RESEARCH PAPER

Small GTPases 4:4, 221–230; October/November/December 2013; © 2013 Landes Bioscience

The Rac-specific exchange factors Dock1 and Dock5 are dispensable for the establishment of the glomerular filtration barrier in vivo

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Keywords: Dock1, Dock180, Dock5, Elmo, podocyte, Rho GTPase, Rac

Abbreviations: ants, antisocial; BSA, bovine serum albumin; Cdc42, cell division cycle 42; Crk, v-crk avian sarcoma virus CT10 oncogene homolog; Crkl: v-crk sarcoma virus CT10 oncogene homolg (avian)-like; Dock1, dedicator of cytokinesis 1; Dock2, dedicator of cytokinesis 2; Dock3, dedicator of cytokinesis 3; Dock4, dedicator of cytokinesis 4; Dock5, dedicator of cytokinesis 5; E, embryonic day; Fbs, fetal bovine serum; Fcm, fusion competent myoblast; Flx, Floxed allele; Fm, founder myoblast; GEF, guanine exchange factor; Grb2, growth factor receptor bound protein 2; Hbs, hibris; IHC, Immunohistochemistry; INF, interferon; LPS, lipopolysaccharide; Mbc, myoblast city; Nck, non-catalytic region for tyrosine kinase adaptor protein 1; NPHS1, Nephrosis1, congenital, Finnish type (Nephrin); Nphs2, nephrosis 2, podocin; N-Wasp, Wiskott-Aldrich syndrome-like; Pod-Cre+, Podocin-Cre+ transgenic mouse; Rac1, RAS-related C3 botulinum substrate 1; Rac2, RAS-related C3 botulinum substrate 2; Rac3, RAS-related C3 botulinum substrate 3; Sns, sticks and stones; Synpo, synaptopodin; Tanc1, tetratricopeptide repeat, ankyrin repeat and coiled-coil containing 1; Tanc2, tetratricopeptide repeat, ankyrin repeat anc coiled-coil containing 2; Wt1, Wilms tumor 1 homolog; -/-, Knockout; +/+, Wild-type

Podocytes are specialized kidney cells that form the kidney filtration barrier through the connection of their foot processes. Nephrin and Neph family transmembrane molecules at the surface of podocytes interconnect to form a unique type of cell-cell junction, the slit diaphragm, which acts as a molecular sieve. The cytoplasmic tails of Nephrin and Neph mediate cytoskeletal rearrangement that contributes to the maintenance of the filtration barrier. Nephrin and Neph1 orthologs are essential to regulate cell-cell adhesion and Rac-dependent actin rearrangement during *Drosophila* myoblast fusion. We hypothesized here that molecules regulating myoblast fusion in *Drosophila* could contribute to signaling downstream of Nephrin and Neph1 in podocytes. We found that Nephrin engagement promoted recruitment of the Rac exchange factor Dock1 to the membrane. Furthermore, Nephrin overexpression led to lamellipodia formation that could be blocked by inhibiting Rac1 activity. We generated in vivo mouse models to investigate whether *Dock1* and *Dock5* contribute to the formation and maintenance of the kidney filtration barrier. Our results indicate that while *Dock1* and *Dock5* are expressed in podocytes, their functions are not essential for the development of the glomerular filtration barrier. Furthermore, mice lacking *Dock1* were not protected from LPS-induced podocyte effacement. Our data suggest that Dock1 and Dock5 are not the important exchange factors regulating Rac activity during the establishment and maintenance of the glomerular barrier.

Introduction

Glomerular filtration, the first step during urine formation, allows for the passage of water and solutes into the urinary space while retaining plasma proteins. The kidney filtration barrier is formed by three components: a fenestrated endothelium, a glomerular basement membrane and the podocytes.[1] Podocytes are specialized epithelial cells of the kidney that extend microtubule-rich primary processes and actin-rich secondary foot processes that interdigitate and fold around the glomerular capillaries to form the so-called slit diaphragm that restricts the passage of macromolecules.[2] Podocytes insult results in foot process effacement, disappearance of the slit diaphragm, and ultimately loss of blood proteins in the urine.[2] Several studies highlight that the slit diaphragm is not only a unique type of cell-cell junction, but also an important signaling hub that dynamically regulates the cytoskeleton network of podocytes to maintain integrity and plasticity of the kidney filtration barrier.[3]

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Submitted: 10/31/2013; Revised: 12/02/2013; Accepted: 12/04/2013; Published Online: 12/23/2013
http://dx.doi.org/10.4161/sgtp.27430

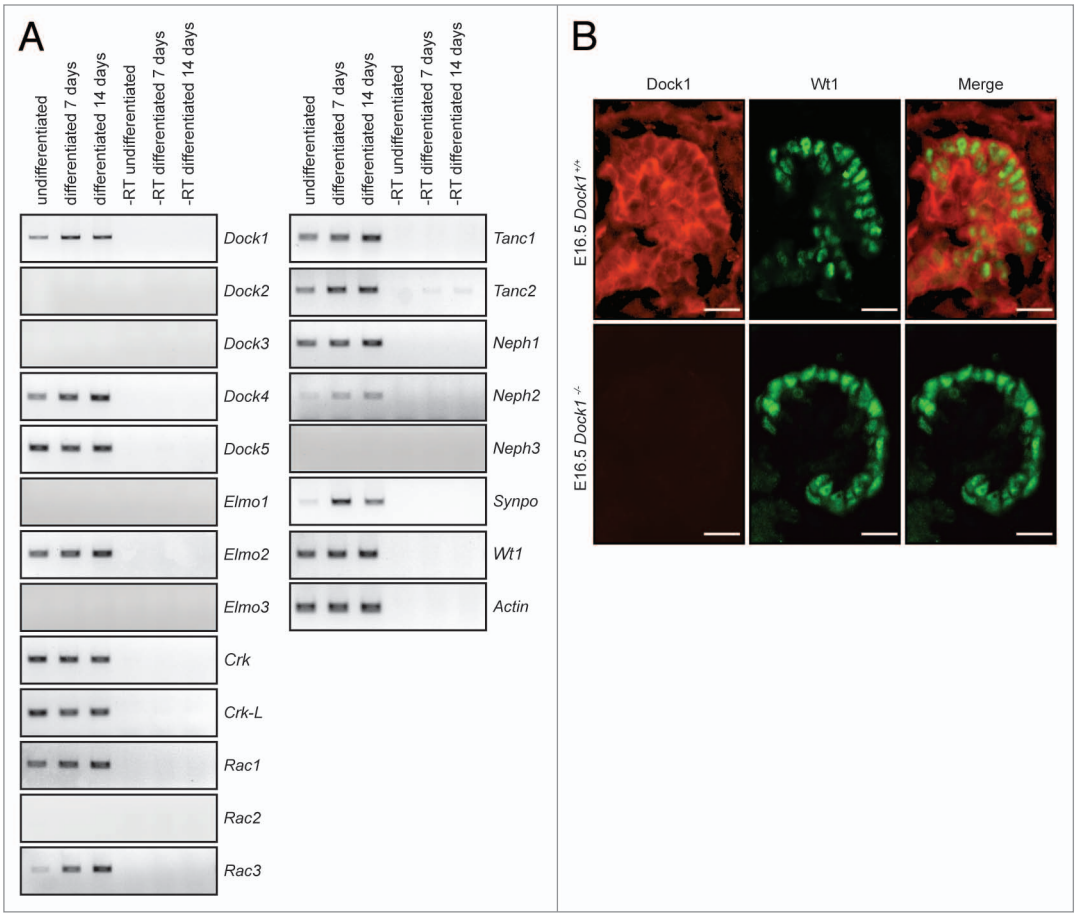

Figure 1. The *Drosophila* myoblast fusion machinery is expressed in mouse podocytes. (**A**) Mouse podocytes were differentiated in vitro for 7 or 14 d or left undifferentiated and expression of components of the *Drosophila* myoblast fusion machinery was evaluated by RT-PCR. (**B**) *Dock1* is expressed in the kidney glomerulus. IHC analyses showing Dock1 (left), Wt1 (middle) and merge (right) expression in E16.5 *Dock1*$^{+/+}$ and *Dock1*$^{-/-}$ glomerulus (Scale bar: 20 μm, 40x).

Table 1. Viability of P21 pups derived from Pod-Cre+Dock1+/flx and Pod-Cre-Dock1+/flx mating

Frequency	***Pod-Cre*$^{-}$*Dock1*$^{+/+}$**	***PodCre*$^{-}$*Dock1*$^{+/flx}$**	***Pod-Cre*$^{-}$*Dock1*$^{flx/flx}$**	***Pod-Cre*$^{+}$ *Dock1*$^{+/+}$**	***Pod-Cre*$^{+}$ *Dock*$^{+/flx}$**	***Pod-Cre*$^{+}$ *Dock1*$^{flx/flx}$**
Expected	12.5%	25%	12.5%	12.5%	25%	12.5%
Observed P21	9% (n = 4)	30% (n = 14)	22% (n = 10)	13% (n = 6)	15% (n = 7)	11% (n = 5)

Nephrin and Neph1 molecules are the transmembrane cores of the slit diaphragm. Trans interaction between their extracellular domains generates the size exclusion molecular sieve that is anchored to the podocytes' cytoskeleton via their cytoplasmic tail. In humans, mutation in the Nephrin gene, *NPHS1*, causes the Congenital Nephrotic Syndrome of the Finnish type characterized by massive proteinuria, highlighting the important contribution of this cell surface protein in podocytes function.[4]

Structures similar to the slit diaphragm are observed in model organisms. Notably, *Drosophila* nephrocytes express and rely on orthologs of Nephrin (sticks and stones; sns and hibris; hbs) and Neph1 (kirre) to achieve filtration of the hemolymphe.[5-7] These *Drosophila* receptors also mediate myoblasts cell-cell adhesion and are fundamental components of the fly myoblast fusion machinery that regulates the formation of multi-nucleated muscle fibers.[8-11] Mechanistically, trans interaction between the extracellular domains of kirre, expressed by founder myoblasts (Fm), and sns and hbs, both expressed by fusion competent myoblasts (Fcm), connects the cells membranes and activates signaling pathways that converge toward reorganization of the actin cytoskeleton which was shown to be essential for cell fusion.[11] In Fm, kirre couples to the adaptor protein antisocial (ants) and acts upstream of the Rac GTPase to remodel the actin cytoskeleton.[12] In Fcm, sns and hbs recruit the molecular adaptor Crk; this interaction is proposed to regulate Wasp-mediated actin polymerization by the Arp2/3 complex.[13] Sns and hbs also mediate Rac activation and signaling through the Rac guanine exchange factor (GEF) myoblast city (mbc) and its protein partner dElmo.[14,15] Until recently, it was unclear whether molecules identified as part of the fly myoblast fusion machinery

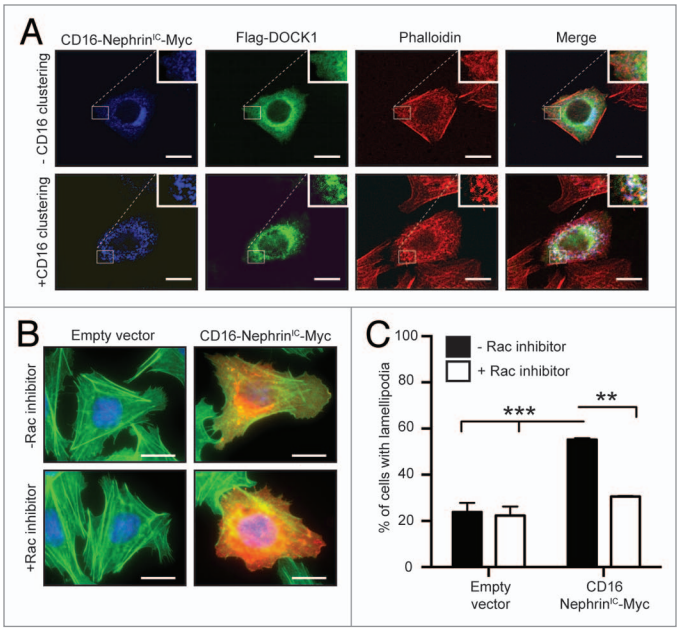

Figure 2. Dock1 is recruited to actin comet tails upon clustering of Nephrin molecules. (**A**) DOCK1 is recruited to actin comet tails upon clustering of chimeric CD16-Nephrin molecules. CHO.KI cells were transfected with CD16-Nephrin^{IC}-Myc and Flag-DOCK1 and clustering of Nephrin molecules was induced by an anti-CD16 antibody treatment. Immunofluorescence analyses showing the distribution of CD16-Nephrin^{IC}-Myc (blue), Flag-DOCK1 (green) and actin (red; phalloidin) molecules in non-treated and CD16-treated cells (Scale bar: 20 μm, 60x). (**B**) Expression of CD16-Nephrin^{IC}-Myc induces lamellipodia formation in cells through Rac activation. CHO.KI cells were transfected with CD16-Nephrin^{IC}-Myc and left untreated or treated with a Rac1 inhibitor. Immunofluorescence analyses showing expression of CD16-Nephrin^{IC}-Myc (red) and actin (green; phalloidin) (Scale bar: 20 μm, 60x). (**C**) Quantification of the % of cells with lamellipodia in B (n = 3).

would play similar roles in mammals. We identified the Rac GEF Dock1, the mammalian ortholog of mbc, as the first example of an important orchestrator of myoblast fusion in mammals: *Dock1*-null embryos exhibited a severe block in myoblast fusion.[16] Similarly, inactivation of *Rac1* and *N-Wasp* in mice revealed an essential role for these molecules during fusion.[17,18] These studies highlight the important degree of conservation between the mechanism of myoblast fusion and cytoskeleton regulation in these cells between species. Nevertheless, Nephrin and Neph1 molecules are surprisingly not major regulators of myoblast fusion in mammals. Yet, signaling occurring on the cytosolic portion of Nephrin and Neph1 in podocytes controls the formation, maintenance and remodeling of foot processes through a significant regulation of the cytoskeleton, notably by controlling the activation status of Rho GTPases.[1]

Based on the central role of Nephrin and Neph1 in fly myoblast fusion, we tested here the hypothesis that signaling molecules of the myoblast fusion machinery are expressed in kidney podocytes to control the formation and maintenance of the filtration barrier. More specifically, we investigated if expression of *Dock1* and *Dock5* is essential in vivo for cytoskeletal regulation during the formation of the slit diaphragm or if *Dock1* contributes to foot process effacement in disease. Our results with mutant mice suggest that *Dock1* and *Dock5* are not essential for the establishment and maintenance of the filtration barrier.

Results

The *Drosophila* myoblast fusion machinery is expressed in murine podocytes

In *Drosophila*, the orthologs of Nephrin and Neph promote myoblast cell-cell adhesion and fusion by activating signaling cascades that control cytoskeletal dynamics.[11] Because Nephrin is a central cell-surface signaling protein in podocytes and is essential for the biology of these cells, we took advantage of a conditionally immortalized mouse podocyte cell line to investigate whether orthologs of *Drosophila* genes necessary for myoblast fusion in *Drosophila* are also expressed in podocytes.[19,20] These podocyte progenitors proliferate rapidly when maintained under permissive conditions (33 °C; +INF-γ) while non-permissive conditions (37 °C; -INF-γ) induce their growth arrest and extensive remodeling of their cytoskeleton in a manner reminiscent of mature podocytes in vivo.[19,20] Podocytes were cultured under permissive and non-permissive conditions and RNA was extracted at various time points to evaluate expression of molecules of the myoblast fusion machinery by RT-PCR. As expected, both undifferentiated and differentiated podocytes expressed *Wt1* as a marker of the podocyte lineage (**Fig. 1A**). Both *Neph1* and *Neph2,* but not *Neph3,* were also detectable and expression of the slit diaphragm protein *Synaptopodin* (*Synpo*) was induced upon INF-γ withdrawal, as previously reported,[19] indicating optimal cell differentiation conditions (**Fig. 1A**).

Expression of the *Dock*-A/B subgroups of the *Dock* GEFs family was examined. *Dock1*, *Dock4*, and *Dock5* were expressed in podocytes regardless of whether they were differentiated or not, while expression of *Dock2* and *Dock3* was not detected (**Fig. 1A**). Mouse podocytes also expressed the *Dock*-A/B members-binding partner *Elmo2*, while expression of *Elmo1* and *Elmo3* was not detectable (**Fig. 1A**). The SH3 adaptors *Crk* and *Crk-L*, which form a physical complex with DOCK1,[21] were also expressed in podocytes. In *Drosophila* myoblasts, Sns (Nephrin) connects to the adaptor protein Ants to promote cell-cell fusion.[12] We observed that orthologs of *Ants*, *Tanc1* and *Tanc2*, are both expressed in podocytes and their expression increased with differentiation time (**Fig. 1A**). Finally the Rho GTPase *Rac1* and *Rac3* were expressed while expression of *Rac2* was not detected (**Fig. 1A**).

Since Dock1 was reported to play a similar role to mbc during myoblast fusion, we hypothesized that Dock1 is a candidate GEF to promote Rac activation and cytoskeleton dynamics downstream of Nephrin/Neph1 cell surface proteins in podocytes.[16] We examined the expression pattern of Dock1 during kidney development in vivo by immunohistochemistry (IHC) analyses using a Dock1 specific antibody and found this GEF to be highly expressed in E16.5 mouse embryonic kidney (**Fig. 1B**). The absence of staining of the Dock1 antibody in *Dock1-null* embryos confirmed the specificity of the observed expression pattern (**Fig. 1B**). Co-staining with Wt1, a marker of

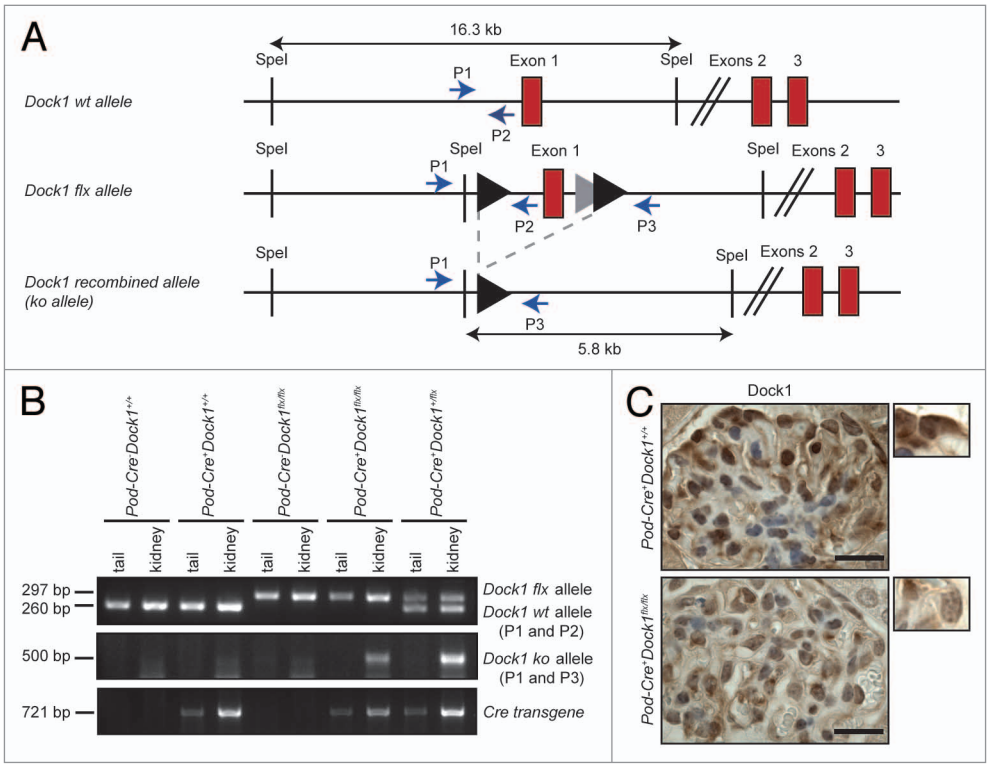

Figure 3. Podocyte-specific ablation of *Dock1* expression. (**A**) Partial schematic representation of the *Dock1 wt* allele, the *Dock1 flx* allele and the *Dock1* recombined (*ko*) allele after exposure to the Cre recombinase. Positions of PCR primers used for genotyping analyses are indicated. (**B**) PCR analyses using P1, P2, P3 and Cre primer sets on genomic DNA extracted from mice kidneys or tails of the indicated genotype. A recombined *Dock1 flx* allele (*ko* allele) is detected in the kidney of transgenic *Pod-Cre*$^{+}$ mice. (**C**) IHC analyses showing the absence of *Dock1* expression in the podocytes of *Pod-Cre*$^{+}$*Dock1*$^{flx/flx}$ mice (Scale bar: 20 μm, 100x).

Table 2. Viability of embryos and P21 pups derived from Pod-Cre+Dock1+/flxDock5-/- and Pod-Cre-Dock1+/flxDock5-/- mating

Frequency	***Pod-Cre*$^{-}$*Dock1*$^{+/+}$ *Dock5*$^{-/-}$**	***Pod-Cre*$^{-}$*Dock1*$^{+/flx}$ *Dock5*$^{-/-}$**	***Pod-Cre*$^{-}$*Dock1*$^{flx/flx}$ *Dock5*$^{-/-}$**	***Pod-Cre*$^{+}$ *Dock1*$^{+/+}$ *Dock5*$^{-/-}$**	***Pod-Cre*$^{+}$ *Dock1*$^{+/flx}$ *Dock5*$^{-/-}$**	***Pod-Cre*$^{+}$ *Dock1*$^{flx/flx}$ *Dock5*$^{-/-}$**
Expected	12.5%	25%	12.5%	12.5%	25%	12.5%
Observed E18.5	15% (n = 13)	21% (n = 18)	15% (n = 13)	9% (n = 8)	19% (n = 16)	20% (n = 17)
Observed P21	7% (n = 3)	34% (n = 15)	21% (n = 9)	7% (n = 3)	21% (n = 9)	11% (n = 5)

the podocyte lineage, indicated that Dock1 is expressed in these cells. Notably, we could also detect strong expression of this GEF in the capillary loop of the developing glomerulus (**Fig. 1B**). Our previous studies demonstrated a role for Dock1 in endothelial cell motility.[22] Collectively, these results indicate that molecules of the myoblast fusion machinery are found in podocytes and that *Dock1* is expressed in mouse podocytes during development.

Dock1 is recruited to actin foci upon Nephrin clustering

Clustering of Nephrin molecules in cells was previously shown to promote phosphorylation of its cytoplasmic tail and induce signaling that mediates the formation of distinguishable actin comet tails at sites of clustering.[23] We took advantage of this model to examine whether Dock1 could signal downstream of Nephrin molecules. CHO.K1 cells were transfected with a construct coding for the intracellular domain of Nephrin fused to CD16 (CD16-Nephrin^{IC}-Myc) and Flag-DOCK1. Clustering of Nephrin by an anti-CD16 antibody induced the formation of actin comet tails that were visualized by phalloidin staining (**Fig. 2A**; bottom). In non-treated cells, DOCK1 was distributed in the cytosplasm (**Fig. 2A**; top). Upon anti-CD16-mediated Nephrin clustering, CD16-Nephrin^{IC}-Myc and DOCK1 co-localized at site of actin foci (**Fig. 2A**; bottom) suggesting that DOCK1 might be required to mediate Nephrin signaling. Moreover, overexpression of CD16-Nephrin^{IC}-Myc in CHO. K1 promoted lamellipodia formation, which could be prevented by treatment of cells with a Rac1 inhibitor (**Fig. 2B-C**). These in vitro experiments suggest that Dock1 is a candidate GEF to mediate Rac1 activation downstream of Nephrin.

Generation of Podocyte-specific *Dock1* knockout mice

Our data indicates that *Dock1* is expressed in multiple cell types of the kidney glomerulus. Moreover, *Dock1* knockout mice die at birth and present several developmental abnormalities.[16,22] Like *mbc* mutants, *Dock1-null* animals display a block in myoblast fusion but also present cardiovascular defects.[16,22] Consequently,

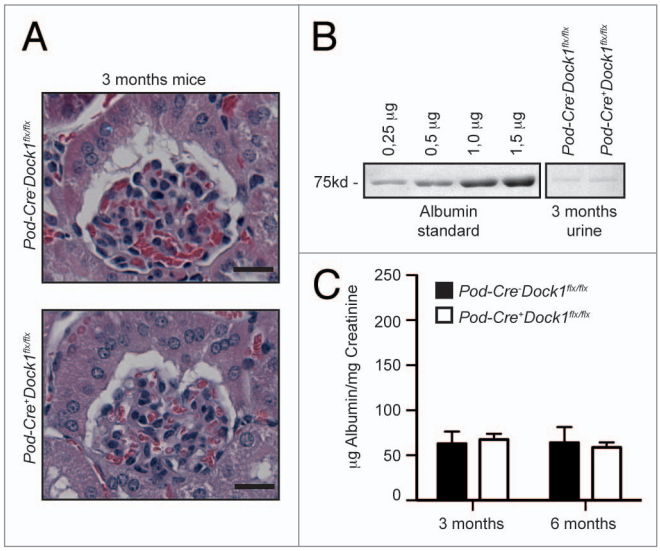

Figure 4. *Pod-Cre*$^{+}$*Dock1*$^{flx/flx}$ mice display normal glomerular structure and function. (**A**) H&E-staining showing the normal glomerular development of 3 mo old *Pod-Cre*$^{-}$*Dock1*$^{flx/flx}$ and *Pod-Cre*$^{+}$*Dock1*$^{flx/flx}$ mice (Scale bar 20 µm, 40x). (**B**) Absence of proteinuria in the urine of 3-mo old *Pod-Cre*$^{-}$*Dock1*$^{flx/flx}$ and *Pod-Cre*$^{+}$*Dock1*$^{flx/flx}$ mice. Coomassie stained protein gel showing BSA standards (left) and the albumin content from the urine (right) of 3 mo old *Pod-Cre*$^{-}$*Dock1*$^{flx/flx}$ and *Pod-Cre*$^{+}$*Dock1*$^{flx/flx}$ mice. (**C**) Normal renal function in the absence of *Dock1* expression in podocytes. Quantification of the average albumin-to-creatinine ratios in the urine of *Pod-Cre*$^{-}$*Dock1*$^{flx/flx}$ and *Pod-Cre*$^{+}$*Dock1*$^{flx/flx}$ mice at 3 and 6 mo after their birth (n = 5).

to examine the role of *Dock1* in the formation and/or maintenance of the kidney filtration barrier in vivo, we generated a mouse with conditional deletion of *Dock1* expression by crossing *Dock1 floxed* (*flx*) animals (**Fig. 3A**) with *Podocin-Cre*$^{+}$ (*Pod-Cre*$^{+}$; also known as *Nphs2-Cre*$^{+}$) transgenic mice that express the Cre recombinase specifically in podocytes.[24,25] Using PCR analyses on mice tails and kidney, we confirmed that expression of the *Cre* transgene led to the recombination of the *Dock1 flx* allele in the kidney (**Fig. 3B**). Furthermore, Cre-mediated genetic ablation of *Dock1* reduced the level of Dock1 protein expression in podocytes of *Pod-Cre*$^{+}$*Dock1*$^{flx/flx}$ animals as verified by IHC analysis (**Fig. 3C**).

Loss of *Dock1* does not impair podocyte development and kidney function

Based on the observation that DOCK1 can be recruited to Nephrin and that Nephrin induces Rac-dependent cytoskeletal changes (**Fig. 2A**), we investigated whether Dock1 could be involved in the development of the glomerular filtration barrier in vivo. *Pod-Cre*$^{+}$*Dock1*$^{flx/flx}$ animals were born and present at weaning (P21) with the expected Mendelian ratio (**Table 1**) and survived into adulthood. Moreover, gross examinations suggest that *Pod-Cre*$^{+}$*Dock1*$^{flx/flx}$ mice are indistinguishable from control littermates. Histological analyses demonstrated that the kidneys and glomeruli develop normally in the absence of *Dock1* expression (**Fig. 4A**). To evaluate the level of proteinuria, urine was collected, using metabolic cages, and fractionated on SDS-PAGE next to an albumin standard (0,25 to 1,5 µg). At 3 mo of age, *Pod-Cre*$^{+}$*Dock1*$^{flx/flx}$ mice did not develop proteinuria in comparison to control animals as revealed by the trace amount of albumin present in the urine of both genotypes (**Fig. 4B**). The level of proteinuria was further quantified and expressed as a ratio between urinary albumin and creatinine and these analyses confirmed that *Pod-Cre*$^{+}$*Dock1*$^{flx/flx}$ do not develop proteinuria at 3 and 6 mo of age (**Fig. 4C**). Older mutant animals did not develop proteinuria, deterioration in heath or reduction in weight (data not shown). These findings indicate that *Dock1* is not essential in podocytes for the formation and maintenance of the filtration barrier.

Loss of *Dock5* does not impair glomerular function

Dock1, *Dock2* and *Dock5* are part of the *Dock-A* subgroup of *Dock* GEFs. While *Dock2* expression is mostly restricted to cells of the hematopoietic lineages,[26] *Dock5* was previously shown to act redundantly with *Dock1* during the fine-tuning of muscle fiber formation.[16] In addition, *Dock5* was identified as one of the gene highly expressed in adult podocytes in comparison to its expression in the kidney cortex.[27] To test the possibility that *Dock2* or *Dock5* GEFs could act redundantly with *Dock1* during the establishment of the filtration barrier, we further confirmed their expression profiles in podocytes in vivo. Using *Dock2-GFP* knock-in mice, we confirmed our RT-PCR analyses by observing that Dock2 is not expressed in Podocalyxin-positive podocytes in vivo (**Fig. 5A**, top).[28] In contrast, Dock2-GFP was readily detectable in the spleen, where an antibody against MOMA-1 marked resident macrophages in *Dock2-GFP* knock-in mice (**Fig. 5A**, bottom). As previously described,[27] Dock5 expression was robustly detectable in adult mice podocytes as revealed by IHC using a Dock5 specific antibody (**Fig. 5B**). These data are in agreement with Dock5 mRNA expression pattern in cultured mouse podocyte cells in vitro (**Fig. 1A**). Dock5-positive podocytes were spatially localized in the glomerulus similarly to Wt1-positive podocytes (**Fig. 5B**).

We previously demonstrated the absence of Dock5 protein expression in *Dock5* mutant mice, which are viable and fertile and do not present gross developmental defects.[16] To test the hypothesis that *Dock5* could act redundantly with *Dock1* in podocytes, we generated mice with deletion of these two GEFs by crossing *Dock5*$^{-/-}$ mice with *Pod-Cre*$^{+}$*Dock1*$^{flx/flx}$ animals. We first tested if ablation of *Dock5* expression alone affects glomerular filtration by testing the level of proteins in the urine of *Pod-Cre*$^{+}$*Dock1*$^{+/+}$*Dock5*$^{-/-}$ mice in comparison to *Pod-Cre*$^{+}$*Dock1*$^{+/+}$*Dock5*$^{+/+}$ animals. Quantification of the ratio between urinary albumin and creatinine confirmed that deletion of *Dock5* expression does not induce proteinuria (**Fig. 5C**). *Pod-Cre*$^{+}$*Dock1*$^{flx/flx}$*Dock5*$^{-/-}$ animals were viable and mice were born and present at weaning (P21) with the expected Mendelian ratio (**Table 2**) and survived into adulthood (data not shown). Moreover, gross examination revealed that *Pod-Cre*$^{+}$*Dock1*$^{flx/flx}$*Dock5*$^{-/-}$ mice were indistinguishable from their control littermates. To evaluate the level of proteinuria in double mutants, urine was collected and fractionated on SDS-PAGE next to an albumin standard (0,2 to 1,5 µg) (**Fig. 5D**). *Pod-Cre*$^{+}$*Dock1*$^{flx/flx}$*Dock5*$^{-/-}$ mice did not develop proteinuria in comparison to control animals as revealed by the trace amount of albumin present in their urine (**Fig. 5D**). Moreover, *Pod-Cre*$^{+}$*Dock1*$^{flx/flx}$*Dock5*$^{-/-}$ mice did not

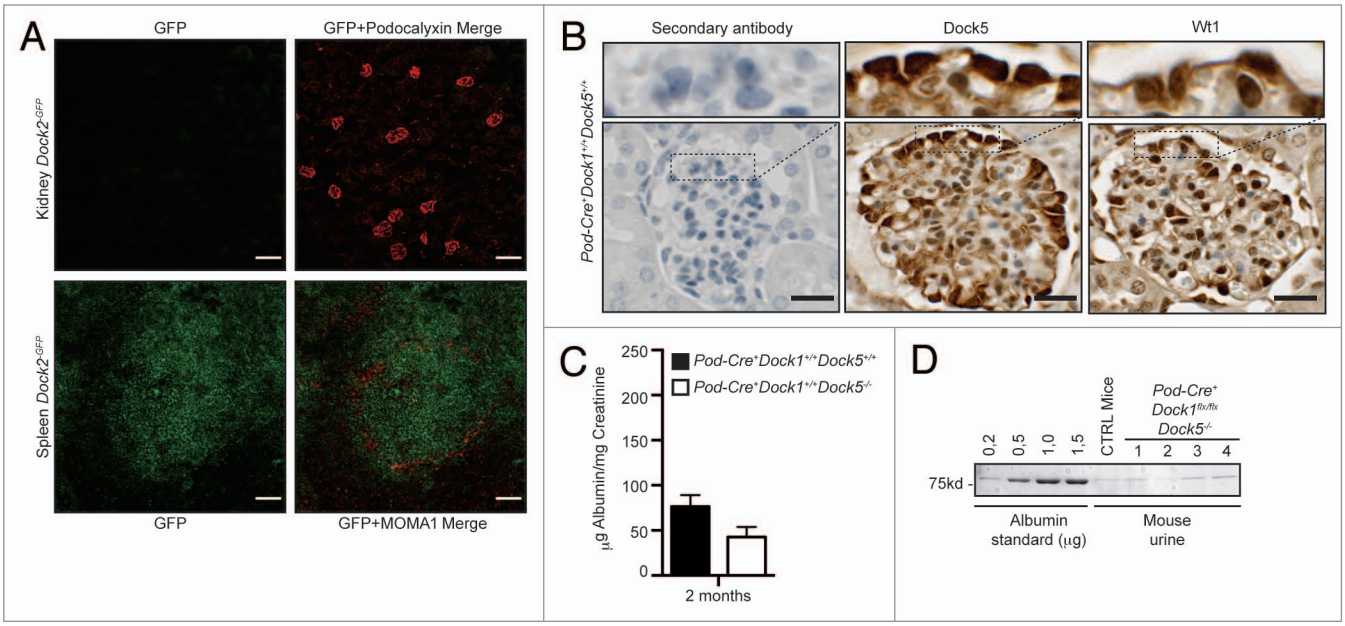

Figure 5. *Dock5* is not essential to glomerular function. (**A**) *Dock2* is not expressed in the kidney. *Dock2-GFP* expression is absent in the kidney (top), but can be detected in the spleen (bottom) (Scale bar: 100 µm, 20x). (**B**) IHC analyses showing the expression of *Dock5* and Wt1 in the podocytes of *Pod-Cre*$^{+}$*Dock1*$^{+/+}$*Dock5*$^{+/+}$ mice (Scale bar: 25 µm, 100x). (**C**) Normal renal function in the absence of *Dock5* expression in mice. Quantification of the average albumin-to-creatinine ratio in the urine of *Pod-Cre*$^{+}$*Dock1*$^{+/+}$*Dock5*$^{+/+}$ and *Pod-Cre*$^{+}$*Dock1*$^{+/+}$*Dock5*$^{-/-}$ 2 mo old mice (n = 3). (**D**) Absence of proteinuria in the urine of P21 *Pod-Cre*$^{-}$*Dock1*$^{flx/flx}$*Dock5*$^{+/+}$ and *Pod-Cre*$^{+}$*Dock1*$^{flx/flx}$*Dock5*$^{-/-}$ mice. Coomassie stained protein gel showing BSA standards (left) and the albumin content from the urine of a control mouse and 4 *Pod-Cre*$^{+}$*Dock1*$^{flx/flx}$*Dock5*$^{-/-}$ mutant mice.

show deterioration in health or reduction in weight overtime (not shown). These findings indicate that *Dock5* does not compensate for *Dock1* loss in podocytes and that elimination of both *Dock1* and *Dock5* expression in these cells does not lead to aberrant formation nor contribute to maintenance of the glomerular filtration barrier.

Loss of *Dock1* does not protect against LPS-induced proteinuria

A common response to podocyte insult includes a profound remodeling of the actin cytoskeleton, which induces foot processes to fuse and efface, a process that mimics lamellipodia formation and spreading in cultured cells.[2] Foot process effacement has important clinical manifestations as it results in the malfunctioning of the slit diaphragm and the lost of blood proteins in the urine. Intraperitoneal injection of low-dose LPS was described as a powerful model to induce transient foot process effacement and proteinuria in mice.[20] Moreover, several proteins were successfully identified as contributing to cytoskeleton rearrangement and therefore their inactivation in mice would protect them from LPS-induced proteinuria.[29] We used the LPS model to test if Dock1 contributes to foot process effacement. Upon LPS injection, the general glomerular histology of control and *Pod-Cre*$^{+}$*Dock1*$^{flx/flx}$ animals was not affected as reported for this model (**Fig. 6A**).[29] To evaluate the level of proteinuria, mouse urine was collected before and after LPS injection and fractionated on SDS-PAGE next to an albumin standard (0,2 to 1,5 µg). (**Fig. 6B**). Both, *Pod-Cre*$^{+}$*Dock1*$^{+/+}$ and *Pod-Cre*$^{+}$*Dock1*$^{flx/flx}$ mice developed proteinuria after LPS injection as revealed by the large amount of albumin present in their urine (**Fig. 6B**). The level of proteinuria before (t = 0) and 28 h after LPS injection (t = 28) was further quantified and expressed as a ratio between urinary albumin and creatinine; this confirmed that both *Pod-Cre*$^{+}$*Dock1*$^{+/+}$ and *Pod-Cre*$^{+}$*Dock1*$^{flx/flx}$ animals develop similar proteinuria after LPS injection (**Fig. 6C**). These findings indicate that Dock1 does not contribute to foot process effacement and that inactivation of *Dock1* in podocytes does not protect mice from LPS-induced proteinuria as previously observed for other Rac pathway activators or regulators.[29]

Discussion

Podocytes have a highly dynamic and complex actin cytoskeleton that must be tightly regulated for their ability to exert glomerular filtration.[2] We presented evidence that the *Drosophila* molecular machinery, involved in the control of the actin cytoskeleton during myoblast fusion, is expressed in mouse podocytes and that Nephrin clustering promotes recruitment of the Rac GEF Dock1 to the membrane where it colocalizes with actin comets. Nephrin engagement promotes the phosphorylation of its cytoplasmic tails that induces Nck recruitment, a crucial step to promote the formation of these actin structures.[23] A direct interaction between Dock1 and Nck-2 has been reported.[30] These in vitro evidences suggested that Dock1 could promote signaling downstream of Nephrin. However, our in vivo data clearly indicates that expression of *Dock1* is not required for the formation and maintenance of the filtration barrier by podocytes. Moreover, we observed that expression of the closely related GEF *Dock5* is also dispensable

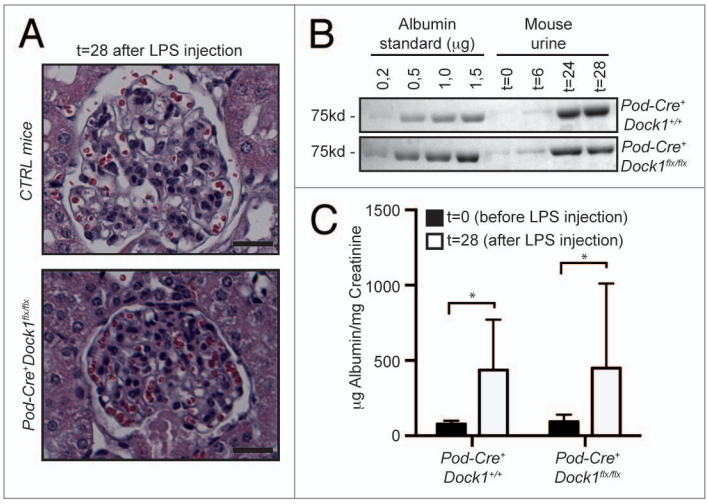

Figure 6. Loss of *Dock1* expression in podocytes does not protect against LPS-induced proteinuria. (**A**) H&E-staining showing the glomerulus of 6 wk old *Control* and *Pod-Cre*$^{+}$*Dock1*$^{flx/flx}$ female mice 28 h (t = 28) after LPS injection (Scale bar 20 μm, 40x). (**B**) LPS injection induces significant proteinuria in 6 wk old *Pod-Cre*$^{+}$*Dock1*$^{+/+}$ and *Pod-Cre*$^{+}$*Dock1*$^{flx/flx}$ mice. Coomassie stained protein gel showing BSA standards (left) and the albumin content in the urine (right) collected from *Pod-Cre*$^{+}$*Dock1*$^{+/+}$ and *Pod-Cre*$^{+}$*Dock1*$^{flx/flx}$ mice before (t = 0) and 6, 24 and 28 h (t = 6, t = 24, t = 28) after LPS injection. (**C**) Quantification of the average albumin-to-creatinine ratio in the urine of *Pod-Cre*$^{-}$*Dock1*$^{flx/flx}$ and *Pod-Cre*$^{+}$*Dock1*$^{flx/flx}$ before (t = 0) and 28 h (t = 28) after LPS injection.

and that it does not compensate for the lack of *Dock1* expression in mice. This was particularly surprising as complete ablation of *Dock1* and *Dock5* in mice completely prevented embryonic development such that no *Dock1*$^{-/-}$*Dock5* $^{-/-}$ mutants were ever recovered at E14.5.[16] Similarly, Neph1 crosslinking promoted the recruitment of Grb2, another adaptor protein that can binds Dock1,[31,32] to induce actin polymerization in cells.[33] Much like we observed here for Dock1 and Dock5, podocyte-specific inactivation of *Grb2* did not interfere with the formation of a functional glomerular filtration barrier in vivo.[34] The expression data in mouse podocytes also revealed expression of *Dock4* and a previous study identified this GEF as one of the gene highly expressed in podocytes in comparison to the rest of the kidney cortex.[27] Therefore, it will be interesting in the future to generate a mouse model for *Dock4* to address its biological role in vivo and more specifically to investigate its role in podocyte.

Two recent independent studies highlighted that expression of *Rac1* is dispensable for podocytes-mediated glomerular filtration; these studies further substantiate our conclusions here that elimination of two Rac1 GEFs in podocytes does not induce proteinuria as a result of a defect in podocyte function.[35,36] Altogether, these results are intriguing since podocytes need to first spread on the glomerular membrane of endothelial capillaries before extending primary and secondary processes. The expression profile in a mouse podocyte cell line suggests that *Rac3* expression is increased upon differentiation of podocytes in vitro. It will be interesting to address in the future the role of this GTPase in mice and evaluate its potential redundancy with *Rac1* during development. The role of the other prototypic Rho GTPases was also recently addressed in mice. While expression of RhoA is dispensable for podocytes physiological role, inactivation of Cdc42 leads to congenital nephropathy and massive proteinuria quickly after birth.[35,36] Therefore, it will be important to identify the GEF(s) needed to specifically activate Cdc42 during the establishment of the glomerular filtration barrier.

Several studies now point toward Rac1 activity as playing a toxic role in podocytes such that it contributes to foot process effacement, a pathological state associated with an increase in cell motility. In a *RhoGDIα* mutant mouse, Rac1 was shown to be hyper-activated and these animals developed proteinuria which was reversible by administration of a Rac inhibitor.[37,38] In addition, the inducible expression of an active form of the GTPase in podocytes induces the rapid unset of proteinuria.[39] Moreover, a mutation that prevents Rac1 inactivation in the *ARHGAP24* was identified in patients with focal segmental glomerulosclerosis.[40] Using protamine sulfate injection as an experimental model to induced foot process effacement in mice, it was observed that loss of *Rac1* expression as well as the inactivation of its upstream signaling intermediates *Crk1/2* in podocytes could counteract foot process effacement.[36,41] In a model where mice were challenged with LPS to induce foot process effacement, podocytes lacking expression of vitronectin, uPar or β3 integrin failed to adopt a motile phenotype and mice were protected from proteinuria.[29] Dock1 was an attractive candidate to mediate Rac1 activation in podocytes since a previous study in breast cancer cell lines showed that signaling downstream of vitronectin/uPar/β3 integrin induces the recruitment of CrkII/Dock1 by p130Cas and was required to promote breast cancer migration.[42] Nevertheless, our study using LPS injection in mice indicates that Dock1 is not the GEF regulating Rac1-mediated foot process effacement at least in this model of transiently induced proteinuria. Altogether, these studies further highlight the importance of identifying activators and effectors of Rac1 in podocytes since they could represent important therapeutic targets to prevent effacement of foot processes in several kidney diseases.

Materials and Methods

Animal care and mouse strains

Mice with a floxed *Dock1* allele and *Dock5* total mutant mice were previously described.[16,24] *Dock1*$^{flx/flx}$*Dock5*$^{-/-}$ mice were generated by inter-crossing *Dock1*$^{flx/flx}$ mice with *Dock5* mutants. Inactivation of *Dock1* in podocytes was generated by crossing *Dock1*$^{flx/flx}$ animals with *Podocin-Cre*$^{+}$ transgenic mice (*Pod-Cre*$^{+}$; also known as *Nphs2-Cre*) mice.[25] All mice were maintained on a mixed genetic background. Genomic DNA from mice tails and kidneys was extracted using standard procedures and mice were genotyped by PCR (see **Table S1** for Primers). All mice were housed in a Specific Pathogen Free (SPF) Facility at the IRCM. Mouse experiments were approved by the IRCM Animal Care Committee and complied with the Canadian Council of Animal Care guidelines.

Cell culture

Podocytes were culture as previously reported.[19,43] Briefly, cells were grown on dishes coated with 0.1 mg/ml Collagen type I and maintained undifferentiated in growth medium [10% FBS (Gibco), 100 U/ml Pen/Strep (Wisent), RPMI 1640 (Gibco)] supplemented with 50 U/ml INF-γ (for the first two passages) or 10 U/ml INF-γ (for the following passages) (#11276905001; Roche) in an incubator set at 33 °C. Podocyte differentiation was induced by splitting the cell at low density, removing INF-γ from the growth media and putting the cells in an incubator set at 37 °C. CHO.K1 cells were maintained in F-12 media (Gibco) containing 10% FBS (Gibco) and 100 U/ml Pen/Strep (Wisent).

CD16 Clustering experiment and immunocytochemistry

$1.5x10^5$ CHO.K1 cells were seeded in a 4-well Nunc Labtek glass chamber (BD Falcon). The following day, cells were transfected with CD16-Nephrin^{IC}-Myc (gift from Dr. A.J. Pawson) and Flag-DOCK1 (gift from Dr. M. Matsuda) plasmids using lipofectamine 2000 reagents (#11668–019; Invitrogen). Eighteen hours following transfection, clustering of Nephrin molecules was initiated by incubating cells with 1 μg/ml mouse anti-CD16 antibody for 15 min (#SC-19620; SantaCruz Biotechnology) as previously described.[23] Cells were then washed with F-12 media and incubated with 1 μg/ml anti-mouse 350 antibody for 15 min (A-11045; Molecular Probes). Cells were washed 3 times with PBS and fixed in 4% Paraformaldehyde (PFA) for 10 min. Cells were washed again with PBS, permeabilized with PBST (0,1% Triton-X100 in PBS) and blocked with PBST 1% BSA for 1 h. Cells were stained using rabbit anti-Flag antibody diluted in PBST 1% BSA (#F7425; Sigma) for 1 h, washed 3 times with PBST and stained with anti-rabbit 488 antibody (1:1500; A-21441; Molecular Probes) for 30 min. Cells were washed 3 times with PBST and stained with Alexa Fluor 633 Phalloidin 633 (1:200; A-22284; Molecular Probes) for 20 min. Cells were washed again 3 times with PBST and slides were mounted using Mowiol reagent. Empty vector or CD16-Nephrin-^{IC}-Myc transfected cells were treated with Rac1 inhibitor (#80602–892; EMD) for 30 min. Cells were then stained as described above.

RNA isolation and RT-PCR

Total RNA from undifferentiated mouse podocyte cells or cells differentiated for 7 or 14 d was extracted using TRIZOL reagent (#15596026; Invitrogen) and DNaseI (#18068015; Invitrogen) treatment was performed according to the recommended procedures. cDNAs were generated using Superscript II (#18064022; Invitrogen) and random primers (#48190011; Invitrogen) as recommended by the manufacturer. The expression of genes from the myoblast fusion machinery was tested by RT-PCR (see **Table S1** for gene list and primer information) as previously described.[24]

Histology and Immunohistochemistry (IHC)

For histological analysis, kidneys were isolated and fixed in 4% PFA, embedded in paraffin, sectioned at 5 μm and stained with H&E. For IHC analysis of E16.5 embryonic kidney, tissues were embedded in OCT and sectioned at 5 μm. For IHC analyses, paraffin sections were deparaffinized in xylene and rehydrated in ethanol gradients while OCT sections were rinsed in PBS. Both paraffin and OCT sections were treated for antigen retrieval according to standard procedures in 10 mM sodium citrate buffer (pH6). Endogenous peroxidase activity was blocked on paraffin sections with 3% H_2O_2. Paraffin and OCT sections were permeabilized with IHC buffer (0,5% Triton X-100; 0,02% Tween-20 in PBS) and blocked for 1 h in IHC buffer containing 1% BSA. Primary antibodies were diluted in IHC buffer containing 1% BSA and incubated overnight. Sections were washed 3 times for 5 min with IHC buffer and incubated with either secondary or biotinylated secondary antibody diluted in IHC buffer containing 1% BSA for 1 h. Sections were washed 3 times with IHC buffer. OCT sections were mounted with Mowiol (#80058–440; VWR) and analyzed; paraffin sections were incubated with Streptavidin-HRP (1:1000; #554066; BD Biosciences) for 30 min. Paraffin sections were washed again 3 times with IHC buffer. The staining was revealed using DAB peroxidase substrate kit (#SK-4100; Vector Laboratories) and slides were counterstained with Hematoxylin. Antibodies used in IHC analysis include Dock1 (1:100; C19 #Sc-6167; Santa Cruz Biotechnology), Dock5 (1:100; from Dr Alan Hall), Wt1 (1:100; C19 #Sc-192; Santa Cruz Biotechnology), Podocalyxin (1:50; #MAB1556; R&D Systems) and MOMA-1 (1:200; #T-2011; BMA Biomedicals). Secondary antibodies used in IHC analyses include anti-goat 568 (1:1000; #A11057; Molecular Probes), anti-rabbit 488 (1:1000; #A21441; Molecular Probes), anti-goat biotinylated (1:150; #BA9500; Vector Laboratories) and anti-rabbit biotinylated (1:150; #BA1000; Vector Laboratories).

Baseline urine proteinuria analysis and LPS mouse model

For baseline proteinuria analyses, mouse urine was collected in metabolic cages at different time points. The urine albumin content was determined by running 10 μl of urine on SDS-PAGE gels. Gels were then stained with Coomassie blue and protein bands were quantified according to the BSA standard using the Fiji software. The model of intraperitoneal injection of LPS was used to induce foot process effacement and transient proteinuria in mice exactly as previously described.[20,29] Briefly, urine from 6 wk old female mice was collected (t = 0) before they were injected intraperitoneally with 10 μg per gram of mouse weight using a LPS solution (1 mg/ml in sterile PBS; #tlrl-eblps; Invivogen). Urine was collected at 6, 24 and 28 h (t = 6, t = 24, t = 28) after LPS injection. At t = 28, mice were euthanized and the kidneys were collected and processed for histological analyses. The albumin content in the urine from LPS-injected mice was quantified by fractionating 5 μl of urine on SDS-PAGE gels, staining them with Coomassie blue and quantifying the bands using Fiji software. The albumin-creatinine ratio was determined by using creatinine ELISA kits (#C500701; Cayman Chemical).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr Tomoko Takano (McGill University) for helpful discussions and Dr M. Patel for critical reading of

the manuscript. We are grateful to Drs Tony Pawson (CD16-Nephrin-Myc construct), Alan Hall (anti-Dock5 polyclonal antibody), Michiyuki Matsuda (Flag-DOCK1 construct) and Susan E. Quaggin (Podocin-Cre (*Nphs2-Cre)* transgenic mice) for generous gifts of reagents. We also acknowledge Dr Peter Mundel for providing the immortalized mouse podocyte cell line. J.F. C. is a recipient of a Senior Investigator award from the Fonds de Recherche du Québec-Santé (FRQ-S). This work was funded by an operating grant from the Canadian Institutes of Health Research (CIHR: MOP-77591) to J.F.C.

Supplemental Material

Supplemental material may be found here: http://www.landesbioscience.com/journals/smallgtpases/article/27430

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