TRPC6 Binds to and Activates Calpain, Independent of Its Channel Activity, and Regulates Podocyte Cytoskeleton, Cell Adhesion, and Motility

Louise K. Farmer,[1] Ruth Rollason,[1] Daniel J. Whitcomb,[2] Lan Ni,[1] Alexander Goodliff,[1] Abigail C. Lay,[1] Lutz Birnbaumer,[3,4] Kate J. Heesom,[5] Shang-Zhong Xu,[6] Moin A. Saleem,[1] and Gavin I. Welsh[1]

[1]Bristol Renal, Bristol Medical School, [2]Henry Wellcome Laboratories for Integrative Neuroscience and Endocrinology, Bristol Medical School, and [5]Proteomics Facility, University of Bristol, Bristol, United Kingdom; [3]Neurobiology Laboratory, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina; [4]Faculty of Medical Sciences, Institute of Biomedical Research, Catholic University of Argentina, Buenos Aires, Argentina; and [6]Centre for Cardiovascular and Metabolic Research, Hull York Medical School, University of Hull, Hull, United Kingdom

ABSTRACT

Background Mutations in the transient receptor potential channel 6 (*TRPC6*) gene are associated with an inherited form of FSGS. Despite widespread expression, patients with *TRPC6* mutations do not present with any other pathologic phenotype, suggesting that this protein has a unique yet unidentified role within the target cell for FSGS, the kidney podocyte.

Methods We generated a stable *TRPC6* knockout podocyte cell line from *TRPC6* knockout mice. These cells were engineered to express wild-type *TRPC6*, a dominant negative *TRPC6* mutation, or either of two disease-causing mutations of *TRPC6*, G109S or K874*. We extensively characterized these cells using motility, detachment, and calpain activity assays; immunofluorescence; confocal or total internal reflection fluorescence microscopy; and western blotting.

Results Compared with wild-type cells, *TRPC6*$^{-/-}$ podocytes are less motile and more adhesive, with an altered actin cytoskeleton. We found that TRPC6 binds to ERK1/2 and the actin regulatory proteins, caldesmon (a calmodulin- and actin-binding protein) and calpain 1 and 2 (calcium-dependent cysteine proteases that control the podocyte cytoskeleton, cell adhesion, and motility *via* cleavage of paxillin, focal adhesion kinase, and talin). Knockdown or expression of the truncated K874* mutation (but not expression of the gain-of-function G019S mutation or dominant negative mutant of *TRPC6*) results in the mislocalization of calpain 1 and 2 and significant downregulation of calpain activity; this leads to altered podocyte cytoskeleton, motility, and adhesion —characteristics of *TRPC6*$^{-/-}$ podocytes.

Conclusions Our data demonstrate that independent of TRPC6 channel activity, the physical interaction between TRPC6 and calpain in the podocyte is important for cell motility and detachment and demonstrates a scaffolding role of the TRPC6 protein in disease.

JASN 30: 1910–1924, 2019. doi: https://doi.org/10.1681/ASN.2018070729

FSGS is a devastating form of nephrotic syndrome.[1,2] The cause of primary FSGS is still unknown but inherited forms of the disease are now providing revolutionary clues to the underlying pathogenesis and the target of damage, the glomerular podocyte.[3] Transient receptor potential channel 6 (TRPC6) is a widely expressed, nonselective cation channel. Mutations in TRPC6 are associated

Received July 16, 2018. Accepted June 17, 2019.

Published online ahead of print. Publication date available at www.jasn.org.

Correspondence: Dr. Gavin I. Welsh, Bristol Renal, Bristol Medical School, University of Bristol, Dorothy Hodgkin Building, Whitson Street, Bristol, BS1 3NY, UK. Email: g.i.welsh@bristol.ac.uk

with an inherited form of FSGS[4–6] and upregulation of TRPC6 expression has been identified in a number of acquired forms of proteinuric kidney diseases.[7] TRPC6 interacts with the podocyte-specific proteins nephrin and podocin, both of which have been shown to regulate its activity and/or localization. Indeed, podocin has been reported to have opposing effects on the gating of TRPC6 channels evoked by membrane stretch or diacylglycerol.[8,9] The mutant forms of TRPC6 have been shown to activate NFAT-dependent transcription *in vitro via* calcium influx and activation of calcineurin and to regulate the activity of ERK.[10–12] TRPC6 has been shown to have several functions in the podocyte. The TRPC6 agonist angiotensin II (AngII) increases podocyte motility.[13] Nephrin, which has a role in podocyte adhesion, has been shown to inhibit TRPC6 activation, and some disease-causing mutants have decreased nephrin-binding capability.[8] TRPC6 associates with the podocyte actin cytoskeleton and there is strong evidence that TRPC6 directly affects podocyte signaling and cytoskeletal organization in these cells.[14–16] Indeed, recently TRPC6 activity has been linked to increased calpain 1 and calcineurin activity leading to podocyte injury.[17]

FSGS-causing TRPC6 mutations, for example, G109S, have traditionally been reported to be gain of function, and this increased calcium conductance is thought to be responsible for pathology.[6] However, several reported disease-causing mutations show no change in, or even decreased, calcium conductance.[18] For example, the K874STOP (K874*) mutation results in a 57-amino-acid deletion in the C terminus but has no effect on calcium conductance.[4] This suggests that changes in calcium conductance may not be the sole mechanism underlying the pathology. Patients with TRPC6 mutations do not present with any other pathologic phenotype, suggesting that this protein has a singular role within the podocyte which is affected by mutation. Therefore, the most conspicuous question is, what is unique about TRPC6 activity in the podocyte, a cell that is highly dependent on a tightly regulated actin cytoskeleton?[19]

In this study we have developed TRPC6 knockout podocytes from TRPC6 KO mice and used them together with expression of either GFP-tagged wild-type (WT), dominant negative (DN), or the G109S and the K874* disease-causing mutant forms of the receptor to identify novel binding partners of TRPC6 and explore how the mutations alter these interactions and protein activity.

Significance Statement

Mutations in the transient receptor potential channel 6 (*TRPC6*) gene are associated with an inherited form of FSGS. Emerging evidence has linked TRPC6 activity with calpain activation and podocyte injury. In this study, the authors generated a *TRPC6* knockout podocyte cell line from *TRPC6* knockout mice, engineering these cells to express wild-type and various mutations of TRPC6. They show that TRPC6 binds to both ERK 1/2 and calpain, and is important for the localization of calpain to the cell membrane, independent of TRPC6 calcium influx. This interaction is vital for cell motility and detachment and demonstrates a scaffolding role of TRPC6. These findings suggest that calpain activation and trafficking may be novel therapeutic targets in the treatment of FSGS.

METHODS

TRPC6 KO Cell Line and TRPC6 Constructs

Conditionally immortalized control and TRPC6 KO podocyte cell lines were made as previously described.[20]

A GFP tag was inserted into the second extracellular loop of a WT TRPC6 construct in a pcDNA vector after amino acid 561 using site-directed mutagenesis. PCR was used to introduce complementary restriction enzyme sites at amino acid 561 of TRPC6 and both ends of the GFP sequence. The constructs were then restriction digested and GFP was ligated into the TRPC6 construct. GFP integration was confirmed by sequencing (MWG Eurofins, Germany). The G109S and K874* and the DN TRPC6 LFW678–680AAA[21] mutations were introduced into the WT TRPC6-GFP construct through site-directed mutagenesis and confirmed through sequencing. All constructs were subcloned into a lentiviral vector (pWPXL, a gift from Didier Trono [Addgene plasmid # 12257]) for stable expression in the T6K cells. This construct was transfected into HEK 293 cells along with packaging vectors pMDG.2 and psAX2 (pMD2.G and psPAX2 were gifts from Didier Trono [Addgene plasmids # 12259 and # 12260]) to produce virus. T6K podocytes were transduced with the virus and 8 μg/ml polybrene overnight. Expression was confirmed through fluorescence microscopy and western blotting.

Electrophysiology

Whole-cell patch-clamp recordings were performed at room temperature using patch pipette solution containing (mM): 115 CsCl, 10 EGTA, 2 $MgCl_2$, 5 Na_2ATP, 0.1 NaGTP, 10 HEPES, and 5.7 $CaCl_2$; pH was adjusted to 7.2 with CsOH. The standard bath solution contained (mM): 130 NaCl, 5 KCl, 8 D-glucose, 10 HEPES, 1.2 $MgCl_2$, and 1.5 $CaCl_2$; the pH was adjusted to 7.4 with NaOH and was perfused at a flow rate of 2–3 ml/min. Cells were voltage clamped at −60 mV. TRPC6 was activated through perfusion of 10 μM AngII into the recording chamber. Series resistance (R_s) was monitored throughout the experiment and cells showing a >20% change in R_s were excluded from analysis. Recordings were carried out using a MultiClamp 700B amplifier (Axon Instruments, Foster City, CA). Online electrophysiology data acquisition and analysis were performed using WinLTP software. The holding current (I_{hold}) was recorded at 30-second intervals, and the change in I_{hold} measured as the difference in the current required to maintain the holding voltage before Ang II perfusion. All data were normalized to the mean of the pre–drug perfusion baseline.[22] Averaged data from each experimental

condition are represented as the mean and SEM. Data were analyzed using *t* test.

Calcium Imaging

Calcium influx to podocytes was measured using a Rhod-3 calcium-imaging kit according to the manufacturer's instructions (#R10145; ThermoFisher). Cells were seeded into a 96-well plate and differentiated for 10–14 days. They were incubated with 10 mM Rhod-3 AM+2.5 mM probenecid in the dark for 30 minutes before being washed and incubated in 2.5 mM probenecid for a further 30 minutes. Live-cell imaging was performed in PBS using the IN Cell Analyzer (GE Healthcare, Amersham, UK) imaging platform. Quantification was performed using IN Cell Analyzer work station 3.5 software. A baseline calcium intensity reading was taken from each cell in the field of view before addition of AngII at a final concentration of 1 μM. A second reading was taken 5 seconds after AngII addition and compared with the first to see the increase in calcium influx in response to AngII. Cells were defined by locating the DAPI nuclear signal and looking at a 3-μM collar around the nucleus.

Biotinylation

Podocytes stably expressing WT TRPC6-GFP were biotinylated to assess membrane expression. Cells were washed with borate buffer (10 mM boric acid, 154 mM NaCl, 7.2 mM KCl, 7.2 mM $CaCl_2$ pH to 8.6) before being incubated with 2 mg biotin for 20 minutes. Excess biotin was quenched by washes with 0.192 M glycine before cells were lysed in 500 μl Tris-buffered saline+2% NP40+protease inhibitor cocktail and incubated with streptavidin or control agarose beads for one hour at 4°C. Five washes of the beads were carried out with PBS, with a one minute spin at 1000xg between each wash. A "total protein" sample was taken before the incubation. Samples were then detached from the beads in 4% SDS running buffer and heated to 95°C for 10 minutes before being run on a 10% acrylamide gel and probed with anti-TRPC6 or anti-CD99 antibodies.

Motility and Detachment Assays

Motility assays were carried out as previously described.[23] Briefly, cells were seeded in six-well plates and differentiated for 10–14 days at 100% confluency. A cross was then scratched into the monolayer of cells using a pipette tip. The cross was imaged at 0 and 12 hours postscratch and the cell infiltration into the cleared area measured using ImageJ software. The detachment assay was carried out using a modified version of a previously published protocol.[24] Differentiated cells were trypsinized and resuspended in media at 3×10^5 cells/ml. Then, 50 μl cells was added to each well of a 96-well plate along with 50 μl PBS. If an inhibitor was being used, then this was added to the PBS at 2× concentration. Cells were left for 48 hours to adhere. Control wells were fixed with 4% paraformaldehyde to measure 100% attachment. Next, 50 μl trypsin was added to each experimental well for 2 minutes before washing with PBS and adding 50 μl FBS to attenuate trypsinization. Cells were then fixed with 4% paraformaldehyde. After washing with H_2O, cells were stained with 0.1% crystal violet in 2% ethanol for 60 minutes. After further washes the dye was solubilized with 100 μl 10% acetic acid and left on an orbital shaker for 5 minutes. Absorbance was measured at 570 nm using a plate reader. Results were expressed as a percentage of 100% attachment of control cells.

Immunofluorescence

Immunofluorescence was performed as described previously.[25] Images were captured using a Leica AM fluorescence confocal microscope or a Leica AM total internal reflection fluorescence (TIRF) microscopy multicolor system attached to a Leica DMI 6000 inverted epifluorescence microscope equipped with 405-, 488-, 561-, and 635-nm laser lines. All primary antibodies are listed in the table below.

Coimmunoprecipitation and Proteomics

Cells transduced with either WT, G109S, or K874* TRPC6-GFP–expressing lentivirus were lysed in TNE buffer (50 mM Tris, 100 mM NaCl, 0.1 mM EDTA) containing 10% glycerol+1% NP40. GFP and GFP–TRPC6 proteins and interacting proteins were immunoprecipitated using the GFP-Trap system (Chromotek). Proteins were eluted from beads into 50 μl 4% SDS loading buffer. Samples were separated on Nupage 4%–12% precast gels (Invitrogen) and subjected to LC–MS/MS analysis on an Orbitrap Velos (Thermo) mass spectrometer as described previously.[25–27]

Antibodies Used

Calpain Assay

The calpain assay was performed on differentiated podocytes using a Calpain Activity Assay Kit (ab65308; Abcam). Triton X-100 (1%) was added to the provided lysis buffer to detect membrane-associated calpain activity. Briefly, cells were

Antibody	Supplier and Catalog Number
TRPC6	Cell Signaling Technology #16716
Caldesmon-1	Cell Signaling Technology #2980
Calpain 1	Cell Signaling Technology #2556
Calpain 2	Cell Signaling Technology #2539S
Calpain 1	Abcam #ab28258
Talin-1	Cell Signaling Technology #4021
Phospho-p44/p42 (ERK1/2)	Cell Signaling Technology #4370S
FAK	Cell Signaling Technology #3285S
Phospho-FAK (Tyr397)	Cell Signaling Technology #8556S
Synaptopodin	Santa Cruz #sc-515842
WT1	Cell Signaling technology #13580
Podocin	Abcam #ab50339
CD2AP	Cell Signaling #5478
Nephrin	Acris #BP5030
GFP	Sigma #11814460001
CD99	Kind gift from Professor George Banting University of Bristol.
Plcy2	Cell Signaling Technology #3872S

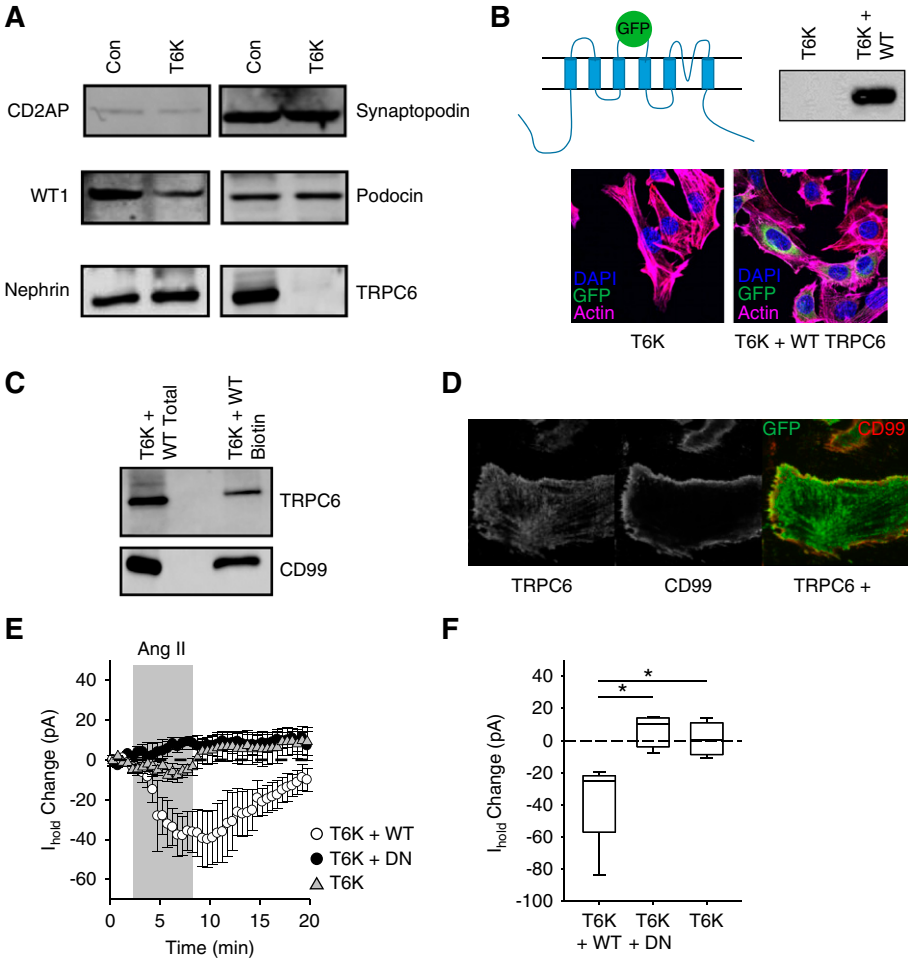


Figure 1. A GFP tagged TRPC6 construct is functional when expressed in a TRPC6 KO podocyte cell line. (A) Podocyte markers CD2-associated protein (CD2AP), synaptopodin, Wills tumor protein 1 (WT1), podocin, and nephrin are expressed in TRPC6 knockout (T6K) cells. TRPC6 is not expressed. (B) A TRPC6 construct with an extracellular green fluorescent protein (GFP) tag at amino acid 561 was generated and reintroduced to the T6K cells. This is demonstrated by western blotting and immunofluorescence (magenta, actin; GFP, green; DAPI, blue). Disease-causing and DN forms of the GFP-tagged TRPC6 construct were also generated and introduced to T6K cells. (C and D) Biotinylation and TIRF microscopy demonstrating that WT TRPC6-GFP was able to traffic to the plasma membrane. CD99 is a membrane protein and was used as a control in both experiments. (E) Patch clamp analysis of channel function. Pooled data of change in I_{hold} caused by 6-minute 1 μM AngII perfusion. AngII perfusion causes a rapid change in I_{hold} in WT (-36.2 ± 10.5 pA at 8-minute timepoint, open symbols, n=5) but not the null mutant (8.7 ± 3.4 pA at 8-minute timepoint, closed symbols, n=4) or T6K (-3.5 ± 4.3 pA at 8-minute timepoint, triangle symbols, n=4) cells. Gray vertical bar represents perfusion of AngII. All symbols represent the mean±SEM. (F) Summary box plot (boxes, 25th–75th percentile; lines, median) showing changes in I_{hold} caused by 1 μM AngII perfusion at the 8-minute timepoint in (E). *$P<0.05$, unpaired t test. Con, control.

seeded into six-well plates and differentiated for 10–14 days. Cells were lysed in the provided lysis buffer and a BSA assay was performed to measure protein concentration. Cell lysate (100 μg) was then loaded into each well of a 96-well plate and incubated with the provided buffer and calpain substrate. Control wells were treated with either active calpain (positive control) or calpain inhibitor (negative control). The plate was incubated at 37°C in the dark for 1 hour before absorbance was measured at Ex/Em=400/505 nm.

Statistical Analyses

All statistics were performed in GraphPad Prism 5.

RESULTS

Expression of a Functional TRPC6 Construct in Knockout Podocytes

Podocytes were isolated from TRPC6$^{-/-}$ mice, and a conditionally immortalized cell line was established as described previously.[20] The TRPC6$^{-/-}$ cell line (T6K cells) was extensively characterized by demonstrating the expression of podocyte markers (CD2AP, synaptopodin, WT1, podocin, and nephrin) and the absence of TRPC6 protein (Figure 1A). The levels of both TRPC3 and 7 did not change in these cells compared with WT cells (Supplemental Figure 1). Using

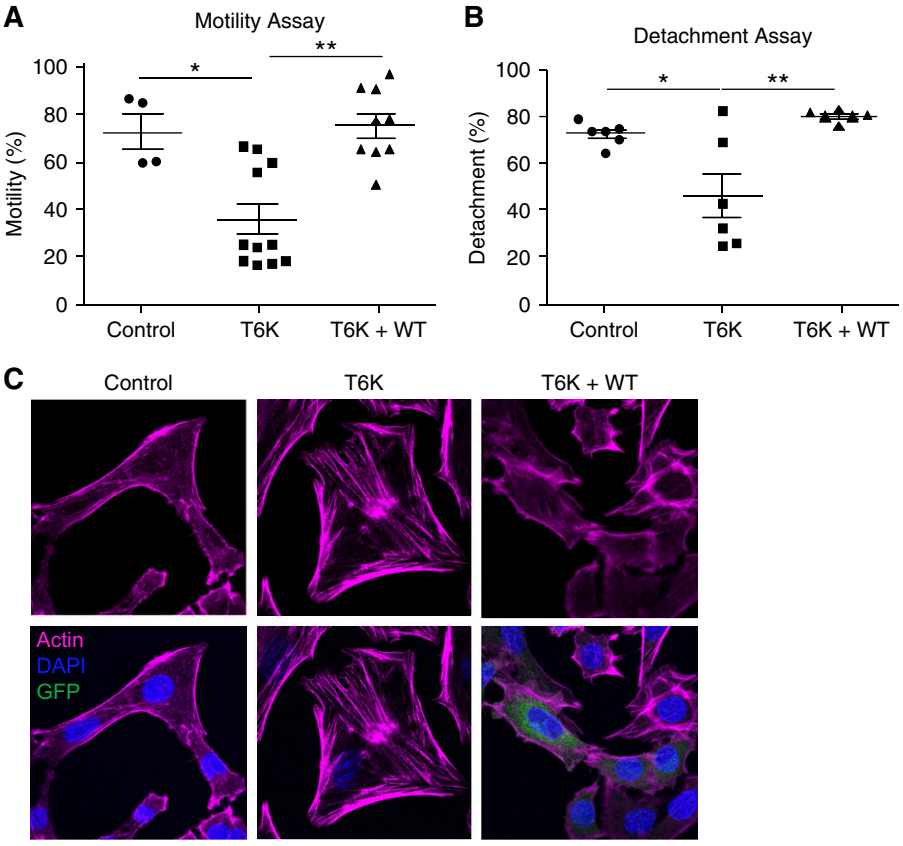


Figure 2. TRPC6 KO cells have altered motility and detachment compared to controls. (A) Motility of the TRPC6 KO (T6K) cell line was measured by scratch assay closure after 12 hours and compared with control and T6K podocytes expressing WT TRPC6-GFP (T6K+WT). T6K cells (38.40%±7.34% closure) were less motile than control podocytes (73.67%±7.56% closure). This was rescued by re-introduction of WT TRPC6-GFP (72.54%±6.82% closure). One-way ANOVA, *$P<0.05$, **$P<0.01$. (B) Detachment was measured as cells lost after 1-minute trypsin treatment. This demonstrated decreased detachment of T6K podocytes (46.35%±9.72%) compared with control and T6K+WT (72.49%±1.93% and 80.08%±0.95%, respectively). One-way ANOVA, *$P<0.05$, **$P<0.01$. (C) There was visible alteration of the actin cytoskeleton in T6K cells compared with control and T6K+WT podocytes. Magenta, phalloidin staining for actin.

lentiviral transduction, TRPC6 constructs were stably expressed in these cells, allowing WT, dominant negative (DN), and disease-causing mutant forms of TRPC6 to be studied without interference of any native TRPC6 channels. To monitor expression of these constructs, an internal GFP tag in the second extracellular loop was added (Figure 1B). There is no significant known role of the second extracellular loop in TRPC6 protein function and thus it was determined that tagging here would cause minimal disruption to protein function. The intracellular N and C termini are known to be involved in trafficking and protein interaction of the channel, and a protein tag placed in this region may have prevented binding. Expression of the GFP-tagged WT TRPC6 construct was confirmed through immunofluorescence and western blotting (Figure 1B). Biotinylation and total internal reflection fluorescence (TIRF) microscopy experiments were also performed to confirm that the construct still trafficked to the plasma membrane despite the presence of the GFP tag (Figure 1, C and D), comparing with the membrane protein CD99. Calcium influx in response to 10 μM AngII was measured in T6K cells and T6K cells expressing either WT (T6K+WT) or the previously described DN (T6K+DN) TRPC6-GFP construct,[21] using rhod-3 calcium imaging and patch clamping,

Experiment	Statistical Analysis	Unit of Analysis
Patch clamp analysis	Unpaired *t* test	Pooled cells
Motility and detachment	One-way ANOVA	Per experiment (three replicates per experiment)
Calpain activity assay	One-way ANOVA	Per experiment (three replicates per experiment)
Calcium assay	One-way ANOVA	Per experiment (three wells imaged per experiment)
Calpain assay with inhibitor	Unpaired *t* test	Per experiment (three replicates per experiment)
Calcium assay with AngII (raw data)	Paired *t* test	Per experiment (three wells imaged per experiment)
Densitometry	One-way ANOVA	Mean of minimum three blots per treatment

A

Protein Name	Peptide no. GFP only	Peptide no. TRPC6-GFP	Peptide ratio TRPC6-GFP GFP only
TRPC6	1	500	500
TRPC7	1	71	71
TRPC3	1	59	59
Calpain 2	1	21	21
Calpain 2 sub	1	20	20
Caldesmon-1	4	36	9
PLCy2	1	7	7

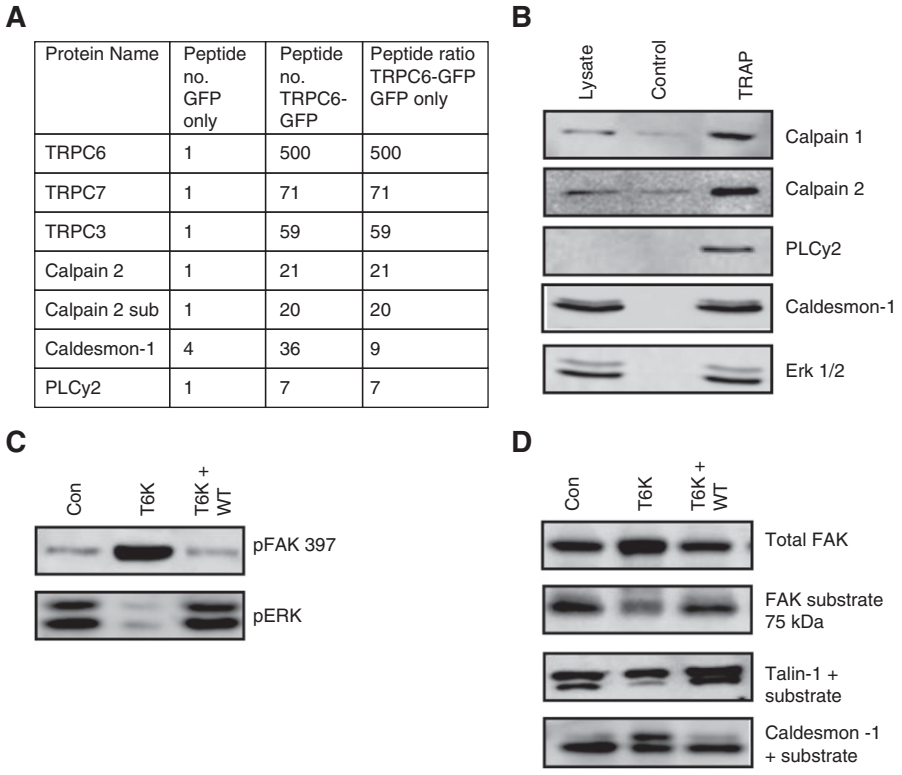


Figure 3. Calpain 1, calpain 2, caldesmon-1 and ERK 1/2 are novel TRPC6 binding partners. (A) Table of proteomics results of TRPC6-binding partners from WT human podocytes overexpressing TRPC6-GFP. TRPC6 was seen to bind to TRPC3, TRPC7, and PLCy2, interactions that have been described previously.[49,50] Novel interactions with calpain 2 and caldesmon-1 were identified. Podocytes expressing the GFP protein only were used as a control. (B) Interactions reported by proteomics were confirmed in TRPC6 KO cells expressing WT TRPC6-GFP (T6K+WT) by coimmunoprecipitation (TRAP lane). Control agarose beads were used to demonstrate that immunoprecipitation was specific to TRPC6 (control lane). Additional interactions with calpain 1 and ERK 1/2 were also identified. On the basis of the proteomics results, the phosphorylation and/or cleavage states of FAK, talin-1, caldesmon-1, and ERK 1/2 were ascertained through western blotting. (C) T6K had increased FAK phosphorylation at Tyr 397 and decreased ERK phosphorylation compared with control and T6K+WT cells. (D) Cleavage of FAK, talin-1, and caldesmon-1 was decreased in T6K cells compared with controls. For densitometry see Supplemental Figure 3. Con, control; TRAP, GFP-TRAP associated pull-down.

demonstrating that the expressed WT TRPC6-GFP was functional (Figure 1, D–F, Supplemental Figure 2A) and importantly that the GFP tag had no significant effect on TRPC6 channel activity compared with an untagged version (Supplemental Figure 2B).

Motility and Adhesion Regulated by TRPC6

Podocyte adhesion and motility are often altered in disease states; therefore, we examined cell motility using a wound healing assay. The WT podocytes and T6K cells transfected with TRPC6 were significantly more motile than the T6K cells, suggesting that the absence of TRPC6 impairs cell motility (Figure 2A).

Adhesion of the cell to, and detachment of the cell from, the culture flask was also studied. There was no significant difference between the control, T6K, and T6K+WT cells in the ability of the cell to adhere to the culture flask (data not shown). However, it was noticed when culturing T6K cells that they required a much longer incubation with trypsin to detach from the culture flask. This was verified using a detachment assay (Figure 2B). The percentage of cells that had detached from the well after a 1-minute trypsin incubation was determined, with significantly fewer T6K cells detaching compared with control or T6K+WT. The decreased motility of T6K cells observed is therefore likely due to an impairment in the cells' ability to deadhere, decreasing their ability to move.

Actin remodeling is closely linked to cell motility and adhesion, and actin reorganization is seen with podocyte foot process effacement. Overexpression of TRPC6 has previously been shown to cause cytoskeletal rearrangement, and inhibition of the receptor has been shown to prevent albumin-induced F-actin cytoskeletal disruption.[7] Control, T6K, and T6K+WT podocytes were stained with phalloidin and imaged on a confocal microscope (Figure 2C). There were more actin stress fibers present in the T6K cells, whereas in the control and T6K+WT podocytes actin was localized to the membrane and evenly spread throughout the cytoplasm (Figure 2C).

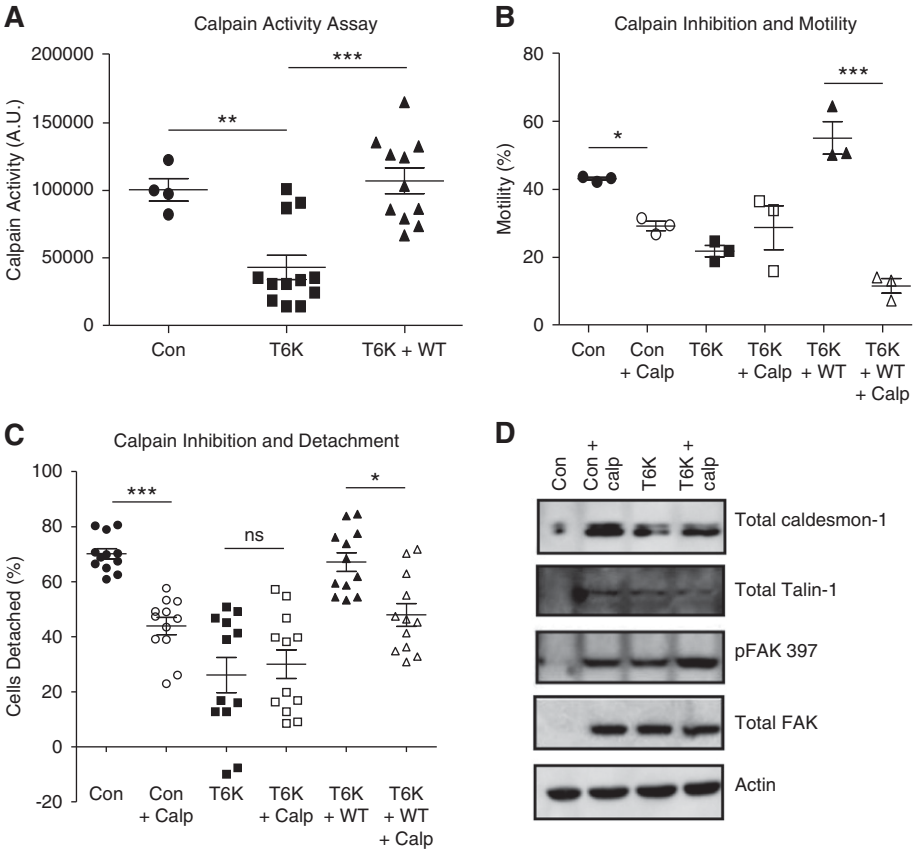


Figure 4. Calpain activity is decreased in T6K cells and calpain inhibition mimics the TRPC6 KO phenotype. (A) Calpain activity was decreased in TRPC6 KO (T6K) cells compared with control (42,609±8900 A.U. versus 99,493±8227 A.U) and recovered to normal levels (106,138±9414 A.U) when WT TRPC6 was reintroduced (T6K+WT). (B) Addition of the calpain inhibitor calpeptin (10 μM) reduced motility of control and T6K+WT podocytes by 67.7% and 20.7%, respectively, but had no effect on T6K cell motility. *$P<0.05$, ***$P<0.001$, unpaired *t* test. (C) Addition of the calpain inhibitor calpeptin on detachment. Treatment with 10 μM calpeptin reduced detachment of control and WT cells by 26.3% and 19.2%, respectively. There was no significant effect on T6K podocytes. (D) Incubation of control cells with 10 μM calpeptin inhibited cleavage of calpain targets and led to phosphorylation of FAK. It had no effect on T6K cells. Unpaired *t* test, **$P<0.01$, ***$P<0.001$. A.U., arbitrary units; Calp, calpeptin; Con, control.

Protein Partners of TRPC6 in Podocytes

To identify novel TRPC6-binding partners, GFP-tagged WT TRPC6-GFP was expressed in podocytes using lentiviral transduction. These and control cells, expressing GFP only, were then lysed and the GFP immunoprecipitated using the highly efficient GFP-Trap method.[28] The precipitated GFP and TRPC6-GFP were separated by SDS-PAGE and interacting proteins analyzed by LC–MS/MS after in-gel tryptic digestion. Three intracellular proteins (calpain 2, caldesmon-1, and PLCy2) and two ion channel proteins (TRPC3 and TRPC7) were identified by MS analysis, and were significantly more abundant in the TRPC6-GFP pulldown compared with the GFP control (Figure 3A). Coimmunoprecipitation experiments were performed to verify the physical interaction of TRPC6 with calpain 2, caldesmon-1, and PLCy2 (Figure 3B). Immunoprecipitation experiments were also carried out for calpain 1 and ERK 1/2, proteins that are known to interact with or be linked to those identified in the proteomic screen, and these proteins were also shown to interact with TRPC6 (Figure 3B, Supplemental Figure 3).

Loss of Calpain Activity in TRPC6 KO Cells

The calpains are a family of calcium-dependent proteases and one of their cleavage targets is focal adhesion kinase (FAK). Given the role of FAK in adhesion and the increased adhesion of the T6K cells, the phosphorylation status of FAK in the control, T6K, and T6K+WT cells was determined. FAK showed increased phosphorylation in T6K cells when compared with T6K+WT and control cells at the Tyr 397 autophosphorylation site (Figure 3C). Because ERK1/2 had also been identified as a TRPC6-binding protein and is known to form a complex with FAK and calpain, its phosphorylation was also studied.[29] We demonstrated decreased ERK1/2 phosphorylation in T6K cells compared with control or T6K+WT podocytes (Figure 3C).

FAK, ERK1/2, and calpain have previously been shown to form a complex[30] and, because all three were coimmunoprecipitated with TRPC6, the calpain cleavage targets talin-1,

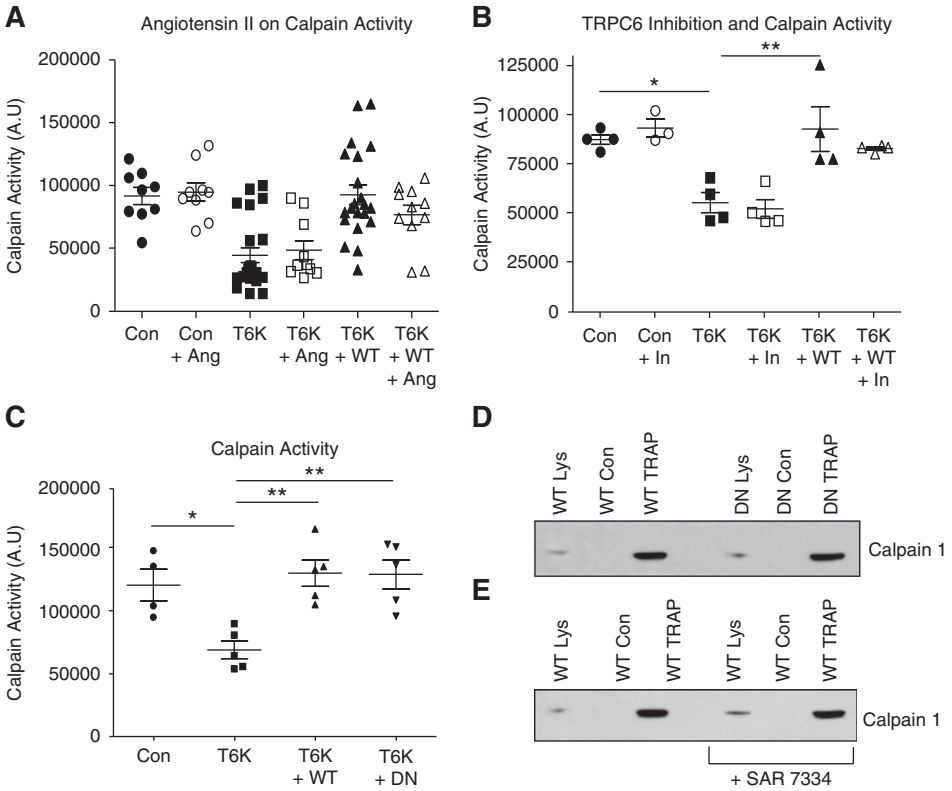


Figure 5. Decreased calpain activity is independent of calcium influx through TRPC6 activation. (A) Addition of the TRPC6 agonist AngII (1 μM), which is known to increase calcium current through the receptor (see Figure 2E, Supplemental Figure 2A), had no effect on calpain activity in control, T6K, or T6K+WT podocytes; unpaired *t* test. (B) Application of TRPC6 inhibitor SAR 7334 (20 nM) to control, T6K, or T6K+WT podocytes had no effect on calpain activity; unpaired *t* test. (C) Calpain activity was at control levels in TRPC6 KO cells expressing a DN mutant and significantly increased compared with T6K (T6K+DN=128,612±11,370 A.U.; control=120,297±12,481 A.U.; T6K+WT=129,619±10,408 A.U.; T6K=68,973±7009 A.U.). *$P<0.05$, **$P<0.01$, one-way ANOVA. (D) GFP TRAP coimmunoprecipitation shows interaction between calpain and TRPC6 in both T6K+WT and T6K+DN podocytes. (E) There was also an interaction between TRPC6 and calpain in cells that had been treated with the TRPC6 inhibitor SAR 7334. Ang, Angiotensin; A.U., arbitrary units; Con, control; Lys, lysate.

caldesmon-1, and FAK were probed for (Figure 3D). Each of these proteins was shown to have increased cleavage in the control, T6K+WT, and T6K+DN podocytes compared with T6K, suggesting that the presence of TRPC6 is important for calpain activity and cleavage of these targets.

Calpain activity assays confirmed a loss of calpain activity in TRPC6 KO cells compared with T6K+WT and control podocytes (Figure 4A). Treatment of control and T6K+WT cells with the calpain inhibitor calpeptin (10 μM) caused motility of the cells to mimic that seen in T6K podocytes but had no effect on the motility of the T6K cells (Figure 4B). Treatment of these cells with calpeptin also decreased detachment and blocked cleavage of talin-1, caldesmon-1, and FAK (Figure 4, C and D). This suggests that the loss of calpain activity is responsible for the decreased motility and detachment of the T6K cells. AngII is a TRPC6 agonist and a calcium assay demonstrated that application of AngII to control and T6K+WT podocytes caused calcium influx into the cell, as shown in Supplemental Figure 2. Neither AngII nor the TRPC6 inhibitor, SAR 7334,[31] had any effect on calpain activity in control, T6K+WT, or T6K podocytes even though SAR 7334 blocked calcium influx into the cells (Figure 5, A and B, Supplemental Figure 2, C–E). Treatment with the TRP channel activator OAG also had no effect on calpain activity (Supplemental Figure 2F). Furthermore, calpain activity was not altered in cells that expressed T6K+DN (Figure 5C) even though this mutant bound to calpain (Figure 5D). These results suggest that increased calcium influx does not alter calpain activity. Calpain binding was also maintained in the presence of SAR 7334 (Figure 5E).

This led us to hypothesize that the effect of TRPC6 knockout on calpain activity was due to altered localization of calpain rather than decreased calcium influx. This was examined using immunofluorescence staining and confocal microscopy (Figure 6A). Calpain 1 appeared to be membrane localized in the control and T6K+WT cells, but not in the T6K cells. This was confirmed by TIRF microscopy, showing that in the T6K+WT, T6K+DN, and control cells calpain was visualized at the

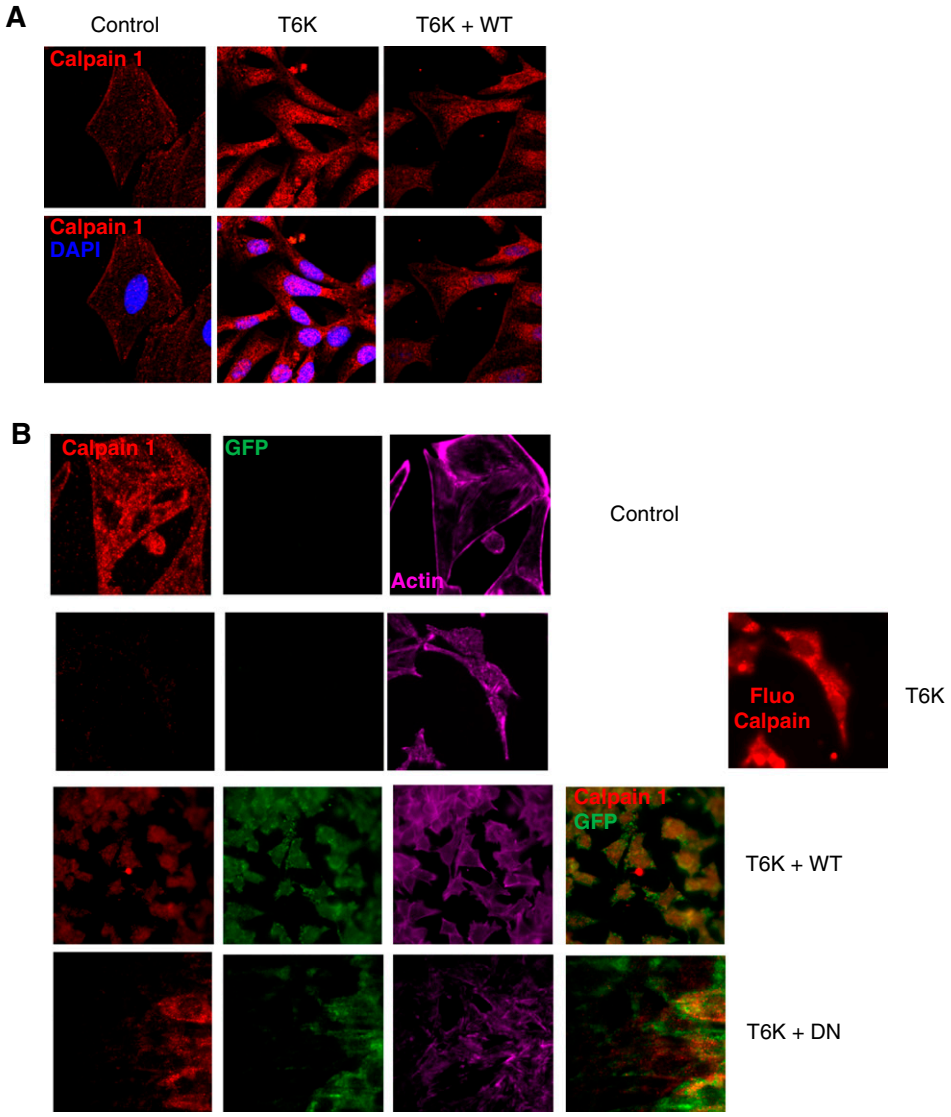
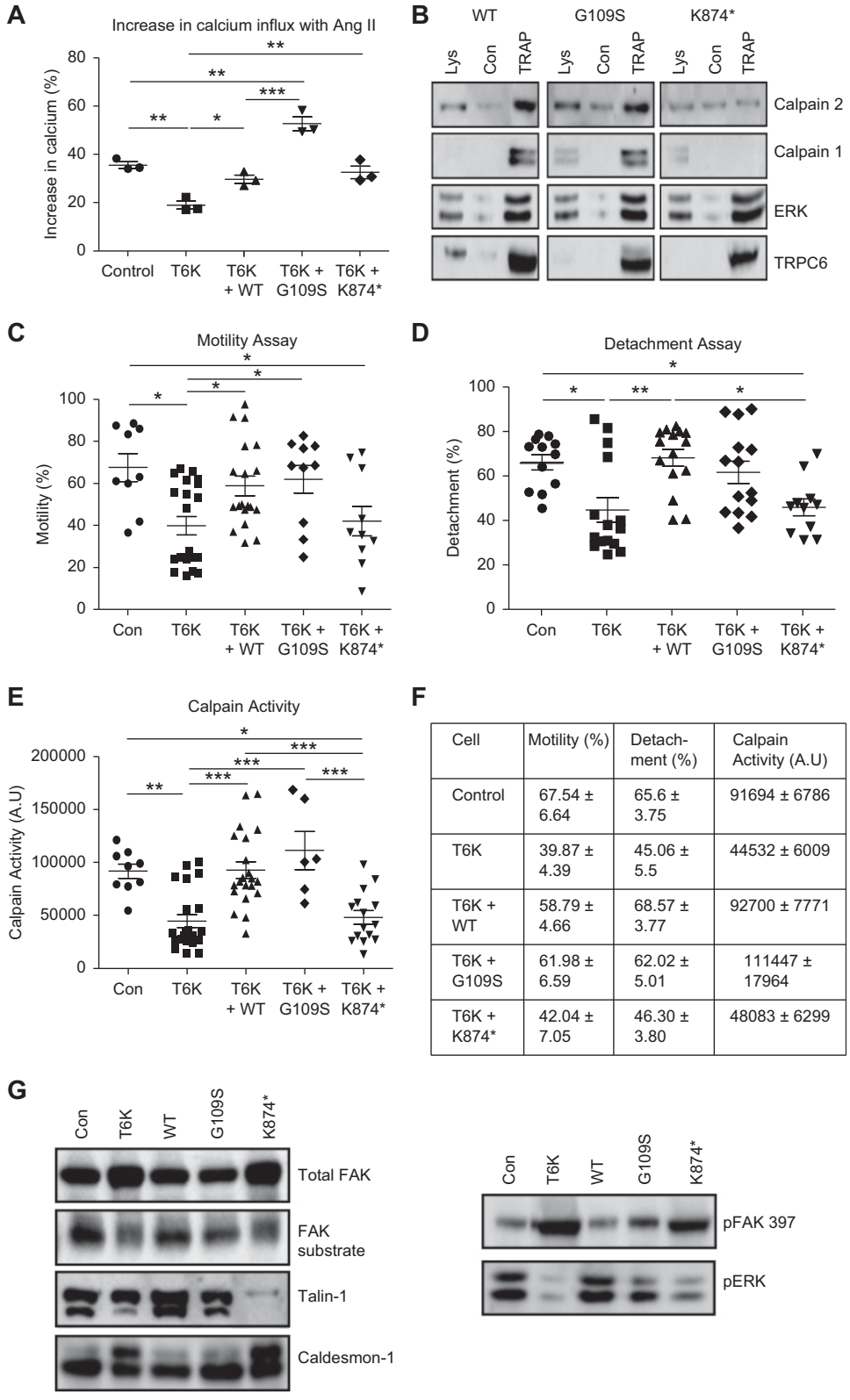


Figure 6. Calpain localization is altered in T6K podocytes. (A) Confocal microscopy showing the localization of calpain 1 and the nuclear marker DAPI in control, TRPC6 KO (T6K), and TRPC6 KO expressing WT TRPC6-GFP (T6K+WT) podocytes. Calpain localization is lost from the membrane in T6K cells, and rescued by the reintroduction of WT TRPC6-GFP. (B) TIRF microscopy showing location of calpain 1, GFP (for TRPC6-GFP), and actin in control, T6K, and T6K+WT podocytes. TIRF microscopy will only image at or very near the cell surface. Calpain can be seen at the surface of control cells, T6K+WT cells, and T6K cells expressing the DN mutant (T6K+DN) but not T6K cells. Fluorescence microscopy has been used on the T6K cells to show that calpain is present in the cell, just not at the surface. Red, calpain; blue, DAPI; green, TRPC6-GFP; pink, actin, in all images.

plasma membrane of the cell, and this membrane localization was lost in the T6K cells (Figure 6B). These results suggest that the interaction between TRPC6 and calpain 1 and 2 is important in the localization and activation of calpain.

TRPC6 Mutants and Calpain Membrane Localization

Disease-causing mutations can have varying effects on the calcium conductance of TRPC6 channels. We investigated whether altered interaction between TRPC6 and calpain could be causing the pathology seen in these patients. Two mutants, G109S, which has been reported to cause an increase in calcium conductance, and K874*, which has no effect on channel conductance, were used.[4,6] As expected, the G109S-expressing podocytes showed increased calcium conductance in response to 1 μM AngII, whereas there was no effect in the K874*-expressing cells (Figure 7A, Supplemental Figure 2A). Both mutants bound ERK 1/2 but there was decreased interaction between TRPC6 K874* and both calpain 1 and calpain 2, whereas the interaction was maintained with the G109S mutant (Figure 7B). The G109S mutant cells mimicked WT cells in their motility, adhesion, protein phosphorylation, and calpain target protein cleavage. In contrast, K874* podocytes



Cell	Motility (%)	Detachment (%)	Calpain Activity (A.U)
Control	67.54 ± 6.64	65.6 ± 3.75	91694 ± 6786
T6K	39.87 ± 4.39	45.06 ± 5.5	44532 ± 6009
T6K + WT	58.79 ± 4.66	68.57 ± 3.77	92700 ± 7771
T6K + G109S	61.98 ± 6.59	62.02 ± 5.01	111447 ± 17964
T6K + K874*	42.04 ± 7.05	46.30 ± 3.80	48083 ± 6299

Figure 7. The TRPC6 mutant K874* does not bind to calpain and podocytes expressing this mutant have a similar phenotype to T6K cells. (A) AngII (1 μM) initiates an increase in calcium in control and in all cell types. Calcium increases were 33.75±4.7%, 19.08±1.6%, 31.20±3.8%, 52.56±2.8%, and 36.62±8.2.6%, for the control, T6K, T6K + WT, T6K + G109S and T6K + K874* podocytes, respectively. The calcium increase is significantly larger in the gain-of-function mutant G109S, and significantly smaller in the

were less motile, more adhesive, and had decreased calpain activity (Figure 7, C–E).

In the K874* mutant podocytes FAK phosphorylation was increased and ERK phosphorylation was decreased, and there was decreased protein cleavage of calpain targets for the K874* mutant (Figure 7F). The K874* mutant therefore mimics the TRPC 6KO cells, despite there being no change in TRPC6 conductance. This suggests that the pathology of the K874* mutant is *via* its altered calpain binding.

As with T6K cells, calpain expression in the K874* podocytes was not seen in the membrane using either confocal (Figure 8A) or TIRF (Figure 8B) microscopy.

DISCUSSION

Mutations in TRPC6, a nonselective cation channel, are associated with an inherited form of FSGS. Despite widespread expression, patients with TRPC6 mutations do not present with any other pathologic phenotype, suggesting that this protein has a unique role within the target cell for FSGS, the kidney podocyte. Although most TRPC6 mutations are reported to cause changes in calcium dynamics, it is unclear how these result in a podocyte-specific phenotype, a cell that is highly dependent on a tightly regulated actin cytoskeleton. To understand the role of TRPC6 in the podocyte and the effect of disease-causing mutations, conditionally immortalized TRPC 6KO podocytes were established from TRPC6 KO mice. These cells were found to be less motile, more adhesive, and to have an altered actin cytoskeleton compared with WT podocytes or knockout podocytes expressing WT TRPC6. This agrees with previous work showing that TRPC 6KO podocytes are less motile, and that this phenotype was rescued by reintroduction of wtTRPC6,[13] but is in contrast to other studies that have reported a role for TRPC6 in inducing Rho activation, stress fiber formation, and decreased motility.[15,32–34] This discrepancy might be due to the level of TRPC6 knockdown because these cells have been developed from a knockout animal compared with the previous studies using siRNA technology, where some preservation of expression is seen.

TRPC6 has been shown to interact with several proteins in the podocyte, including podocin and nephrin.[4] This led us to wonder whether there are other, as yet unidentified, TRPC6 protein interactions that are important in podocyte function. Using GFP TRAP-pulldown coupled with mass spectrometry we identified several TRPC6-binding partners, including TRPC3, TRPC7, and PLC, which are known interactors of TRPC6, thus validating our approach.[8] However, two of the identified interactors, calpain 2 and caldesmon-1, are novel. These interactions were confirmed by immunoprecipitation experiments, which also showed that TRPC6 binds to calpain 1. Because ERK1/2 signaling is known to be required for calpain activation,[35] and because gain-of-function TRPC6 mutations have been shown to increase ERK1/2 activation,[10] we also looked to see whether there was a physical interaction between TRPC6 and ERK 1/2. Importantly, we showed that ERK 1/2 is also a novel TRPC6 interactor. Whether these are direct interactions or *via* a complex with other proteins, such as seen for podocin for the interaction of TRPC6 with NADPH oxidase, is still to be determined.[36]

The calpains are a family of calcium-dependent proteases that have critical functions in controlling the podocyte cytoskeleton, and hence cell adhesion and motility, *via* cleavage of paxillin, FAK, and talin. An increase in calpain activity has previously been reported as contributing to puromycin aminonucleoside–induced podocyte injury.[37] Furthermore, cleavage of talin-1 by calpain has also been hypothesized to promote the pathogenesis of nephrotic syndrome, and calpain 1 has recently been shown to link TRPC6 activity to podocyte injury.[17,38] In neuronal cells and tissue activation of calpain has been reported to lead to TRPC6 degradation, contributing to neuronal damage in cerebral ischemia.[39–41] Calpain activity was significantly downregulated in the TRPC6 knockout cells and treatment of control and T6K+WT cells with the calpain inhibitor calpeptin resulted in decreased motility in the control and T6K+WT cells, mimicking that seen in TRPC6 knockout podocytes. This suggests that the loss of calpain activity is responsible for the decreased motility of the TRPC6 knockout cells. Importantly, although treatment of the control and T6K+WT podocytes but not the TRPC6 knockout cells with AngII caused calcium influx into the cell, there was no effect of treatment on calpain activity. These data indicate that the regulation of calpain activity by TRPC6 is independent of alterations in its calcium conductance. Interestingly, TRPC6 activity has recently been linked to increased calpain 1 and calcineurin activity leading to podocyte injury.[17] However, in contrast to our data this study demonstrated calpain activation upon treatment of mouse podocytes with Adriamycin or the TRP channel activator OAG,[17] suggesting that the mode of calpain activation requires further study.

Knockdown of TRPC6 or expression of the K874*, but not the G109S or TRPC6DN mutant, resulted in increased FAK phosphorylation and decreased ERK phosphorylation. It can

TRPC6 KO podocytes (T6K). One-way ANOVA, *$P<0.05$, **$P<0.01$, ***$P<0.001$. (B) Coimmunoprecipitation shows that calpain 1 and 2 bind to WT and G109S TRPC6 but not K874*. TRAP lane shows protein eluted from TRAP beads; control lane shows protein eluted from control beads. ERK1/2 binds to all three TRPC6 constructs. (C–E) Motility, detachment, and calpain assays for control, T6K, WT, and mutant podocytes. K874* mimicked T6K, whereas G109S mimicked control and WT cells. Values are given in the table in (F). (F) Summary table of values for motility, detachment, and calpain activity. (G) Phosphorylation and cleavage of control, T6K, WT, and mutant podocytes. K874* mimics T6K, whereas G109S mimics WT. For densitometry see Supplemental Figure 3. *$P<0.05$, **$P<0.01$, ***$P<0.001$. A.U., arbitrary units; Con, control; Lys, lysate.

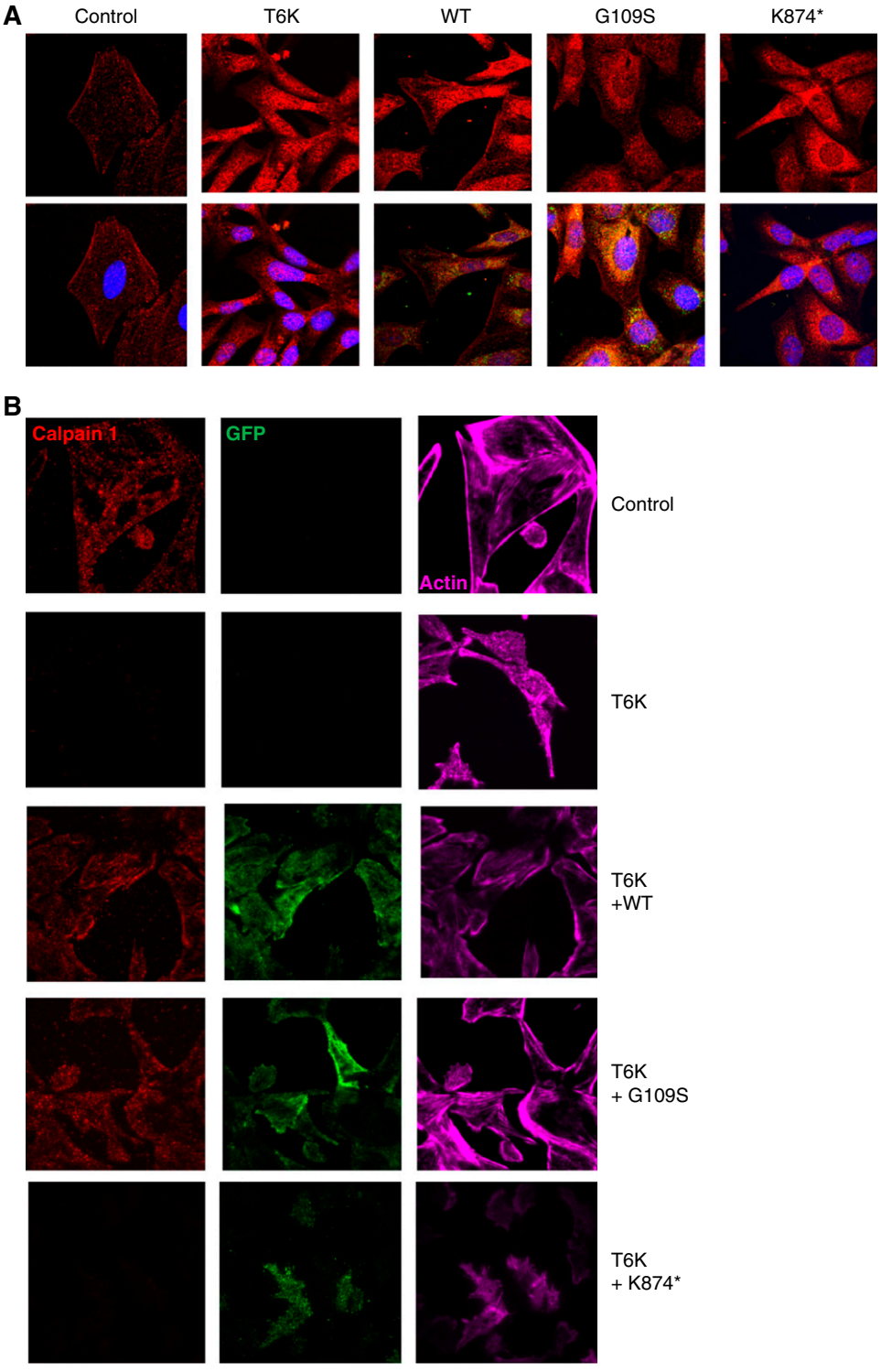


Figure 8. K874* podocytes do not bind to calpain and have a similar phenotype to T6K cells. (A) Confocal microscopy showing localization of calpain 1. Calpain 1 shows some membrane localization in control and WT or G109S TRPC6 expressing TRPC6 KO podocytes (T6K+WT) or (T6K+G109S) cells, but not in T6K or T6K+K874*. GFP has been used to confirm presence of TRPC6 in transfected cells. (B) TIRF microscopy supported this loss of membranous calpain in T6K and K874* cells. Membranous calpain was seen in all other cell types. For all images, red, calpain; green, TRPC6-GFP; pink, actin.

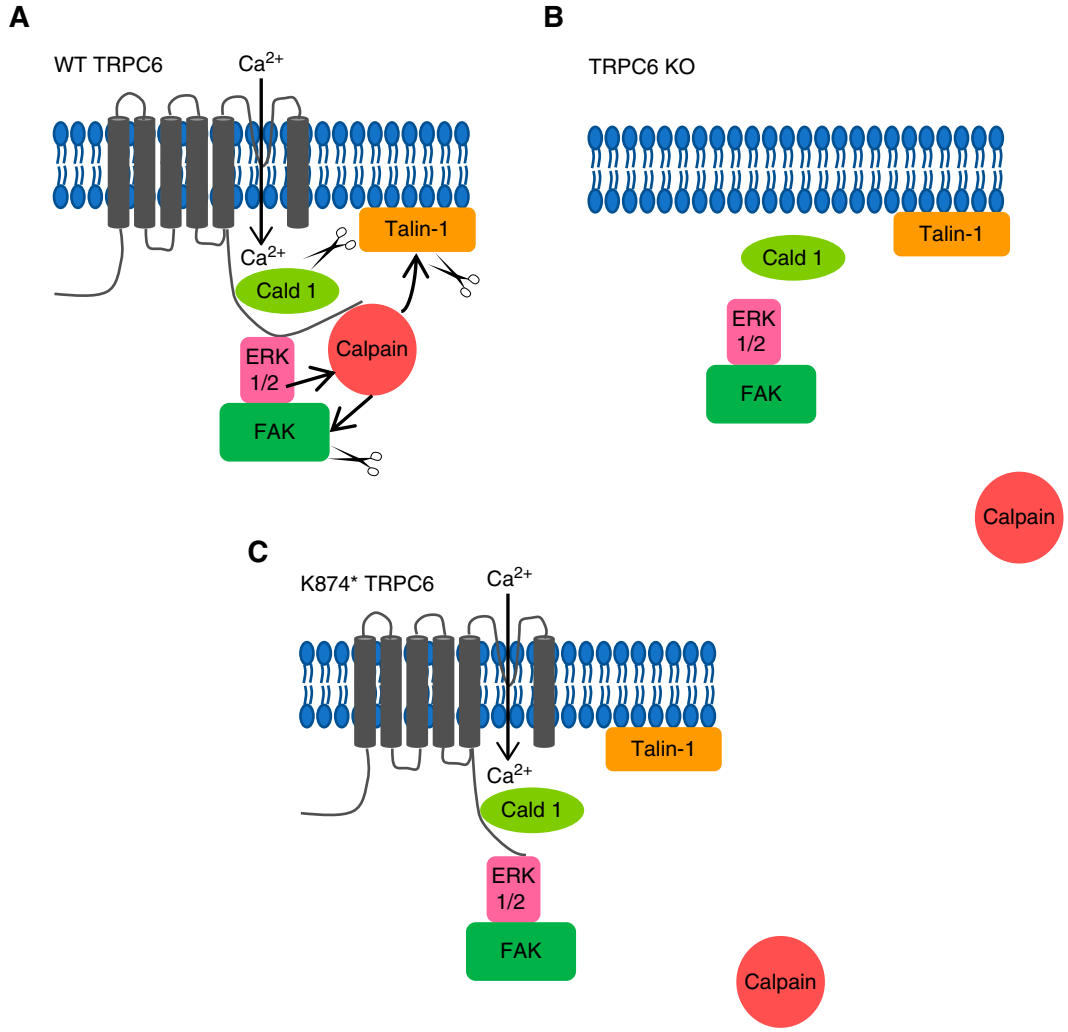


Figure 9. Proposed role of the calpain-TRPC6 interaction in podocytes. (A) WT TRPC6 binds to, and acts as a structural scaffold at the membrane for, caldesmon-1 (cald1), ERK 1/2, and calpain. This keeps calpain localized just below the membrane where it can easily cleave its targets talin-1, FAK, and caldesmon-1. (B) In the absence of TRPC6 there is no calcium influx to the cell and calpain is also not localized to the membrane. This means that there is no cleavage of talin-1, FAK, or caldesmon-1. (C) The disease-causing mutant TRPC6 K874* has a truncation at its C terminus. Calpain no longer binds to this form of TRPC6 and is mislocalized. The mutant allows the same calcium influx as WT TRPC6. There is no cleavage of caldesmon-1, talin-1, or FAK. This suggests that the localization of calpain to the membrane is important in its function in the podocyte.

therefore be deduced that TRPC6 is contributing to the phosphorylation state of ERK1/2 and FAK. Src-induced phosphorylation of FAK has been shown to be required for rapid actin stress fiber assembly and focal adhesion formation. It is also required for the formation of a calpain–FAK–ERK1/2 complex and calpain cleavage of FAK.[42] Furthermore, it has previously been shown that there is an increase in ERK1/2 phosphorylation in podocytes expressing gain-of-function disease-causing TRPC6 mutations.[10] In addition, ERK phosphorylation has been shown to increase calpain activation[43] and cell motility.[44–46] Knockdown of TRPC6 or expression of the K874*mutant also led to decreased cleavage of the calpain targets talin-1, caldesmon-1, and FAK, suggesting that the presence of TRPC6 is important for calpain cleavage of these targets. The cleavage of FAK leads to deadhesion and motility of cells and talin-1 cleavage has been demonstrated to be a rate-limiting step in adhesion disassembly,[47] so decreased cleavage of these proteins is consistent with the increased adhesion, decreased motility, and actin reorganization observed in the T6K and K874* mutant podocytes. Again, the lack of effect of the DN and G109S mutants on calpain activity and cleavage of target proteins suggests that increased calcium conductance is not important in the regulation of calpain by TRPC6. Confocal and TIRF microscopy of these cells demonstrated a mislocalization of calpain away from the plasma membrane, suggesting that interaction of calpain with TRPC6 is critical for its correct localization and regulation. These data support the idea that proteolytic turnover of focal adhesion proteins involving the

calpains is an important driver in the pathogenesis of FSGS, as highlighted in the summary schematic (Figure 9).[48]

Overall, this study shows that TRPC6 plays an important role in the motility and adhesion of podocytes, achieved in part through its physical interaction with calpain 1 and 2, independent of any alteration in calcium conductance, providing a new mechanism for disease pathogenesis in FSGS.

ACKNOWLEDGMENTS

We acknowledge the support of both the Wolfson Bioimaging and Proteomics Facilities, University of Bristol. We thank Dr. Phil Regan for assistance with the patch clamping experiments.

These studies were conceived and funding obtained by Dr. Xu, Prof. Saleem, and Dr. Welsh. Dr. Farmer performed the majority of the experiments. Mrs. Ni established the wild-type and TRPC 6KO cell lines. Proteomic analysis was carried out by Dr. Heesom. Dr. Rollason, Dr. Whitcomb, Dr. Lay, and Mr. Goodliff contributed to data collection. Prof. Birnbaumer provided the TRPC6 KO mice used in this study. All authors reviewed and academically commented on the manuscript.

DISCLOSURES

The authors have nothing to disclose.

FUNDING

This study was supported by Kidney Research UK project grants SP/FSGS1/2013, JF-S/RP/2015/4, and Paed_RP_006_20170929 to Dr. Welsh. Prof. Birnbaumer is supported in part by the Intramural Research Program of the National Institutes of Health (project Z01-ES-101684.

SUPPLEMENTAL MATERIAL

This article contains the following supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2018070729/-/DCSupplemental.

Supplemental Figure 1. TRPC6 channel expression in control, T6K, and T6K + WT podocytes.

Supplemental Figure 2. Effect of Angiotensin II and the TRPC6 inhibitor SAR 7334 on calcium influx to podocytes and of TRPC6 agonists on calpain activity.

Supplemental Figure 3. Full-length blots from all immunoprecipitation experiments.

Supplemental Figure 4. Densitometry on protein cleavage and phosphorylation of WT and mutant podocytes.

REFERENCES

1. Kiffel J, Rahimzada Y, Trachtman H: Focal segmental glomerulosclerosis and chronic kidney disease in pediatric patients. *Adv Chronic Kidney Dis* 18: 332–338, 2011
2. D'Agati VD: Pathobiology of focal segmental glomerulosclerosis: New developments. *Curr Opin Nephrol Hypertens* 21: 243–250, 2012
3. Schell C, Huber TB: New players in the pathogenesis of focal segmental glomerulosclerosis. *Nephrol Dial Transplant* 27: 3406–3412, 2012
4. Reiser J, Polu KR, Möller CC, Kenlan P, Altintas MM, Wei C, et al.: TRPC6 is a glomerular slit diaphragm-associated channel required for normal renal function. *Nat Genet* 37: 739–744, 2005
5. Winn MP, Conlon PJ, Lynn KL, Farrington MK, Creazzo T, Hawkins AF, et al.: A mutation in the TRPC6 cation channel causes familial focal segmental glomerulosclerosis. *Science* 308: 1801–1804, 2005
6. Santín S, Ars E, Rossetti S, Salido E, Silva I, García-Maset R, et al.: FSGS Study Group: TRPC6 mutational analysis in a large cohort of patients with focal segmental glomerulosclerosis. *Nephrol Dial Transplant* 24: 3089–3096, 2009
7. Möller CC, Wei C, Altintas MM, Li J, Greka A, Ohse T, et al.: Induction of TRPC6 channel in acquired forms of proteinuric kidney disease. *J Am Soc Nephrol* 18: 29–36, 2007
8. Kanda S, Harita Y, Shibagaki Y, Sekine T, Igarashi T, Inoue T, et al.: Tyrosine phosphorylation-dependent activation of TRPC6 regulated by PLC-γ1 and nephrin: Effect of mutations associated with focal segmental glomerulosclerosis. *Mol Biol Cell* 22: 1824–1835, 2011
9. Anderson M, Kim EY, Hagmann H, Benzing T, Dryer SE: Opposing effects of podocin on the gating of podocyte TRPC6 channels evoked by membrane stretch or diacylglycerol. *Am J Physiol Cell Physiol* 305: C276–C289, 2013
10. Chiluiza D, Krishna S, Schumacher VA, Schlöndorff J: Gain-of-function mutations in transient receptor potential C6 (TRPC6) activate extracellular signal-regulated kinases 1/2 (ERK1/2). *J Biol Chem* 288: 18407–18420, 2013
11. Schlöndorff J, Del Camino D, Carrasquillo R, Lacey V, Pollak MR: TRPC6 mutations associated with focal segmental glomerulosclerosis cause constitutive activation of NFAT-dependent transcription. *Am J Physiol Cell Physiol* 296: C558–C569, 2009
12. Wang Y, Jarad G, Tripathi P, Pan M, Cunningham J, Martin DR, et al.: Activation of NFAT signaling in podocytes causes glomerulosclerosis. *J Am Soc Nephrol* 21: 1657–1666, 2010
13. Hall G, Rowell J, Farinelli F, Gbadegesin RA, Lavin P, Wu G, et al.: Phosphodiesterase 5 inhibition ameliorates angiontensin II-induced podocyte dysmotility via the protein kinase G-mediated downregulation of TRPC6 activity. *Am J Physiol Renal Physiol* 306: F1442–F1450, 2014
14. Jiang L, Ding J, Tsai H, Li L, Feng Q, Miao J, et al.: Over-expressing transient receptor potential cation channel 6 in podocytes induces cytoskeleton rearrangement through increases of intracellular Ca2+ and RhoA activation. *Exp Biol Med (Maywood)* 236: 184–193, 2011
15. Tian D, Jacobo SM, Billing D, Rozkalne A, Gage SD, Anagnostou T, et al.: Antagonistic regulation of actin dynamics and cell motility by TRPC5 and TRPC6 channels [Erratum appears in Sci Signal. 2010 3(147): er11]. *Sci Signal* 3: ra77, 2010
16. Greka A, Mundel P: Balancing calcium signals through TRPC5 and TRPC6 in podocytes. *J Am Soc Nephrol* 22: 1969–1980, 2011
17. Verheijden KAT, Sonneveld R, Bakker-van Bebber M, Wetzels JFM, van der Vlag J, Nijenhuis T: The calcium-dependent protease calpain-1 links TRPC6 activity to podocyte injury. *J Am Soc Nephrol* 29: 2099–2109, 2018
18. Riehle M, Büscher AK, Gohlke BO, Kaßmann M, Kolatsi-Joannou M, Bräsen JH, et al.: TRPC6 G757D loss-of-function mutation associates with FSGS. *J Am Soc Nephrol* 27: 2771–2783, 2016
19. Welsh GI, Saleem MA: The podocyte cytoskeleton--key to a functioning glomerulus in health and disease. *Nat Rev Nephrol* 8: 14–21, 2011
20. Saleem MA, O'Hare MJ, Reiser J, Coward RJ, Inward CD, Farren T, et al.: A conditionally immortalized human podocyte cell line demonstrating nephrin and podocin expression. *J Am Soc Nephrol* 13: 630–638, 2002
21. Hofmann T, Schaefer M, Schultz G, Gudermann T: Subunit composition of mammalian transient receptor potential channels in living cells. *Proc Natl Acad Sci U S A* 99: 7461–7466, 2002

22. Anderson WW, Collingridge GL: Capabilities of the WinLTP data acquisition program extending beyond basic LTP experimental functions. *J Neurosci Methods* 162: 346–356, 2007
23. Harris JJ, McCarthy HJ, Ni L, Wherlock M, Kang H, Wetzels JF, et al.: Active proteases in nephrotic plasma lead to a podocin-dependent phosphorylation of VASP in podocytes via protease activated receptor-1. *J Pathol* 229: 660–671, 2013
24. Humphries MJ: Cell adhesion assays. *Methods Mol Biol* 522: 203–210, 2009
25. Rollason R, Wherlock M, Heath JA, Heesom KJ, Saleem MA, Welsh GI: Disease causing mutations in inverted formin 2 regulate its binding to G-actin, F-actin capping protein (CapZ α-1) and profilin 2. *Biosci Rep* 36: e00302, 2016
26. Steinberg F, Gallon M, Winfield M, Thomas EC, Bell AJ, Heesom KJ, et al.: A global analysis of SNX27-retromer assembly and cargo specificity reveals a function in glucose and metal ion transport. *Nat Cell Biol* 15: 461–471, 2013
27. Steinberg F, Heesom KJ, Bass MD, Cullen PJ: SNX17 protects integrins from degradation by sorting between lysosomal and recycling pathways. *J Cell Biol* 197: 219–230, 2012
28. Trinkle-Mulcahy L, Boulon S, Lam YW, Urcia R, Boisvert FM, Vandermoere F, et al.: Identifying specific protein interaction partners using quantitative mass spectrometry and bead proteomes. *J Cell Biol* 183: 223–239, 2008
29. Carragher NO, Westhoff MA, Fincham VJ, Schaller MD, Frame MC: A novel role for FAK as a protease-targeting adaptor protein: regulation by p42 ERK and Src. *Current biology* 13: 1442–1450, 2003
30. Carragher NO, Westhoff MA, Fincham VJ, Schaller MD, Frame MC: A novel role for FAK as a protease-targeting adaptor protein: Regulation by p42 ERK and Src. *Curr Biol* 13: 1442–1450, 2003
31. Maier T, Follmann M, Hessler G, Kleemann HW, Hachtel S, Fuchs B, et al.: Discovery and pharmacological characterization of a novel potent inhibitor of diacylglycerol-sensitive TRPC cation channels. *Br J Pharmacol* 172: 3650–3660, 2015
32. Park S, Lee S, Park EJ, Kang M, So I, Jeon JH, et al.: TGFβ1 induces stress fiber formation through upregulation of TRPC6 in vascular smooth muscle cells. *Biochem Biophys Res Commun* 483: 129–134, 2017
33. Singh I, Knezevic N, Ahmmed GU, Kini V, Malik AB, Mehta D: Galphaq-TRPC6-mediated Ca2+ entry induces RhoA activation and resultant endothelial cell shape change in response to thrombin. *J Biol Chem* 282: 7833–7843, 2007
34. Zhang L, Ji T, Wang Q, Meng K, Zhang R, Yang H, et al.: Calcium-sensing receptor stimulation in cultured glomerular podocytes induces TRPC6-dependent calcium entry and RhoA activation. *Cell Physiol Biochem* 43: 1777–1789, 2017
35. Glading A, Uberall F, Keyse SM, Lauffenburger DA, Wells A: Membrane proximal ERK signaling is required for M-calpain activation downstream of epidermal growth factor receptor signaling. *J Biol Chem* 276: 23341–23348, 2001
36. Kim EY, Anderson M, Wilson C, Hagmann H, Benzing T, Dryer SE: NOX2 interacts with podocyte TRPC6 channels and contributes to their activation by diacylglycerol: Essential role of podocin in formation of this complex. *Am J Physiol Cell Physiol* 305: C960–C971, 2013
37. Ding F, Li X, Li B, Guo J, Zhang Y, Ding J: Calpain-mediated cleavage of calcineurin in puromycin aminonucleoside-induced podocyte injury. *PLoS One* 11: e0155504, 2016
38. Tian X, Kim JJ, Monkley SM, Gotoh N, Nandez R, Soda K, et al.: Podocyte-associated talin1 is critical for glomerular filtration barrier maintenance. *J Clin Invest* 124: 1098–1113, 2014
39. Qu Z, Wang Y, Li X, Wu L, Wang Y: TRPC6 expression in neurons is differentially regulated by NR2A- and NR2B-containing NMDA receptors. *J Neurochem* 143: 282–293, 2017
40. Zhang J, Mao X, Zhou T, Cheng X, Lin Y: IL-17A contributes to brain ischemia reperfusion injury through calpain-TRPC6 pathway in mice. *Neuroscience* 274: 419–428, 2014
41. Du W, Huang J, Yao H, Zhou K, Duan B, Wang Y: Inhibition of TRPC6 degradation suppresses ischemic brain damage in rats. *J Clin Invest* 120: 3480–3492, 2010
42. Westhoff MA, Serrels B, Fincham VJ, Frame MC, Carragher NO: SRC-mediated phosphorylation of focal adhesion kinase couples actin and adhesion dynamics to survival signaling. *Mol Cell Biol* 24: 8113–8133, 2004
43. Glading A, Bodnar RJ, Reynolds IJ, Shiraha H, Satish L, Potter DA, et al.: Epidermal growth factor activates m-calpain (calpain II), at least in part, by extracellular signal-regulated kinase-mediated phosphorylation. *Mol Cell Biol* 24: 2499–2512, 2004
44. Han MY, Kosako H, Watanabe T, Hattori S: Extracellular signal-regulated kinase/mitogen-activated protein kinase regulates actin organization and cell motility by phosphorylating the actin cross-linking protein EPLIN. *Mol Cell Biol* 27: 8190–8204, 2007
45. Martinez-Quiles N, Ho HY, Kirschner MW, Ramesh N, Geha RS: Erk/Src phosphorylation of cortactin acts as a switch on-switch off mechanism that controls its ability to activate N-WASP. *Mol Cell Biol* 24: 5269–5280, 2004
46. Tanimura S, Hashizume J, Arichika N, Watanabe K, Ohyama K, Takeda K, et al.: ERK signaling promotes cell motility by inducing the localization of myosin 1E to lamellipodial tips. *J Cell Biol* 214: 475–489, 2016
47. Franco SJ, Rodgers MA, Perrin BJ, Han J, Bennin DA, Critchley DR, et al.: Calpain-mediated proteolysis of talin regulates adhesion dynamics. *Nat Cell Biol* 6: 977–983, 2004
48. Rinschen MM, Huesgen PF, Koch RE: The podocyte protease web: Uncovering the gatekeepers of glomerular disease. *Am J Physiol Renal Physiol* 315: F1812–F1816, 2018
49. Hofmann T, Schaefer M, Schultz G, Gudermann T: Subunit composition of mammalian transient receptor potential channels in living cells. *Proceedings of the National Academy of Sciences of the United States of America* 99: 7461–7466, 2002
50. Kanda S, Harita Y, Shibagaki Y, Sekine T, Igarashi T, Inoue T, et al.: Tyrosine phosphorylation-dependent activation of TRPC6 regulated by PLC-gamma1 and nephrin: effect of mutations associated with focal segmental glomerulosclerosis. *Molecular biology of the cell* 22: 1824–1835, 2011