of band observed in the input sample. The ratio of this value in test samples to the value obtained in control samples was calculated and represents the amount of surface Integrin β1 in indicated test groups relative to control.

Husbandry of Dok1/2 Knockout Mice

Dok1-/- Dok2 -/- mice on the inbred C57BL/6J background (originally described by Pandolfi and colleagues(*59*)) were obtained from Dr. Pascale Duplay (Université du Québec, QC). Both male and female mice were used in the studies, as indicated. All experimental procedures were approved by University of Guelph Animal Care Committee and carried out in accordance with Canadian Council on Animal Care protocols

Genotyping

Mice were genotyped by PCR using DNA extracted from ear notches. Dok1 WT primers: (5'-GCCAGTTCCCTCCTCTAGATCTTC) and (3'-AGTGAAGGCCGTGACATCACCTG). Dok1 KO primers: (5'GCCAGTTCCCTCCTCTAGATCTTC) and (3'- CGCTCCCGATTCGCAGCGCATC). Dok2 WT primers: (5'- ATGGAGAGTCTGGCTGTGCC) and (3'- AAGCCAACAGGCAGATGGCC). Dok2 KO primers: (5' AACCACTACCTGAGCACCCAGTCC) and (3'- CGCTCCCGATTCGCAGCGCATC). PCR cycling conditions used for Dok1 were: 95°C for 5 minutes, then 40 cycles of 94°C (30 s), 58°C (30 s), 68°C (60 s) followed by a final extension at 68°C for 7 minutes. PCR cycling conditions used for

Dok 2 were: 95°C for 5 minutes, then 35 cycles of 94°C (30 s), 58°C (30 s), 72°C (45 s) followed by a final extension at 72°C for 7 minutes. DNA products were examined on 2% agarose gels.

Mouse Kidney Tissue Histology

For histology, kidneys were halved and fixed in 10% buffered formalin overnight before being embedded in paraffin. 4µm sections were cut and stained with PAS. Slides were viewed on a Leica DM100. Images were prepared for presentation using Adobe Photoshop CS5. For transmission electron microscopy (TEM), small pieces of kidney tissue were fixed in 0.1M sodium cacodylate buffer containing 4% paraformaldehyde and 2% gluteraldehyde (all from Electron Microscopy Sciences) and post fixed in 1% OsO_4, then dehydrated through graded ethanols. TEM samples were embedded in Quetol-Spurr resin. Ultrathin sections were cut and stained with uranyl acetate and lead citrate and viewed using a FEI CM100 TEM.

For quantitative analysis of kidney histology, glomeruli from each specimen were assessed on PAS-stained sections, and the level of glomerulosclerosis in each glomerulus was semi-quantitatively scored as follows: 0, no sclerosis; 1, sclerosis in less than 10% of glomeruli; 2, sclerosis in 10% to approximately 25% of glomeruli; 3, sclerosis in 25% to approximately 50% of glomeruli; 4, sclerosis in more than 50% of glomeruli(*60*). A total of 30 glomeruli were counted from 3 sections in 3-8 mice.

Induction of NTS nephritis

Cages, nestlets, food and water were sterilized by autoclaving before use. 6–8 week old mice were anesthetized using isoflurane followed by retro-orbital injection of 20-70 µl of an anti-GBM serum (rabbit anti-mouse glomeruli serum procured from Probetex INC, PTX-001).

Urine samples were collected every 24 hours after antiserum injection. On day 7, the mice were sacrificed by CO_2 and cervical dislocation, and kidneys were removed.

Evaluation of Proteinuria

Mice were placed into a clean cage without bedding until they urinated freely. Urine samples (5µL) were diluted in 2x SDS Sample buffer, then run on 10% SDS-PAGE gels and stained with Coomassie brilliant blue R. The urinary albumin/creatinine ratio (ACR) was determined using the Albuwell M ELISA (Exocell) and creatinine companion (Exocell) kits according to the manufacturer's instructions.

Nephroseq Analysis

Human Dok1 and Dok2 expression data were downloaded from Ju *et al.* and Hodgin *et al.* data sets using the Nephroseq data mining platform (www.nephroseq.org, 2017; University of Michigan, Ann Arbor, MI).

Statistics

Values are presented as mean ± S.E.M. Statistical analysis was performed using 2-tailed Student's *t* test or 1-way ANOVA (Prism). Graphs were prepared using Graphpad Prism version 5.0 (Graphpad Software). $P < 0.05$ was considered statistically significant.

CHAPTER 3: RESULTS

3.1 Profiling Dok1/2 expression using open access human databases

To first examine the distribution of Dok1 and Dok2 in human kidney, we utilized the Human Protein Atlas (HPA) Tissue Atlas, which is an open access online database featuring antibody-based imaging of proteins across 44 different human tissues, including the kidney. All antibodies used are controlled for cross-reactivity and western blotting is performed to ensure antibody specificity. Analysis of Dok expression in kidney sections in the HPA shows prominent staining in glomeruli in a pattern consistent with that of nephrin in podocytes for Dok1 but not Dok2 (Figure 8). Specifically, for Dok1, cells in glomeruli show medium staining with moderate intensity, with signal localized to the cytoplasm, while cells in tubules show low staining. This pattern is consistent across all three samples provided on the HPA, which are a mixture of females and males and age groups. For Dok2, no expression is detected in glomeruli, tubules or total kidney, across six samples ranging in age and sex.

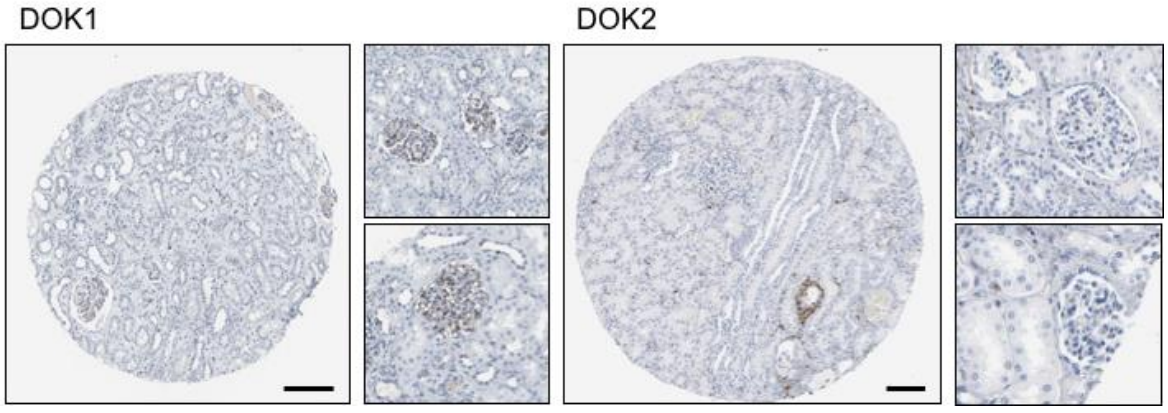
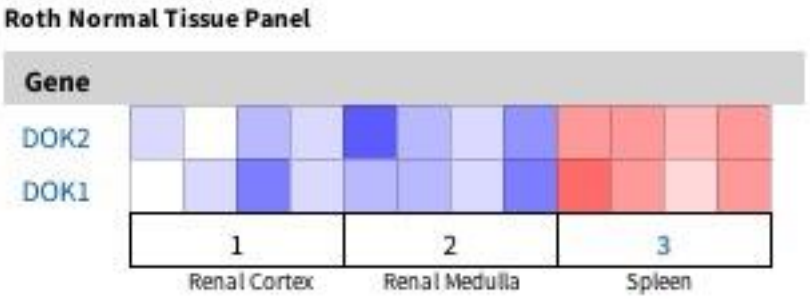


Figure 8: Staining of Dok1 and Dok2 in kidney glomeruli *in vivo.* These images, taken directly from the online HPA database, show staining of Dok staining in kidney glomeruli. Dok1 staining is observed in kidney glomeruli, whereas, there is no Dok2 staining observed. All staining was in normal kidney tissue of a variety of ages and sexes. Adapted from Human Protein Atlas. Scale bars represents 200um.

Next, we used Nephroseq, which is a web-based database of multiple genome-wide expression studies of patients with kidney disease as well as healthy donors. Consistent with the HPA, *Dok1* and *Dok2* are not highly expressed in normal kidney in either the cortex or medulla compared to spleen (Figure 9a). Interestingly however, both *Dok1* and *Dok2* are overexpressed in multiple datasets representing several different forms of kidney disease including lupus, IgA nephropathy, and focal segmental glomerulosclerosis, with upregulation in glomeruli (Appendix Table 1). As an example, in the Ju CKD Glom dataset (199 CKD patients) in the lupus disease subgroup (32 CKD patients) *Dok2* is within the top 1% of overexpressed genes (1.82 fold change, p=5^-12), while *Dok1* is within the top 4% (1.18 fold change, p=8.78^-6) (Figure 9b). We observed expression of Dok1 and Dok2 in our mouse podocyte cell line (Figure 10a). However, we saw very little expression of Dok1 and Dok2 in glomerular lysate (Figure 10a). Our mouse podocyte cell lines are a pure culture, whereas the glomerular lysate contains multiple cell types. Together these findings suggest that Dok1 and Dok2 are minimally expressed in normal glomeruli, but upregulated in kidney disease.

A



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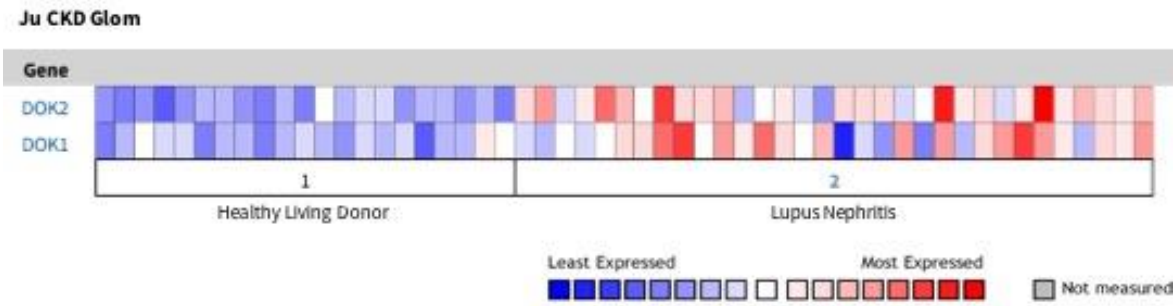


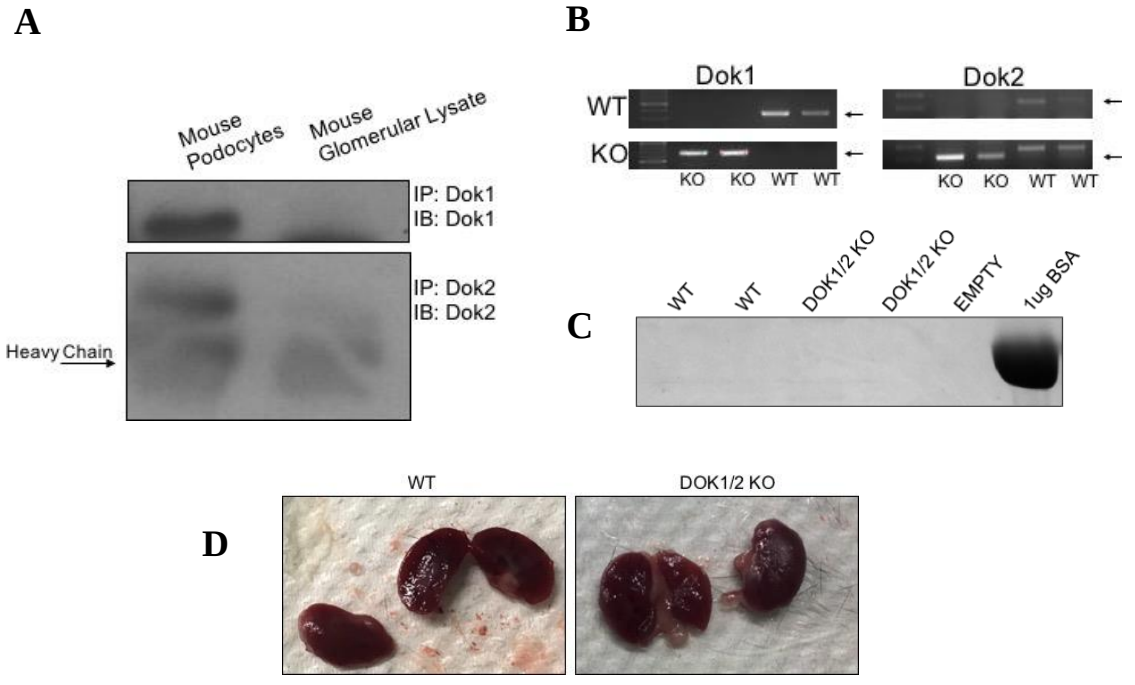
Figure 9: Heat maps of Dok1 and Dok2 expression in normal tissue panels and disease panels. A) Dok1 and Dok2 are not highly expressed in normal tissue panels. Expression of Dok1 and Dok2 are high in normal spleen. Roth Normal Tissue Panel consists of 353 samples. Human microarray of 19,574 measure genes in multiple tissue types. B) In Lupus Nephritis, expression of Dok1 and Dok2 is upregulated and expressed. Ju CKD Glom Panel consists of 199 samples. Human microarray of 17,379 measured genes in glomeruli. (Adapted from Nephroseq).

3.2 Dok1/2 knockout mice show no overt kidney phenotypes

To investigate the role of Dok1 and Dok2 in kidney homeostasis, we analyzed the phenotype of compound Dok1/Dok2 knockout mice. These mice harbour a total body deletion of both Dok1 and Dok2, as no floxed lines currently exist to perform podocyte-specific knockout studies. Dok1/2 KO mice on the inbred C57BL/6 background were genotyped according to established protocols using Dok1 and Dok2 specific primers (Figure 10b).

General phenotypic features of mice null for Dok1 and Dok2 were described previously. An analysis of 324 mice starting at 11 months of age, revealed that double mutant genotypes develop lung adenocarcinomas at a medium to high penetrance. In addition, these double knockout animals displayed a survival defect, with animals beginning to die at approximately 1

year(*50*). We investigated this phenotype in our mice, and observed no such phenotype, even at 1.5 years of age. However, to take into account previous findings and to minimize effects of these late onset phenotypes, mice were analyzed at 6 months of age. Urine analysis on Dok1/2 KO mice using SDS-PAGE followed by Coomassie blue staining demonstrated no albuminuria (Figure 10c). Additionally, urine analysis on Dok1/2 KO mice at 12 months of age showed no baseline albuminuria (figure not shown). Whole kidney analysis showed no overt changes in weight, shape or colour (Figure 10d). To determine if deletion of Dok1 and Dok2 can affect glomerular morphology, kidney samples at 6 months of age were processed for histological and ultrastructural analysis. Light microscopy (periodic acid Schiff staining) showed normal glomerular and tubular morphology in both WT and Dok1/2 KO at 6 months of age (Figure 10e). Transmission electron micrographs showed comparable appearance of the glomerular filtration barrier in both WT and Dok1/2 KO mice, with normal foot processes, distinct slit diaphragms, non-thickened GBM and intact endothelium (Figure 10f).



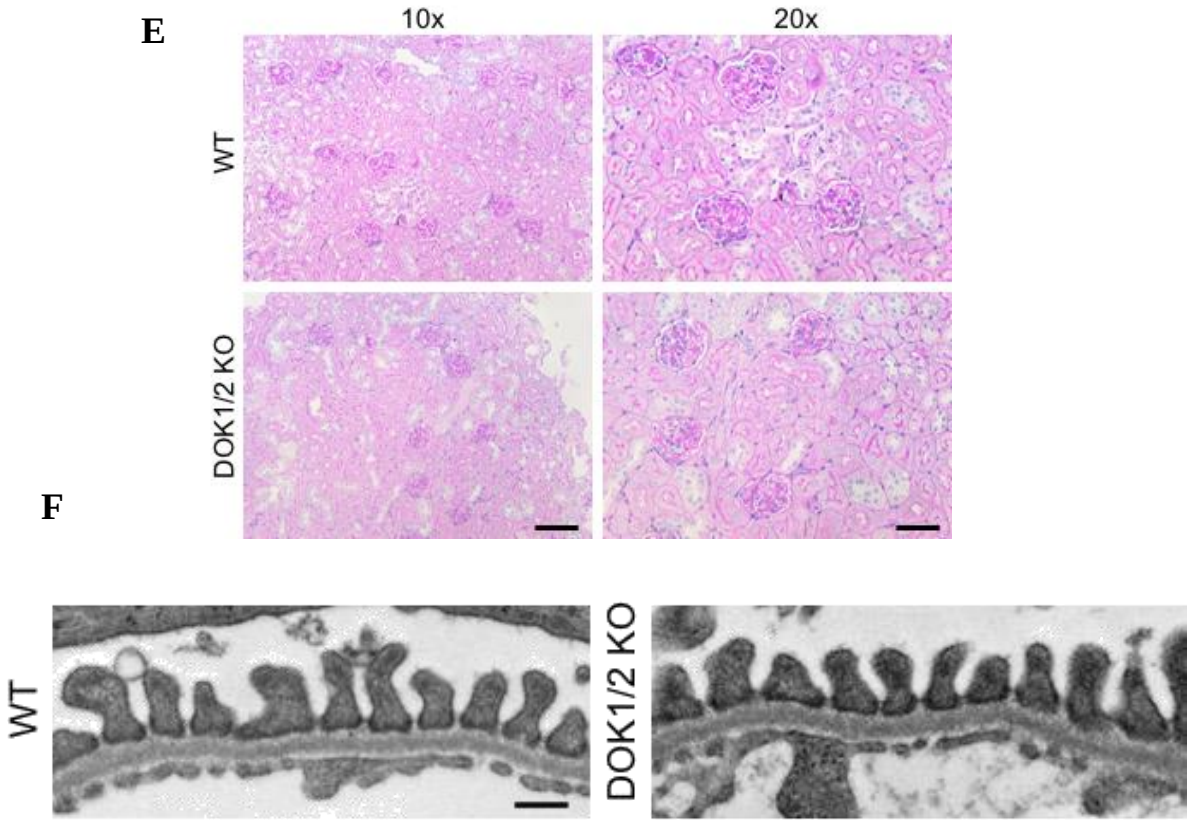


Figure 10: Dok1/2 KO mice show no overt renal phenotype. A) Dok1 and Dok2 are expressed in mouse podocyte cells, but not in glomerular lysate. B) Dok1/2 KO mice genotypes were confirmed using standardized PCR protocols. C) Coomassie-stained polyacrylamide gel of 6 month old mice showing no signs of proteinuria. D) Whole mount and cross section photos of WT and Dok1/2 KO mice showing no physical abnormalities. E) PAS stains of WT and Dok1/2 KO mice show absence of protein casts or glomerular abnormalities. Scale bar represents 100um (10x) and 50um (20x) F). TEM of both genotypes confirms normal glomerular filtration barrier morphology. Scale bar represents 250nm.

3.3 Dok1/2 KO attenuates proteinuria after glomerular injury

Given that Dok1/2 KO mice showed no obvious glomerular abnormalities even with aging, we next determined whether Dok1/2 loss might impact response to podocyte injury. We used the nephrotoxic nephritis model, which involves injection of antibodies against antigens from the GBM. Rabbit anti-mouse GBM antibodies (also known as nephrotoxic serum [NTS]) or normal IgG (as a control) are injected. In this model, proteinuria peaks at 24 hours post-NTS injection, and resolves within 72-96 hours later(*61*). We first used a low dose protocol, where Dok1/2 KO and WT male mice received 1ug/ul of NTS at 8 weeks of age. Urine was collected passively from mice prior to and at 24 hour intervals after injection for 96 hours. Dok1/2 KO and WT mice that received NTS showed a marked increase in albumin excretion detected in urine samples collected at 24 hours post injection (Figure 11). Intriguingly however, Dok1/2 KO mice displayed lower levels of albuminuria at 24 hours post injection, and by 48 hours, Dok1/2 KO mice showed little evidence of albuminuria compared to WT controls (Figure 11). By 96 hours post injection, both Dok1/2 KO and WT mice were fully recovered.

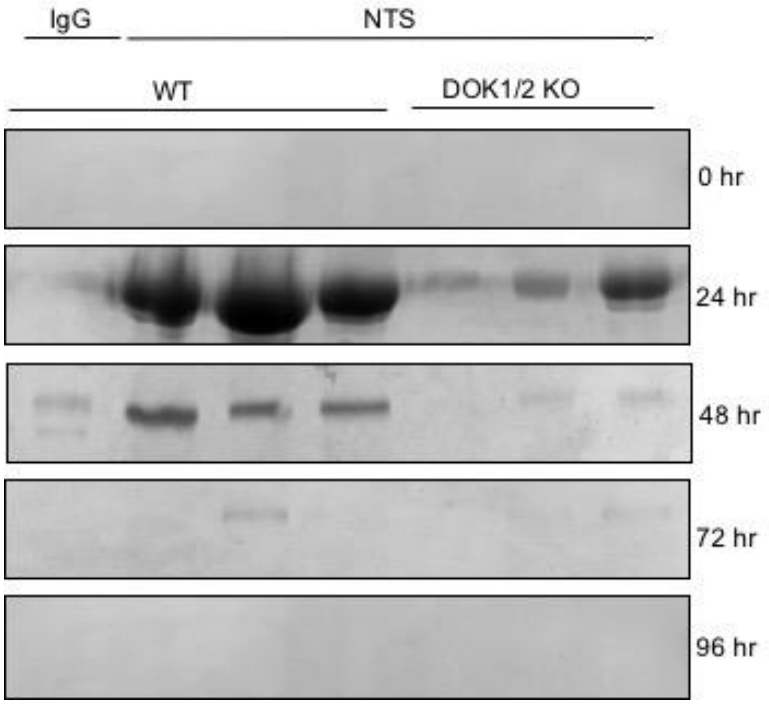
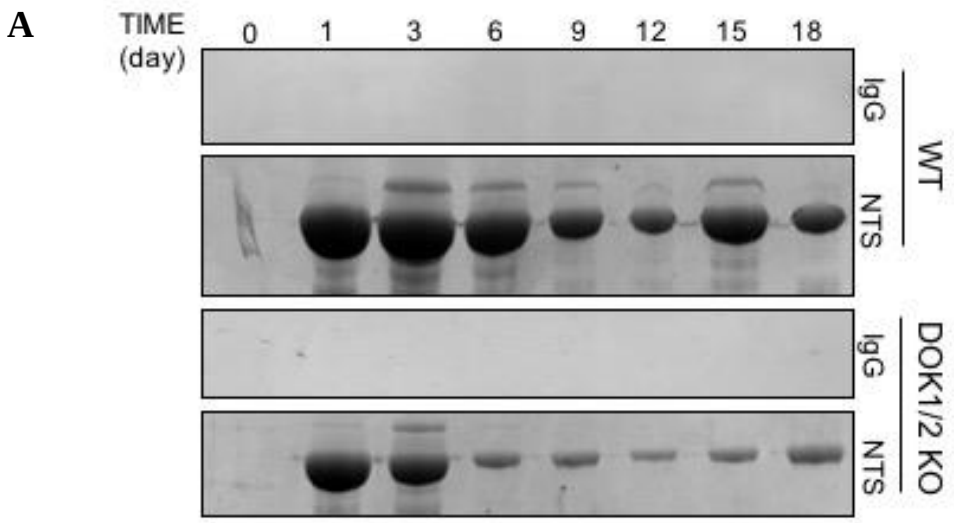


Figure 11: Coomassie urine gel of NTS 1ug/ul reveals Dok1/2 KO mice may be protected from injury. Urine samples were prepared and resolved on 10% gels, incubated in Coomassie stain and eventually destained to reveal protein in urine. At 24 hours, Dok1/2 KO mice did not exhibit high proteinuria levels relative to WT. Dok1/2 KO mice were able to recover fully by 48 hours, however, it took WT mice 72 hours to recover. Coomassie-stained polyacrylamide gels are a representation of 4 WT mice and 5 Dok1/2 KO mice.

We next investigated the effects of a higher dose of NTS, which induces more severe and sustained proteinuria(*62*). Mice were challenged using 2.13ug/ul of NTS and monitored for

18 days post injection, and urine was collected passively every 3 days. Similar to the low dose injection, Dok1/2 KO mice had decreased levels of albuminuria throughout the 18 day period in the high dose injection. At day 1 post injection, both Dok1/2 KO and WT mice presented with high levels of albuminuria (Figure 12a). By day 6 post injection, Dok1/2 KO mice exhibit decreased levels of albuminuria compared to WT mice (Figure 12a). At day 18 post injection, both Dok1/2 KO mice and WT mice presented with decreased levels of albuminuria relative to day 1, however, these mice still showed physical signs of being sick. Albuminuria demonstrated by Coomassie Blue staining was quantitated using urine albumin/creatinine ratios (ACR). ACR is calculated by dividing albumin concentrations by creatinine concentrations, where creatinine numbers stay relatively constant. At day 6 post injection, significant proteinuria was measured for WT mice but not for Dok1/2 KO mice relative to time 0 respectively for each genotype (Figure 12b,c). Statistical significance was measured using a one-way ANOVA for each genotype. A significant proteinuric difference was observed between WT and Dok1/2 KO at 144 hours using a two-way ANOVA (Figure 12d)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(day)
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IgG
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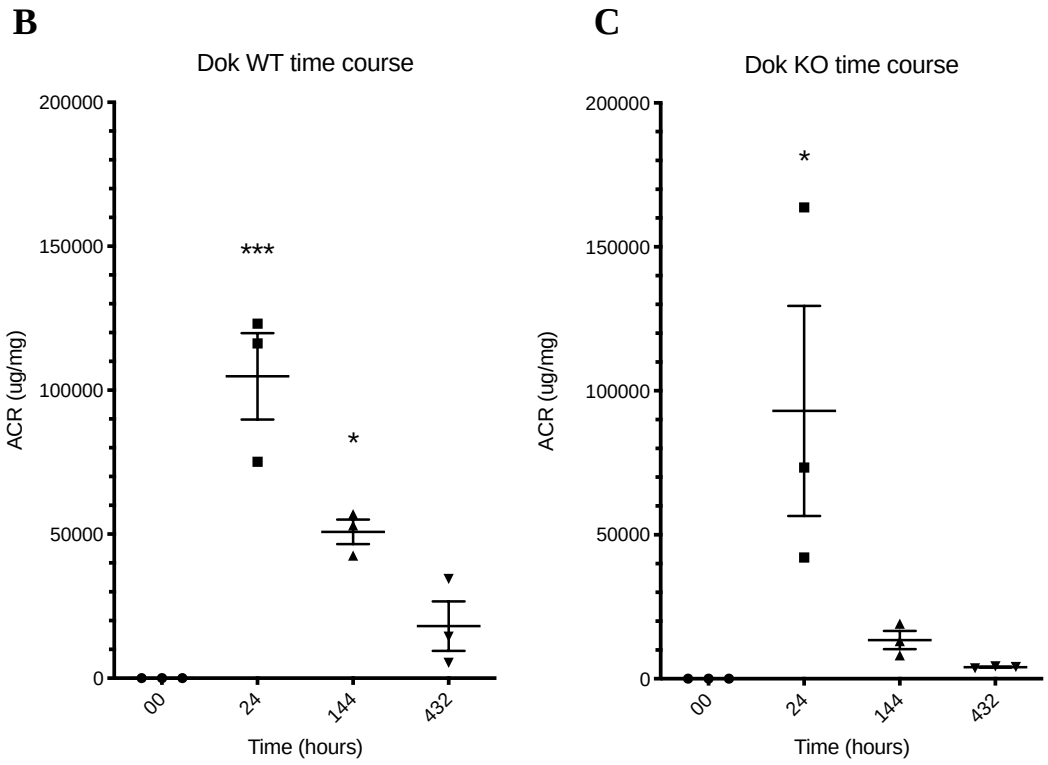
DOK1/2 KO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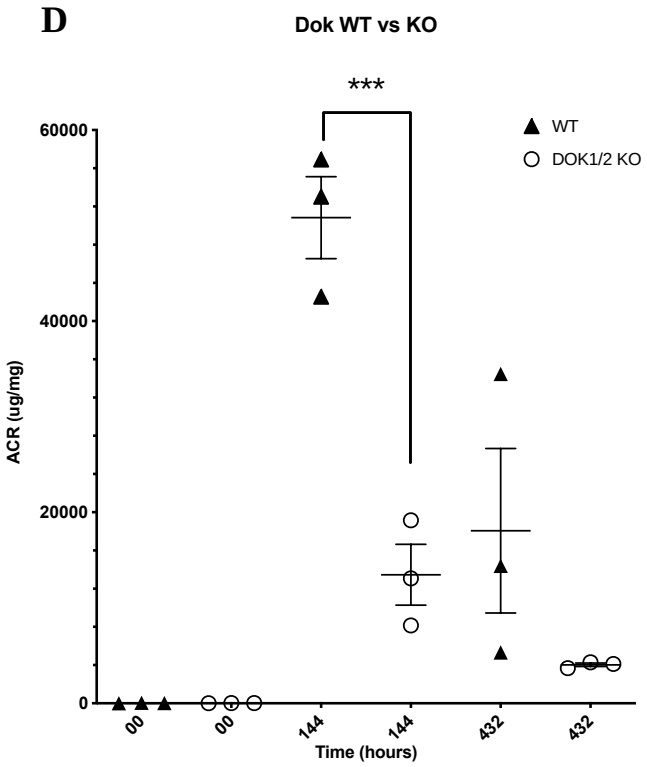
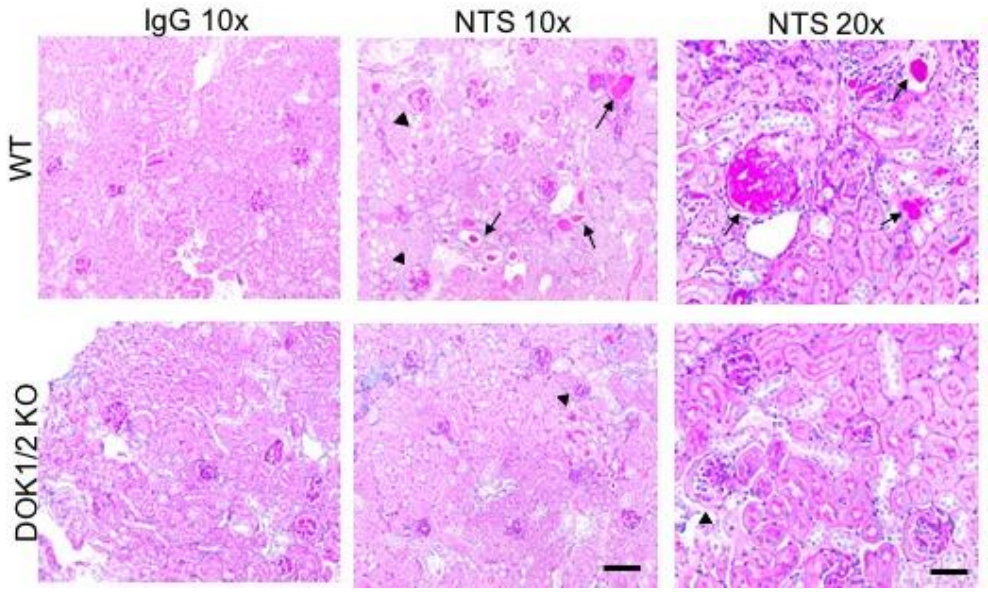


Figure 12: Coomassie urine gel of NTS 2.13ug/ul reveals Dok1/2 KO mice show an attenuated injury time course. A) Urine samples were prepared and resolved on 10% SDS-PAGE gels, incubated in Coomassie stain and eventually destained to reveal protein in urine. At day 1, both WT and Dok1/2 KO mice exhibit proteinuria. By day 6, Dok1/2 KO mice start to recover, and low levels of protein are detected in the urine. However, at day 6 for WT mice, there are still high levels of protein in the urine. 0.1 ul of urine was loaded. Coomassie-stained polyacrylamide gel are a representation of 3 WT mice and 4 Dok1/2 KO mice. B) ACRs results reveal significance using one-way ANOVA (* = $p<0.05$). C) ACRs results reveal significance using two-way ANOVA comparing WT and Dok1/2 KO at 144 hours (*** = $p<0.001$)

At 18 days post injection, both genotypes still showed signs of disease. Mice were allowed a week more to recover, however, by day 25 post injection, no full recovery was made for these animals, as Coomassie-stained polyacrylamide gels still showed levels of albuminuria (figure not shown). Dok1/2 KO and WT animals were sacrificed for tissue processing to assess

glomerular damage. Histology results revealed severe phenotypes for WT mice. PAS staining highlighted protein casts in the tubules of WT mice, but not in the Dok1/2 KO mice (Figure 13a). PAS staining also revealed a large number of glomerular crescents in Dok1/2 KO animals, and some WT mice samples (Figure 13a). Glomerular scarring was assessed analyzing 30 glomeruli at various sections from three mice. Glomerular scarring quantification was conducted as previously described in literature[60]. All scoring was conducted in 10x field view, analyzing glomerular damage. Dok1/2 KO mice scored a sclerosis index of 1.86, whereas WT mice scored a sclerosis index of 3.30. A normal glomerular sclerosis index ranges from 0-1[60]. These were observed in our IgG animals for both genotypes.

A



B

Sclerosis Index		
	WT	**DOK1/2 KO**
IgG	0.86	0.77
NTS	3.30	1.86

Figure 13: Dok1/2 display no protein casts and score a relatively low sclerosis index. A) PAS staining of NTS (2.13) injected mice revealed high levels of protein casts (arrows) and glomerular crescents (arrow heads) for WT mice, but not Dok1/2 KO mice. Fewer and less severe glomerular changes were observed in Dok1/2 KO mice. Scale bar represents 100um (10x) and 50um (20x). B) PAS images were quantified to obtain a sclerosis index, based on the amount of glomerular scarring observed. WT mice scored a higher sclerosis index.

3.4 Loss of Dok1/2 results in a modest integrin hyperactivation

Given the potential for Dok proteins to regulate integrin activity, we next explored expression and localization of a prominent integrin subunit in podocytes, integrin β1. Glomeruli were purified from collagenase-digested kidney, incubated in biotin buffer, then lysed and subjected to streptavidin agarose precipitation to enrich for surface expressed protein. Biotin bound integrin β1 in Dok1/2 glomerular lysate was compared to WT mice, all relative to total (input) integrin β1. Dok1/2 KO animals had higher levels of surface integrin β1, relative to WT, though no differences in total integrin β1 were seen between genotypes (Figure 14). Dok1/2 KO

biotin bound integrin β1 was significantly ($p<0.05$) increased compared to WT animals relative to input integrin β1 using a Two-Tailed Student T test.

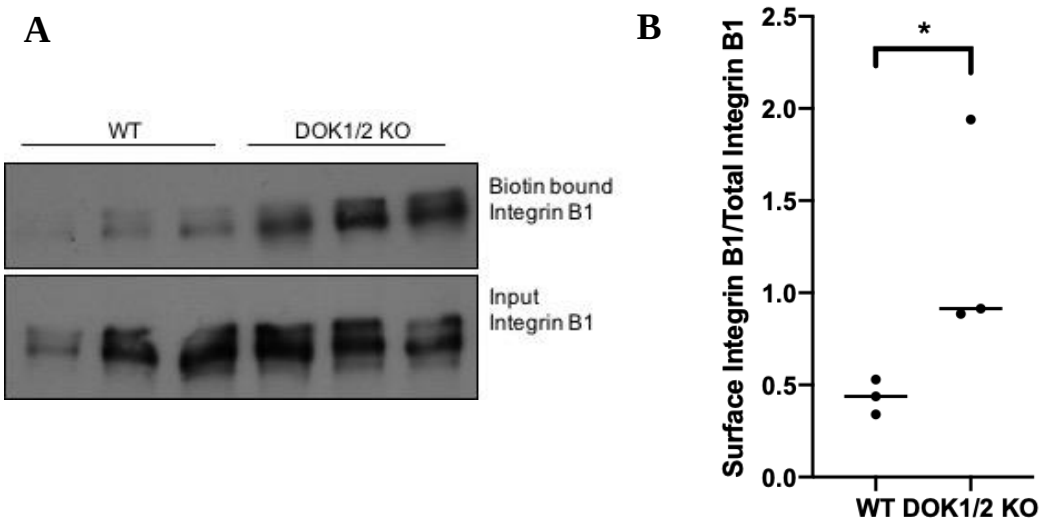


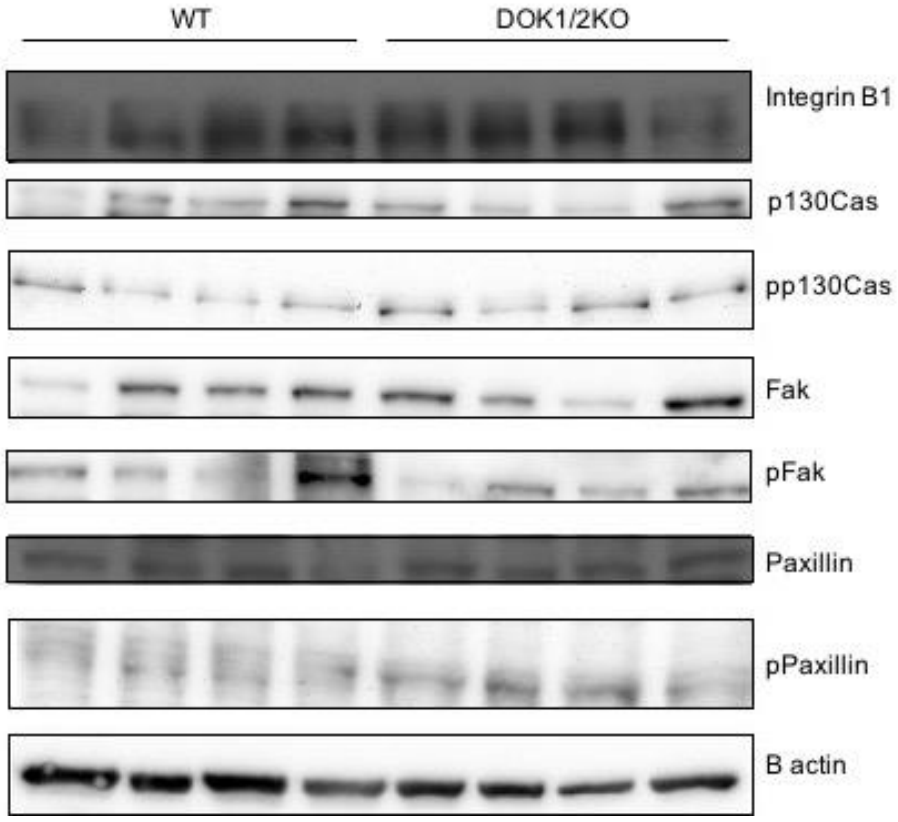
Figure 14: Biotinylation assay of glomerular lysate uncovered high levels of biotin bound integrin β1 for Dok1/2 KO mice relative to total integrin β1. A) Dok1/2 KO glomerular lysates had increased levels of biotin bound integrin β1 relative to WT. Biotin bound Integrin β1 were compared over input, or total integrin β1 and graphed. B-actin was used as a loading control. B) Densitometry results revealed significance using a Two-Tailed student T-Test (* = $p<0.05$).

3.5 Loss of Dok1/2 alters focal adhesion signaling pathways

Finally, as Dok1/2 KO mice show alterations in surface integrin levels, we explored whether this might correspond with changes in expression and/or phosphorylation of focal adhesion proteins. Western blotting analysis of WT and Dok1/2KO glomerular lysates was performed, followed by densitometric quantitation. No significant changes in total or phosphorylated p130cas or FAK were observed between genotypes (Figure 15a-c). By contrast,

phosphorylation of paxillin was significantly increased in glomerular lysates from Dok1/2 KO animals relative to WT controls (Figure 15a,d).

A



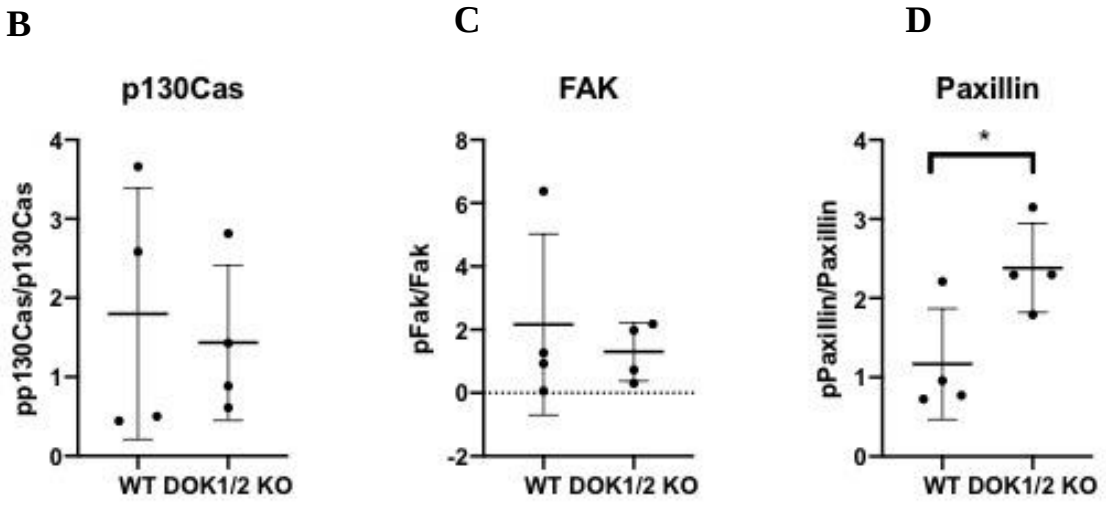


Figure 15: pPAX is significantly increased in Dok1/2 KO mice compared to WT. A) Glomerular lysate was obtained, prepared with 2X SDS and resolved on a 10% SDS-PAGE gel. A BCA assay ensured equal protein loading and B-actin was used as a loading control. 20ug of protein was loaded per lane. N=4 per sample group. B,C) No statistical significance was observed using Two-tailed student T test (unpaired) for p130Cas or FAK when comparing phosphorylation levels. D) Phosphorylation of paxillin in Dok1/2 KO mice was significantly increased compared to WT (P = 0.05).

CHAPTER 4: DISCUSSION AND FUTURE DIRECTIONS

The Dok family of adaptors, specifically Dok1, Dok2 and Dok3 are well characterized negative regulators of many tyrosine-based signaling pathways(*47*, *50*, *63*). They are phosphorylated by different kinases and involved in processes such as cell differentiation, proliferation, spreading and migration. Dok1 and Dok2 act as negative regulators of the Ras-Erk pathway downstream of many immunoreceptor mediated signaling systems. The biological functions of Dok1 and Dok2 have been studied primarily in the hematopoietic system, as these proteins are highly expressed in the spleen. However, the functional roles of Dok1 and Dok2 have yet to be studied in podocytes. In this study, we found that Dok1 and Dok2 may have a role in maintaining podocyte integrity. We report the following: (i) Dok1/2 deletion attenuates proteinuria upon glomerular injury, (ii) Dok1/2 KO mice have increased levels of surface integrin β1 and (iii) there is altered focal adhesion signaling in Dok1/2 KO mice. Overall, our findings reveal the potential importance of Dok1 and Dok2 in regulating podocyte homeostasis.

Analyses from the HPA illustrate Dok1 expression in glomeruli, but not for Dok2. Interestingly, both Dok1 and Dok2 are overexpressed in diseased states, such as lupus nephritis, but not in a healthy state. Although our studies focused primarily on Dok1/2 whole body knockout animals, our results may highlight an important role for Dok1 only, as it is expressed in glomeruli, whereas Dok2 is not. These assumptions are based primarily on the HPA, and further investigations within our lab have to be conducted. We will conduct antibody staining (Dok1 or Dok2 and podocin as a podocyte marker) of normal and kidney diseased human tissue. Concurrently, a recent mRNA and miRNA transcriptional profile with quantitative proteomic analyses revealed highly specific gene regulation in podocytes(*53*). Dok1 expression was detected within the *in vivo* podocyte transcriptome and proteome.

Mice deficient in Dok1 and Dok2 do not display any significant glomerular changes, though under podocyte injury, specifically nephrotoxic serum injections, Dok1/2 KO mice show attenuated proteinuria. We observed that Dok1/2 KO mice were susceptible to injury, but were quicker relative to WT mice to recover, in both low and high dose models. Glomerular dysmorphisms were characterized by protein casts and glomerular crescents. Nephrotoxic serum nephritis pathogenesis has been shown to occur in two phases: heterologous and autologous(*65*). The heterologous phase is mediated in part by the complement system, specifically complement C3(*65*). NTS studies conducted in mice deficient in C3 showed that these mice develop less severe injury in an acute heterologous model(*65*). On the other hand, the autologous phase is mediated by the adaptive immune system, which relies on B and T cells(*65*). Several studies in two different Dok1/2 KO mouse lines have looked at the role of these proteins in immune signalling, specifically within T cells. The Yamanashi group demonstrated that Dok1/2 KO mice show elevated responses to T cell receptor signalling and they conclude that the negative regulatory effect of Dok1 and Dok2 may be important for immune tolerance, as the KO mice develop a lupus-like nephritis characterized by immune complex deposition (including complement C3)(*57*). An additional study conducted by the Duplay group, however, showed that Dok1/2 KO mice exhibited diminished CD8+ T cell response during acute infection and impaired generation of memory CD8+ T cells(*66*). The Dok1/2 KO mice we have used in this study originate from the Duplay line, and though this is the same genetic background (C57BL/6J) as the Yamanashi line, we have not observed development of glomerulonephritis in the mice, even at 1.5 years of age. Nonetheless, due to the relationship between Dok1 and Dok2 expression in immune related disorders, and the

dependency of NTS on the complement system to cause glomerular disease, we cannot rule out the possibility that the attenuated phenotype is due to potential alterations in immune signalling rather than disruption in focal adhesion dynamics.

As such, to verify the role of Dok1/2 in podocyte injury, it will be important to use another injury model. Protamine sulfate (PS) perfusion is well established in our lab and used to model rapid and transient foot process effacement. Kidneys are perfused with PS to neutralize the negative surface charge of the podocyte, which is reversible upon subsequent perfusion with heparin sulfate. This is an excellent system for monitoring the contribution of early cell signaling events such as tyrosine phosphorylation to remodel the podocyte actin cytoskeleton, with no association to immunity. This data would validate and strengthen the results we have already observed in our Dok1/2 KO animals. In parallel, it will be prudent to make a podocyte-specific Dok1/2 KO (or just Dok1 conditional KO). Similar investigations as we conducted in this study should be replicated in these mice, and, if the same phenotypes are observed, we can confirm that Dok1 does have an integral signaling role in podocytes.

Although we have a strong attenuating phenotype in the Dok1/2 KO mice upon podocyte injury, the signaling pathways for adhesion remain unclear. Biotin labeling of integrin β1 in Dok1/2 KO glomerular lysate revealed increased surface levels. Therefore, loss of Dok1/2 resulted in integrin hyperactivation. Dok1 is a known negative regulator of integrin activation. Under phosphorylation conditions, Dok1 can outcompete talin to bind the cytoplasmic region of integrin β1(*48*). In turn, Dok1 inactivates integrin β1 by altering the conformational state of the integrin complex such that bi-directional signaling can no longer occur. In the absence of Dok1, talin would have no competition and can continually bind integrin β1, increasing the

number of focal adhesions, and in turn activating integrin signaling. Integrins lack intrinsic enzymatic activity, therefore active integrins recruit a number of adaptor and effector proteins. Talin dependent recruitment of further proteins, like FAK and vinculin, causes the formation of focal adhesions. Podocyte specific KO of talin causes severe glomerular dysfunction and early kidney failure(*31*). Therefore, we believe that our Dok1/2 KO mice have hyperlocalization of integrin β1 on the surface because talin is continually allowing bi-directional signaling.. Podocytes are constantly fluid structures, always needing to adjust to constant hemodynamic pressures. Focal adhesion turnover is vital to allow podocyte contractility and maintenance of the filtration barrier. In the absence of Dok, there is no negative control of integrin activation, resulting in increased focal adhesions enhancing the strength of the filtration barrier and barring podocyte detachment. This may be the reason we see Dok1/2 KO mice attenuate proteinuria. In line with our findings, the drug Abatacept has been shown to induce remission of proteinuria in patients with kidney disease, presumably through stabilization of integrin β1 activation(*67*),

Integrity and function of the podocyte is dependent on both cell-to cell and cell-to-matrix adhesion. The interplay between integrin and adhesion molecules dictates the glomerular basement interface. To address the signaling mechanisms at play, we compared Dok1/2 KO animal glomerular lysate protein levels relative to WT. Although there were no distinct difference observed in the Western blot analyses, we did observe a significant increase in the phosphorylation of paxillin in our Dok1/2 KO mice. Paxillin is a 70-kD protein localized to focal adhesions and dependent on integrin activation. Phosphorylation of paxillin upon integrin-dependent cell adhesion involves Src-FAK activity and is linked to the control of adhesion

turnover(*68*). Paxillin hyperphosphorylation is the consequence of the obstruction of focal adhesion disassembly. Again, our results suggest that increased levels of surface integrin β1 promotes more focal adhesions, but less turnover, observed by augmented levels of phosphorylated paxillin.

Similarly, FAK and p130cas are also key components of the signal transduction pathways triggered by integrins. Alongside vinculin, FAK is also a core constituent of focal adhesions and regulates turnover. Phosphorylation of FAK acts on paxillin and p130cas. Therefore, if we observed a significant increase in the phosphorylation of paxillin, we believe that it would also directly relate to an increase of phosphorylation of both FAK and p130cas. In order to accurately represent our theory, more glomerular lysate needs to be collected and compared.

An ongoing experiment in the Dok1/2 KO project is to obtain pure podocyte cultures from our WT and Dok1/2 KO mice. Deriving primary podocytes from isolated glomeruli from these mice will present a more accurate representation of the signaling pathways at play. Preliminary studies in our lab have shown that primary podocytes can be cultured on collagen-coated coverslips. The cultured podocytes were not pure, and often were susceptible to contamination within two weeks. However, due to antibody specificity and poor immunofluorescence capability, these cultured podocytes were unable to be viewed. Alongside our Western blot analyses, it is imperative we conduct a cell spreading assay where we can observe adhesion differences. We suspect a loss of Dok1 and Dok2 would result in a modest reduction in cell spreading (as seen in MEFs (*49*)) as a result of integrin hyper-localization at the

surface membrane and decreased levels of focal adhesion turnover defined by phosphorylated paxillin.

Another alternative method to studying Dok1/2 signaling pathways would be to create adenoviruses to knockdown Dok1 and/or Dok2 expression in our mouse podocyte cell lines. Dok1 adenoviruses are available for purchase from Addgene. Preliminary investigation and titer adjustments may need to optimized such that a successful knockdown can be achieved. Western blots may reveal clearer signaling pathways compared to glomerular lysate. Moreover, we would conduct cell spreading assays to determine cell adhesion dynamics to confirm previous results. Cells would also be dual immunofluorescence labelled to observe actin and focal adhesion morphology.

In conclusion, we report that Dok proteins may have a previously unrecognized role in the regulation of podocyte function. Our data suggests that knockout of Dok1/2 may attenuate disease by stabilizing signaling complexes at focal adhesions. Consistent with this, increased expression of both *Dok1* and *Dok2* is observed in human kidney diseases. These studies warrant further investigation to elucidate the molecular regulation of Dok expression in podocytes.

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APPENDIX

Table 1: Nephroseq analyses of Dok1 and Dok2. Adapted from Nephroseq.

Gene	Disease Group	Dataset	Probe ID	Tissue Subset	Gene Rank	Fold Change	P Value
Dok 2	Lupus	Ju CKD Glom	9046	glom	**75/17,379 (top 1%)**	**1.82**	**5^-12**
Dok 1			1796		742/17,379	1.18	8.78^-6
Dok 2	Vasculitis				**101/17,379 (top 1%)**	**1.84**	**5.8^-10**
Dok 1					279/17,379	1.22	6.87^-8
Dok 2	IgAN				**106/17,379 (top 1%)**	**1.55**	**1.32^-8**
Dok 1					**265/17,379 (top 2%)**	**1.20**	**8.11^-7**
Dok 2	DN				**468/17,379 (top 3%)**	**1.6**	**2.41^-4**
Dok 1					2338/17,379	1.08	.061
Dok 2	TBMD				**527/17,379 (top 3%)**	1.52	**.019**
Dok 1					3314/17,379	1.18	.232
Dok 2	Collapsing FSGS	Hodgin FSGS Glom	Hs.71215.0.S2_3p_at	glom	**108/19,139 (top 1%)**	**1.64**	**.003**
Dok 1			G6606314_3p_a_at		12729/19,139	-1.01	.543
Dok 2	FSGS				**313/19,139 (top 2%)**	**1.53**	**.008**
Dok 1					3218/19,139	1.10	.08
Dok 2	IgAN	Reich IgAN Glom	216835_s_at		**221/12,624 (top 2%)**	**1.51**	**1.76^-6**
Dok 1			214054_at		**252/12,624 (top 2%)**	**1.73**	**3.02^-6**
Dok 2	LN	Berthier Lupus Glom	214054_at	glom	**101/12,624 (top 1%)**	**1.86**	**1.02^-11**
Dok 1			216835_s_at		2396/12,624	1.22	.004
Dok 2	LN	ERCB Lupus Glom	ENSG00000147443	glom	**926/22,017 (top 5%)**	**5.83**	.014
Dok 1			ENSG00000115325		6925/22,017	-1.11	.504
Dok 2	DN	ERCB Nephrotic	ENSG00000147443	TubInt	**840/22,017 (top 4%)**	**6.20**	**3.45^-4**

		Syndrome TubInt					
Dok 1			ENSG00000115325		**452/22,017 (top 2%)**	**2.23**	**7.21^-5**
Dok 2	FSGS		ENSG00000147443		**1133/22,017 (top 6%)**	**4.65**	**9.54^-4**
Dok 1			ENSG00000115325		**6925/22,017 (top 32%)**	**1.68**	**.003**
Dok 2	LN	ERCB Lupus TubInt	ENSG00000147443	TubInt	**747/22,017 (top 4%)**	**3.46**	**.003**
Dok 1			ENSG00000115325		4007/22,017	1.31	.148

Table 2: Representation table of low dose NTS trial. Table showing mouse samples, genotype, weight and the dose received. IgG was used as a control. All injections were administered intraocularly.

Mouse	Weight (g)	Receiving	Dose (uL)
71 wt	24.1	**NTS 1.0**	24
72 wt	24.5	**NTS 1.0**	25
73 wt	21.6	**NTS 1.0**	22
74 wt	23.2	**NTS 1.0**	23
75 wt	26.3	**IgG 1.0**	26
77 ko	18.6	**NTS 1.0**	19
78 ko	23.6	**NTS 1.0**	24
79 ko	23.1	**NTS 1.0**	23

Table 3: Representation table of high dose NTS trial. Table showing mouse samples, genotype, weight and the dose received. IgG was used as a control. All injections were administered intraocularly.

Mouse	Weight (g)	Receiving	Dose (uL)
58 ko	25.4	**NTS 2.13**	54.1
85 ko	24.9	**NTS 2.13**	53.0
86 ko	22.6	**NTS 2.13**	48.1
87 ko	21.5	**NTS 2.13**	45.8
88 ko	23.1	**NTS 2.13**	49.2
56 ko	20.9	**IgG 2.13**	44.5
57 ko	28.4	**IgG 2.13**	60.5
63 wt	24.6	**NTS 2.13**	52.4
64 wt	28.9	**NTS 2.13**	61.6
65 wt	28.8	**NTS 2.13**	61.3
66 wt	29.0	**NTS 2.13**	61.8
67 wt	30.9	**NTS 2.13**	65.8
68 wt	30.2	**IgG 2.13**	64.3