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TRPC6 binds and activates calpain independent of its channel activity, to regulate podocyte cytoskeleton, cell adhesion and motility

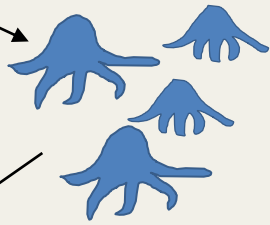
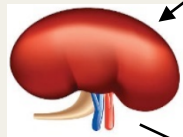
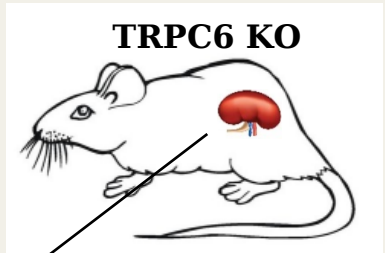
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TRPC6 binds and activates calpain independent of its channel activity regulating podocyte cytoskeleton, cell adhesion and motility

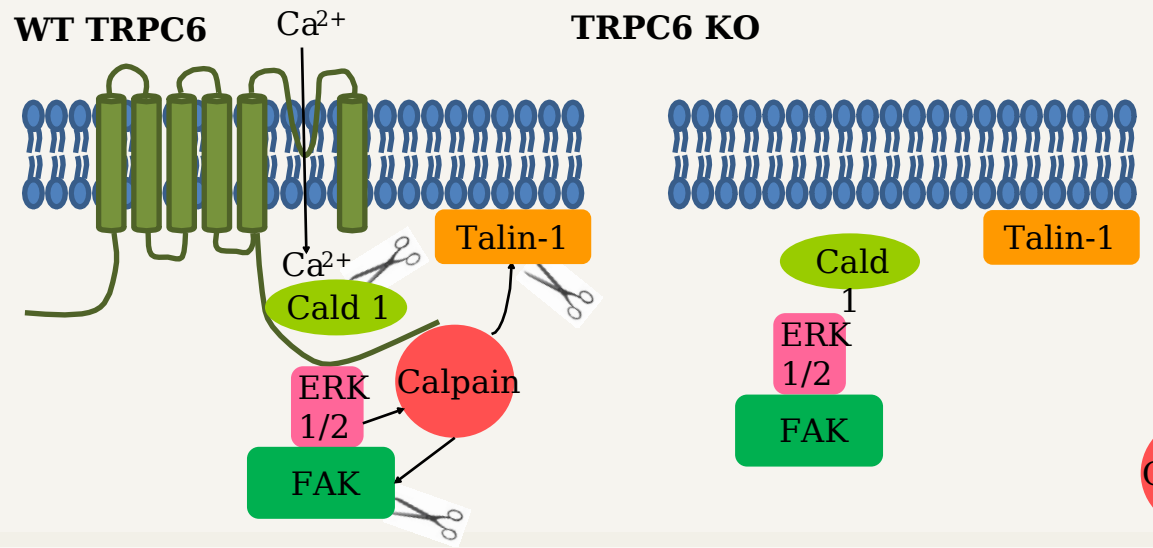
METHODS

OUTCOME Mislocalisation and loss of calpain function in the absence of TRPC6



TRPC6 KO podocyte cell line

Characterise



CONCLUSION TRPC6 binds to calpain and localises it near the membrane in order for it to cleave adhesive proteins and facilitate cell detachment and motility

Significance Statement

Transient Receptor Potential Cation Channel 6 (TRPC6) activity has recently been linked to calpain activation and podocyte injury. Here we show that TRPC6 binds to both ERK 1/2 and calpain and is important for the localisation 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 the TRPC6 protein that is important in cell physiology. Calpain activation and trafficking may therefore be a novel therapeutic target in the treatment of focal segmental glomerulosclerosis.

For Peer Review

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3 **TRPC6 binds to and activates calpain, independent of its channel activity, regulating podocyte**
4 **cytoskeleton, cell adhesion and motility.**
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Significance Statement

Transient Receptor Potential Cation Channel 6 (TRPC6) activity has recently been linked to calpain activation and podocyte injury. Here we show that TRPC6 binds to both ERK 1/2 and calpain and is important for the localisation 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 the TRPC6 protein that is important in cell physiology. Calpain activation and trafficking may therefore be a novel therapeutic target in the treatment of focal segmental glomerulosclerosis.

Abstract

Background

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

Methods

A stable TRPC6 knock out podocyte cell line was generated from TRPC6 knockout mice. These cells were engineered to express wild type, dominant negative or either G109S or K874* disease causing mutants of TRPC6. These cells were extensively characterised via motility, detachment and calpain activity assays, immunofluorescence and confocal or Total Internal Reflection fluorescence (TIRF) microscopy, and western blotting.

Results

TRPC6^{-/-} podocytes are less motile and more adhesive, with an altered actin cytoskeleton compared to wild type cells. Mechanistically, we show that TRPC6 binds to ERK1/2 and the actin regulatory proteins, caldesmon and calpain 1 and 2. Calpains are calcium dependent cysteine proteases, which control the podocyte cytoskeleton, cell adhesion and motility via cleavage of paxillin, focal adhesion kinase and talin. Knockdown or expression of the truncated K874*, but not the gain of function G019S or dominant negative mutant of TRPC6 results in the mislocalization of calpain 1 and 2 and significant down-regulation of calpain activity leading to altered podocyte cytoskeleton, motility and adhesion, a phenocopy of TRPC6^{-/-} podocytes.

Conclusions

Our data demonstrates that the physical interaction between TRPC6 and calpain in the podocyte is important in disease, independent of TRPC6 channel activity.

Introduction

Focal segmental glomerulosclerosis (FSGS) is a devastating form of nephrotic syndrome^{1, 2}. The aetiology 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 podocyte³. Transient receptor potential channel 6 (TRPC6) is a widely expressed, non-selective cation channel. Mutations in TRPC6 are associated with an inherited form of FSGS⁴⁻⁶ and upregulation of TRPC6 expression has been identified in a number of acquired forms of proteinuric kidney diseases⁷. 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 ERK¹⁰⁻¹². TRPC6 has been shown to have several functions in the podocyte. The TRPC6 agonist angiotensin II (Ang II) increases podocyte motility¹³. Nephrin, which has a role in podocyte adhesion, has been shown to inhibit TRPC6 activation, and some disease-causing mutants have decreased nephrin binding capability⁸. TRPC6 associates with the podocyte actin cytoskeleton and there is strong evidence that TRPC6 directly affects podocyte signalling and cytoskeletal organization in these cells¹⁴⁻¹⁶. Indeed recently TRPC6 activity has been linked to increased calpain 1 and calcineurin activity leading to podocyte injury¹⁷

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3 FSGS-causing TRPC6 mutations, for example G109S, have traditionally been reported to be gain of
4 function, and this increased calcium conductance is thought to be responsible for pathology⁶. However,
5 several reported disease-causing mutations, show no change in, or even decreased, calcium conductance
6¹⁸. For example, the K874STOP (K874*) mutation results in a 57 amino acid deletion in the C terminus
7 but has no effect on calcium conductance⁴. This suggests that changes in calcium conductance may not
8 be the sole mechanism underlying the pathology. Patients with TRPC6 mutations do not present with
9 any other pathological phenotype, suggesting that this protein has a singular role within the podocyte
10 which is affected by mutation. Therefore, the most conspicuous question is what is unique about TRPC6
11 activity in podocytes, a cell that is highly dependent on a tightly regulated actin cytoskeleton¹⁹?

12 **In this study we have** developed TRPC6 knockout podocytes **from TRPC6 KO mice** and used them
13 together with expression of either GFP tagged wild type, dominant negative or the G109S and the
14 K874* disease-causing mutant forms of the receptor to identify novel binding partners of TRPC6 and
15 explore how the mutations alter these interactions and protein activity.
16

17 **Methods**

18 **TRPC6 KO Cell Line and TRPC6 Constructs**

19 Conditionally immortalised control and TRPC6 KO podocyte cell lines were made as previously
20 described²⁰.

21 A GFP tag was inserted **into the second extracellular loop of** a wild type (WT) TRPC6 construct in a
22 pcDNA vector **after amino acid 561** using site directed mutagenesis. PCR was used to introduce
23 complementary restriction enzyme sites at amino acid 561 of TRPC6 and both ends of **the GFP**
24 **sequence**. The constructs were then restriction digested and GFP was ligated into the TRPC6 construct.
25 GFP integration was confirmed by sequencing (MWG Eurofins, Germany). The G109S and K874* and
26 the dominant negative TRPC6 LFW678–680AAA (DN)²¹ mutations were introduced into the WT
27 TRPC6-GFP construct through site directed mutagenesis and confirmed through sequencing. All
28 constructs were subcloned into a lentiviral vector (pWPXL, a gift from Didier Trono, Addgene plasmid
29 # 12257) for stable expression in the T6K cells. This construct was transfected into HEK 293 cells along
30 with packaging vectors pMDG.2 and psAX2 [pMD2.G and psPAX2 were a gift from Didier Trono
31 (Addgene plasmids # 12259 and # 12260)] to produce virus. T6K podocytes were transduced with the
32 virus and 8 μ g/ml polybrene overnight. **Expression was confirmed through fluorescence microscopy and**
33 **western blotting.**
34

35 **Electrophysiology**

36 Whole-cell patch-clamp recordings were performed at room temperature using patch pipette solution
37 containing (mM): 115 CsCl, 10 EGTA, 2 MgCl₂, 5 Na₂ATP, 0.1 NaGTP, 10 HEPES, 5.7 CaCl₂; pH
38 was adjusted to 7.2 with CsOH. The standard bath solution contained (mM): 130 NaCl, 5 KCl, 8 D-
39 glucose, 10 HEPES, 1.2 MgCl₂ and 1.5 CaCl₂; the pH was adjusted to 7.4 with NaOH and was perfused
40 at a flow rate of 2-3ml/min. Cells were voltage clamped at -60mV. TRPC6 was activated through
41 perfusion of 10 μ M **Angiotensin II (AngII)** into the recording chamber. Series resistance (R_s) was
42 monitored throughout the experiment and cells showing a >20% change in R_s were excluded from
43 analysis. Recordings were carried out using a MultiClamp 700B amplifier (Axon Instruments, Foster
44 City, CA, USA). On-line electrophysiology data acquisition and analysis were performed using
45 WinLTP software. The holding current (I_{hold}) was recorded at 30 sec intervals, and the change in I_{hold}
46 measured as the difference in the current required to maintain the holding voltage before Ang perfusion.
47 All data were normalised to the mean of the pre-drug perfusion baseline²². Averaged data from each
48 experimental condition are represented as the mean (symbols) and standard error of the mean (S.E.M.).
49 Data were analysed by using Student's *t*-test.
50

51 **Calcium Imaging**

52 Calcium influx to podocytes was measured using a Rhod-3 calcium imaging kit according to the
53 manufacturer's instructions (ThermoFisher #R10145). **Cells were seeded into a 96-well plate and**
54 **differentiated for 10-14 days. They were incubated with 10mM Rhod-3 AM + 2.5mM probenecid in**
55 **the dark for 30 minutes before being washed and incubated in 2.5 mM probenecid for a further 30**
56 **minutes.** Live cell imaging was performed in **phosphate buffered saline (PBS)** using the IN Cell
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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 Angiotensin II at a final concentration of 1 μ M. A second reading was taken 5 seconds after AngII addition and compared to 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 μ M collar around the nucleus.

Biotinylation

Podocytes stably expressing WT TRPC6-GFP were biotinylated as described previously²³. Cells were washed with borate buffer (10mM Boric Acid, 154 mM NaCl, 7.2 mM KCl, 7.2 mM CaCl₂, 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 500ul Tris Buffered Saline (TBS) + 2% NP40 + Protease Inhibitor Cocktail (PIC) and incubated with streptavidin or control agarose beads. A 'total protein' sample was taken before the incubation. Samples were then run on a 10% acrylamide gel and probed with anti-TRPC6 or anti CD99 antibodies.

Motility and Detachment Assays

These were carried out as previously described^{24,25}. For the motility assay cells were seeded in 6 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 post scratch and the cell infiltration into the cleared area measured using ImageJ software. For the detachment assay differentiated cells were trypsinised and resuspended in media at 3x10⁵ cells/ml. 50ul of cells was then added to each well of a 96 well plate along with 50ul PBS. If an inhibitor was being used, then this was added to the PBS at 2x concentration. Cells were left for 48 hours to adhere. Control wells were fixed with 4% paraformaldehyde (PFA) to measure 100% attachment. 50ul trypsin was added to each experimental well for 5 minutes before being washed with PBS and adding 50ul Foetal Bovine Serum (FBS) to attenuate trypsinisation. Cells were then fixed with 4% PFA. After washing with H₂O cells were stained with 0.1% crystal violet in 2% ethanol for 60 minutes. After further washes the dye was solubilised with 100ul 10% acetic acid and left on an orbital shaker for 5 minutes. Absorbance was measured at 570nm using a plate reader. Results were expressed as a percentage of 100% attachment of control cells.

Immunofluorescence

Immunofluorescence was performed as described previously²⁶ Images were captured using a Leica AM fluorescent confocal microscope or Leica AM total internal reflection fluorescence (TIRF) microscopy MC (multi-colour) system attached to a Leica DMI 6000 inverted epifluorescence microscope equipped with 405, 488, 561, 635 nm laser lines. All primary antibodies are listed in the table below.

Co-Immunoprecipitation and Proteomics

Cells transduced with either WT, G109S or K874* TRPC6-GFP expressing lentivirus were lysed in TNE buffer (50mM Tris, 100mM NaCl, 0.1mM EDTA) containing 10% glycerol + 1% NP40 and GFP and GFP-TRPC6 protein and interacting proteins were immunoprecipitated using the GFP-Trap system (Chromotek). Proteins were eluted from beads into 50ul 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 previously²⁶⁻²⁸.

Antibodies used

Antibody	Supplier and catalogue number
TRPC6	Cell Signalling Technology #16716
Caldesmon 1	Cell Signalling Technology #2980
Calpain 1	Cell Signalling Technology #2556
Calpain 2	Cell Signalling Technology #2539S
Calpain 1	Abcam #ab28258
Talin 1	Cell Signalling Technology #4021

Phospho-p44/p42 (ERK1/2)	Cell Signalling Technology #4370S
FAK	Cell Signalling Technology #3285S
Phospho-FAK(Tyr397)	Cell Signalling Technology #8556S
Synaptopodin	Santa Cruz #sc-515842
WT1	Cell Signalling technology #13580
Podocin	Abcam #ab50339
CD2AP	Cell Signalling #5478
Nephrin	Acris #BP5030
GFP	Sigma #11814460001
CD99	Kind gift from Professor George Banting University of Bristol.
Pley2	Cell Signalling Technology #3872S

Calpain Assay

The calpain assay was performed on differentiated podocytes using a Calpain Activity Assay Kit (Abcam ab65308). 1% triton X-100 was added to the provided lysis buffer to detect membrane associated calpain activity. Briefly cells were seeded into 6 well plates and differentiated for 10-14 days. Cells were lysed in provided lysis buffer and a BSA assay was performed to measure protein concentration. 100 ug of cell lysate 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 one hour before absorbance was measured at Ex/Em = 400/505 nm.

Statistics

All statistics were performed in Graphpad Prism 5.

Experiment	Statistical analysis	Unit of analysis
<u>Patch clamp analysis</u>	<u>Unpaired Student's t-test</u>	<u>Pooled cells</u>
<u>Motility and detachment</u>	<u>One-way ANOVA</u>	<u>Per experiment (3 replicates per experiment)</u>
<u>Calpain activity assay</u>	<u>One-way ANOVA</u>	<u>Per experiment (3 replicates per experiment)</u>
<u>Calcium assay</u>	<u>One-way ANOVA</u>	<u>Per experiment (3 wells imaged per experiment)</u>
<u>Calpain assay with inhibitor</u>	<u>Unpaired t-test</u>	<u>Per experiment (3 replicates per experiment)</u>
<u>Calcium assay with ang II (raw data)</u>	<u>Paired t-test</u>	<u>Per experiment (3 wells imaged per experiment)</u>
<u>Densitometry</u>	<u>One-way ANOVA</u>	<u>Mean of minimum 3 blots per treatment</u>

Results

Expression of a Functional TRPC6 Construct in Knockout Podocytes

Podocytes were isolated from TRPC6^{-/-} mice, and a conditionally immortalised cell line was established as described previously²⁰. 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 (Fig.1A). The levels of both TRPC3 and 7 did not change in these cells compared to wild type cells (Fig.S1). Using lentiviral transduction, TRPC6 constructs were stably expressed in these cells, allowing WT, dominant negative 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 (Fig.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

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3 and C termini are known to be involved in trafficking and protein interaction of the channel and a
4 protein tag placed in this region may have prevented binding. Expression of the GFP tagged WT TRPC6
5 construct was confirmed through immunofluorescence and western blotting (Fig.1B). Biotinylation and
6 TIRF experiments were also performed to confirm that the construct still trafficked to the plasma
7 membrane despite the presence of the GFP tag (Fig. 1C and D) comparing with the membrane protein
8 CD99. Calcium influx in response to 10 μ M AngII was measured in T6K and T6K cells expressing
9 either wild type (T6K+WT) or the previously the described dominant negative (T6K+DN) TRPC6-GFP
10 construct²¹, using rhod-3 calcium imaging and patch clamping, demonstrating that the expressed wild
11 type TRPC6-GFP was functional (Fig.1D-F and S2A) and importantly that the GFP tag has no
12 significant effect on TRPC6 channel activity compared to an untagged version (Fig S2B).

13 Motility and adhesion regulated by TRPC6

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

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

26 Actin remodelling is closely linked to cell motility and adhesion and actin reorganisation is seen with
27 podocyte foot process effacement. Overexpression of TRPC6 has previously been shown to cause
28 cytoskeletal rearrangement and inhibition of the receptor has been shown to prevent albumin induced
29 F-actin cytoskeletal disruption⁷. Control, T6K and T6K+WT podocytes were stained with phalloidin
30 and imaged on a confocal microscope (Fig.2C). There were more actin stress fibres present in the T6K
31 cells whereas in the control and T6K+WT podocytes actin was localised to the membrane and evenly
32 spread throughout the cytoplasm (Fig.2C).

33 Protein partners of TRPC6 in podocytes

34 To identify novel TRPC6 binding partners GFP-tagged wild type TRPC6-GFP was expressed in
35 podocytes using lentiviral transduction. These and control cells, expressing GFP only, were then lysed
36 and the GFP immunoprecipitated using the highly efficient GFP-Trap method²⁹. The precipitated GFP
37 and TRPC6-GFP were separated by SDS/PAGE and interacting proteins analysed by LC-MS/MS after
38 in-gel tryptic digestion. Three intracellular proteins (calpain 2, caldesmon-1 and PLCy2) and two ion
39 channel proteins (TRPC3 and TRPC7) were identified by MS analysis, which were significantly more
40 abundant in the TRPC6-GFP pulldown compared with the GFP control (Fig.3A). Co-
41 immunoprecipitation experiments were performed to verify the physical interaction of TRPC6 with
42 calpain 2, caldesmon-1 and PLCy2 (Fig.3B). Immunoprecipitation experiments were also carried for
43 calpain 1 and ERK 1/2, proteins that are known to interact with or be linked to those identified in the
44 proteomic screen and these proteins were also shown to interact with TRPC6 (Fig 3B, S3).

45 Loss of calpain activity in TRPC6 KO cells

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

54 FAK, ERK1/2 and calpain have previously been shown to form a complex³⁰ and as all three were co-
55 immunoprecipitated with TRPC6, the calpain cleavage targets talin-1, caldesmon-1, and FAK were
56 probed for (Fig.3D). Each of these proteins was shown to have increased cleavage in the control
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3 T6K+WT and T6K+DN podocytes compared to T6K, suggesting that the presence of TRPC6 is
4 important for calpain activity and cleavage of these targets.

5 Calpain activity assays confirmed a loss of calpain activity in TRPC6 KO cells compared to T6K+WT
6 and control podocytes (Fig.4A). Treatment of control and T6K+WT cells with the calpain inhibitor
7 calpeptin (10 uM) caused motility of the cells to mimic that seen in T6K podocytes but had no effect
8 on the motility of the T6K cells (Fig.4B). Treatment of these cells with calpeptin also decreased
9 detachment and blocked cleavage of talin-1, caldesmon-1, and FAK (Fig 4C and D). This suggests that
10 the loss of calpain activity is responsible for the decreased motility and detachment of the T6K cells.
11 AngII is a TRPC6 agonist and a calcium assay demonstrated that application of AngII to control and
12 T6K+WT podocytes caused calcium influx into the cell as shown in Fig.S2. Neither AngII nor the
13 TRPC6 inhibitor, SAR 7334³¹, had any effect on calpain activity in control, T6K+WT or T6K
14 podocytes even though SAR 7334 blocked calcium influx into the cells (Fig.5A, 5B and S2C-E).
15 Treatment with the TRP channel activator OAG also had no effect on calpain activity (Fig.S2F).
16 Furthermore, calpain activity was not altered in cells that expressed T6K+DN (Fig.5C) even though this
17 mutant bound to calpain (Fig.5D). These results suggest that increased calcium influx does not alter
18 calpain activity. Calpain binding was also maintained in the presence of SAR 7334 (Fig 5E).

19 This led us to hypothesise that the effect of TRPC6 knockout on calpain activity was due to altered
20 localisation of calpain rather than decreased calcium influx. This was examined using
21 immunofluorescence staining and confocal microscopy (Fig.6A). Calpain 1 appeared to be membrane
22 localised in the control and T6K+WT cells, but not in the T6K cells. This was confirmed by total internal
23 reflection microscopy (TIRFM) showing that in the T6K+WT, T6K+DN and control cells, calpain was
24 visualised at the plasma membrane of the cell and this membrane localisation was lost in the T6K cells
25 (Fig.6B). These results suggest that the interaction between TRPC6 and calpain 1 and 2 is important in
26 the localisation and activation of calpain.

27 TRPC6 mutants and calpain membrane localization

28 Disease-causing mutations can have varying effects on the calcium conductance of TRPC6 channels.
29 We investigated if altered interaction between TRPC6 and calpain could be causing the pathology seen
30 in these patients. Two mutants, G109S, which has been reported to cause an increase in calcium
31 conductance, and K874* which has no effect on channel conductance were used^{4,6}. As expected, the
32 G109S expressing podocytes showed increased calcium conductance in response to 1 uM AngII whilst
33 there was no effect in the K874* expressing cells (Fig.7A and S2A). Both mutants bound ERK 1/2 but
34 there was decreased interaction between TRPC6 K874* and both calpain 1 and calpain 2, whilst the
35 interaction was maintained with the G109S mutant (Fig.7B). The G109S mutant cells mimicked WT
36 cells in their motility, adhesion, protein phosphorylation and calpain target protein cleavage. In contrast
37 K874* podocytes were less motile, more adhesive and had decreased calpain activity (Fig.7C-E).

38 In the K874* mutant podocytes FAK phosphorylation was increased, ERK phosphorylation decreased
39 and there was decreased protein cleavage of calpain targets for the K874 mutant (Fig.7F). The K874*
40 mutant therefore mimics the TRPC6KO cells, despite there being no change in TRPC6 conductance.
41 This suggests that the pathology of the K874* mutant is via its altered calpain binding.

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

44 Discussion

45 Mutations in TRPC6, a non-selective cation channel, are associated with an inherited form of
46 focal segmental glomerulosclerosis (FSGS). Despite widespread expression, patients with TRPC6
47 mutations do not present with any other pathological phenotype suggesting that this protein has a unique
48 role within the target cell for FSGS, the kidney podocyte. Although most TRPC6 mutations are reported
49 to cause changes in calcium dynamics, it is unclear how these result in a podocyte specific phenotype,
50 a cell that is highly dependent on a tightly regulated actin cytoskeleton. To understand the role of
51 TRPC6 in the podocyte and the effect of disease causing mutations conditionally immortalized
52 TRPC6KO podocytes were established from TRPC6 KO mice. These cells were found to be less motile,
53 more adhesive and have an altered actin cytoskeleton compared to wild type podocytes or knockout
54 podocytes expressing wild type TRPC6. This agrees with previous work showing that TRPC6KO
55 podocytes are less motile, and this phenotype was rescued by re-introduction of wtTRPC6¹³ but is in

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3 contrast to other studies which have reported a role for TRPC6 in inducing Rho activation, stress fibre
4 formation and decreased motility^{15, 35-37}. This discrepancy might be due to the level of TRPC6
5 knockdown as these cells have been developed from a knockout animal compared to the previous
6 studies using SiRNA technology, where some preservation of expression is seen.

7 TRPC6 has been shown to interact with several proteins in the podocyte including podocin and
8 nephrin⁴. This led us to wonder if there are other, as yet, unidentified TRPC6 protein interactions that
9 are important in podocyte function. Using GFP TRAP-pulldown coupled with mass spectrometry we
10 identified several TRPC6 binding partners including TRPC3, TRPC7 and PLC γ which are known
11 interactors of TRPC6 thus validating our approach⁸. However, two of the identified interactors, Calpain
12 2 and caldesmon are novel. These interactions were confirmed by immunoprecipitation experiments,
13 which also showed that TRPC6 binds to calpain 1. As ERK1/2 signalling is known to be required for
14 calpain activation³⁸ and as gain of function TRPC6 mutations have been shown to increase ERK1/2
15 activation¹⁰ we also looked to see if there was a physical interaction between TRPC6 and ERK 1/2.
16 Importantly we showed that ERK 1/2 is also a novel TRPC6 interactor. Whether these are direct
17 interactions or via a complex with other proteins such as seen for podocin for the interaction of TRPC6
18 with NADPH Oxidase is still to be determined³⁹.

19
20 The calpains are a family of calcium dependent proteases that have critical functions in
21 controlling the podocyte cytoskeleton and hence cell adhesion and motility via cleavage of paxillin,
22 focal adhesion kinase (FAK) and talin. An increase in calpain activity has previously been reported as
23 contributing to puromycin aminonucleoside-induced podocyte injury⁴⁰. Furthermore, cleavage of talin-
24 1 by calpain has also been hypothesised to promote the pathogenesis of nephrotic syndrome and
25 **Calpain-1 has recently been shown to link TRPC6 Activity to Podocyte Injury**^{17, 41}. In neuronal cells
26 and tissue activation of calpain has been reported to lead to TRPC6 degradation contributing to neuronal
27 damage in cerebral ischemia⁴²⁻⁴⁴. Calpain activity was significantly downregulated in the TRPC6
28 knockout cells and treatment of control and T6K+WT cells with the calpain inhibitor calpeptin resulted
29 in decreased motility in the control and T6K+WT cells, mimicking that seen in TRPC6 knockout
30 podocytes. This suggests that the loss of calpain activity is responsible for the decreased motility of the
31 TRPC6 knockout cells. Importantly, although treatment of the control and T6K+WT podocytes but not
32 the TRPC6 knockout cells with Ang II caused calcium influx into the cell, there was no effect of
33 treatment on calpain activity. This data indicates that the regulation of calpain activity by TRPC6 is
34 independent of alterations in its calcium conductance. Interestingly TRPC6 activity has recently been
35 linked to increased calpain 1 and calcineurin activity leading to podocyte injury⁴⁵. However in contrast
36 to our data this study demonstrated calpain activation upon treatment of mouse podocytes with
37 Adriamycin or the TRP channel activator OAG¹⁷ suggesting that the mode of calpain activation requires
38 further study.

39
40 Knockdown of TRPC6 or expression of the K874* but not the G109S nor TRPC6DN mutant,
41 resulted in increased FAK phosphorylation and decreased ERK phosphorylation. It can therefore be
42 deduced that TRPC6 is contributing to the phosphorylation state of ERK1/2 and FAK. Src induced
43 phosphorylation of FAK has been shown to be required for rapid actin stress fibre assembly and focal
44 adhesion formation. It is also required for the formation of a calpain-FAK-ERK1/2 complex and calpain
45 cleavage of FAK⁴⁶. Furthermore, it has previously been shown that there is an increase in ERK1/2
46 phosphorylation in podocytes expressing gain of function disease-causing TRPC6 mutations¹⁰. In
47 addition, ERK phosphorylation has been shown to increase calpain activation⁴⁷ and cell motility⁴⁸⁻⁵⁰.
48 Knockdown of TRPC6 or expression of the K874*mutant also led to decreased cleavage of the calpain
49 targets talin-1, caldesmon-1, and FAK suggesting that the presence of TRPC6 is important for calpain
50 cleavage of these targets. The cleavage of FAK leads to de-adhesion and motility of cells and talin-1
51 cleavage has been demonstrated to be a rate-limiting step in adhesion disassembly⁵¹ so decreased
52 cleavage of these proteins is consistent with the increased adhesion, decreased motility and actin
53 reorganisation observed the T6K and K874*mutant podocytes. Again, the lack of effect of the DN and
54 G109S mutant on calpain activity and cleavage of target proteins suggests that increased calcium
55 conductance is not important in the regulation of calpain by TRPC6. Confocal and TIRF microscopy of
56 these cells and glomeruli from TRPC6KO mice demonstrated a mislocalization of calpain away from
57 the plasma membrane suggesting that interaction of calpain with TRPC6 is critical for its correct
58 localization and regulation. **This data supports the idea that proteolytic turnover of focal adhesion**
59 **proteins involving the calpains is an important driver in the pathogenesis of FSGS as highlighted in the**
60

summary schematic ⁵².

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.

Author Contributions

These studies were conceived, and funding obtained by SZX, MAS and GIW. LF performed the majority of the experiments. LN established the wild type and TRPC6KO cell lines. Proteomic analysis was carried out by KH. All authors reviewed and academically commented on the manuscript.

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Disclosures

None

Supplemental Material Table of contents

Figure S1 TRPC6 channel expression in control, T6K and T6K + WT podocytes

Figure S2 Effect of Angiotensin II and the TRPC6 inhibitor SAR 7334 on calcium influx to podocytes and of TRPC6 agonists on calpain activity

Figure S3 Full length blots from all immunoprecipitation experiments

Figure S4 Densitometry on protein cleavage and phosphorylation of WT and mutant podocytes

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Figure Legends

Figure 1. Generation of a TRPC6 KO podocyte cell line and functional GFP tagged TRPC6 construct

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(A) Podocyte markers CD2 associated prorotein (CD2AP), synaptopodin, Wills tumour 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 re introduced to the T6K cells. This is demonstrated by Western blotting and immunofluorescence (magenta = actin, GFP = green, DAPI = blue). Disease causing and dominant negative (DN) forms of the GFP tagged TRPC6 construct were also generated and introduced to T6K cells. (C, D) Biotinylation and Total Internal Reflection Fluorescence (TIRF) microscopy

demonstrating that wild type (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 holding current (I_{hold}) caused by 6 min 1 μ M angiotensin II (Ang II) perfusion. Ang II perfusion causes a rapid change in I_{hold} in WT (-36.2 ± 10.5 pA at 8 min timepoint, open symbols, n=5) but not the null mutant (8.7 ± 3.4 pA at 8 min timepoint, closed symbols, n=4) or T6K (-3.5 ± 4.3 pA at 8 min timepoint, triangle symbols, n=4) cells. Grey vertical bar represents perfusion of Ang II. All symbols represent the mean \pm S.E.M. **(F)** Summary box plot (boxes, 25th-75th percentile; lines, median) showing changes in I_{hold} caused by 1 μ M Ang II perfusion at the 8 min timepoint in E. *P<0.05, unpaired Student's t-test.

Figure 2. Altered motility and detachment of T6K cells compared to controls

(A) Motility of the TRPC6 KO (T6K) cell line was measured by scratch assay closure after 12 hours and compared to 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. It demonstrated decreased detachment of T6K podocytes (46.35 ± 9.72 %) compared to 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 to control and T6K + WT podocytes. Magenta = phalloidin staining for actin.

Figure 3. Identification of novel TRPC6 binding partners

(A) Table of proteomics results of TRPC6 binding partners from wild type (WT) human podocytes overexpressing TRPC6-GFP. TRPC6 was seen to bind to TRPC3, TRPC7, and PLCy2, interactions that have been described previously. 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 co-immunoprecipitation (TRAP lane). Control agarose beads were used to demonstrate immunoprecipitation was specific to TRPC6 (control lane). Additional interactions with calpain 1 and ERK 1/2 were also identified. **(C)** Based on the proteomics results the phosphorylation and/or cleavage state of focal adhesion kinase (FAK), talin-1, caldesmon-1 and ERK 1/2 was ascertained through Western blotting. T6K had increased FAK phosphorylation at Tyr 397 and decreased ERK phosphorylation compared to control and T6K + WT cells. Cleavage of FAK, talin-1 and caldesmon-1 was decreased in T6K cells compared to controls. For densitometry see supplementary figure 3.

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 to control (42609 ± 8900 vs 99493 ± 8227 A.U) and recovered to normal levels (106138 ± 9414 A.U) when wild type (WT) TRPC6 was reintroduced (T6K + WT). **(B)** Addition of the calpain inhibitor calpeptin (10uM) 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 10uM calpeptin reduced detachment of control and WT cells by 26.3% and

19.2% respectively. There was no significant effect on T6K podocytes. Unpaired t-test, ** $P < 0.01$, *** $P < 0.001$

Figure 5. Decreased calpain activity is independent of calcium influx through TRPC6 activation.

(A) Addition of the TRPC6 agonist Angiotensin II (Ang II, 1 μ M) which is known to increase calcium current through the receptor (see figure 2E and S2A) had no effect on calpain activity in control, T6K or T6K + WT podocytes, unpaired T-test. (B) Application of a TRPC6 inhibitor SAR 7334 (20nM) 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 dominant negative (DN) mutant and significantly increased compared to T6K (T6K + DN = 128612 ± 11370 , Control = 120297 ± 12481 , T6K + WT = 129619 ± 10408 , T6K = 68973 ± 7009). * $P < 0.05$, ** $P < 0.01$, One-way ANOVA. (D) GFP TRAP co-immunoprecipitation shows interaction between calpain and TRPC6 in both T6K + WT and T6K + DN podocytes (left panel). (E) There was also an interaction between TRPC6 and calpain in cells that had been treated with the TRPC6 inhibitor SAR 7334 (right panel).

Figure 6. Calpain localisation is altered in T6K podocytes

(A) Confocal microscopy showing the localisation of calpain 1 and the nuclear marker DAPI in control, TRPC6 KO (T6K) and TRPC6 KO expressing WT TRPC6-GFP (T6K + WT) podocytes. Calpain localisation is lost from the membrane in T6K cells, and rescued by the reintroduction of WT TRPC6-GFP. (B) Total Internal Reflection Fluorescence (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, T6K + WT cells and T6K cells expressing the dominant negative mutant (T6K + DN) but not T6K. 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.

Figure 7. The TRPC6 mutant K874* does not bind to calpain and podocytes expressing this mutant have a similar phenotype to T6K cells

(A) 1 μ M angiotensin II initiates an increase in calcium in control in all cell types. Calcium increases were 33.75 ± 4.7 , 19.08 ± 1.6 , 31.20 ± 3.8 , 52.56 ± 2.8 and $36.62 \pm 8.2.6$ respectively. This calcium increase is significantly larger in the gain of function mutant G109S, and significantly less in the 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 is protein eluted from control beads. ERK1/2 binds to all three TRPC6 constructs. (C,D) Motility, detachment and calpain assays for control, T6K, WT and mutant podocytes. K874* mimicked T6K, while G109S mimicked control and WT cells. Values are given in the table in panel E. (E) Summary table of values for motility, detachment and calpain activity. (F) Phosphorylation and cleavage of control, T6K, WT and mutant podocytes. K874* mimics T6K, while G109S mimics WT. For densitometry see supplementary figure 3. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 8. K874* podocytes do not bind to calpain and have a similar phenotype to T6K cells

(A) Confocal microscopy showing localisation of calpain 1. Calpain 1 shows some membrane localisation in control and TRPC6 KO podocytes expressing either WT TRPC6 (T6K + WT) or G109S TRPC6 (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

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3 and K874* cells. Membranous calpain was seen in all other cell types. For all images red = calpain,
4 green = TRPC6-GFP, pink = actin.
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6 **Figure S1 TRPC channel expression in control, T6K and T6K + WT podocytes.** There was no
7 significant upregulation seen in TRPC6 KO podocytes of either TRPC3 (A) or TRPC7 (B) One-way
8 ANOVA.
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10 **Figure S2 Effect of Angiotensin II and the TRPC6 inhibitor SAR 7334 on calcium influx to**
11 **podocytes and of TRPC6 agonists on calpain activity.** (A) Raw calcium data showing calcium influx
12 in response to the TRPC6 agonist 1 μ M Ang II at control and mutant podocytes. Data was recorded
13 from the same cells before (closed symbols) and after (open symbols) Ang II application. Paired t-test
14 * P < 0.05, ** P < 0.01, *** P < 0.001 (B) The presence of the GFP tag had no effect on calcium influx
15 through the TRPC6 receptor. HEK 293 cells were transfected with either TRPC6 or TRPC6-GFP and
16 the both baseline calcium and calcium influx in response to AngII (1 μ M) recorded. For the untagged
17 construct baseline calcium influx was 466537 ± 51502 and was 631216 ± 60690 AU. The tagged
18 construct had a baseline calcium influx of 496910 ± 64007 which rose to 630364 ± 72799 with Ang II
19 application (C-E) Calcium influx in response to 1 μ M Ang II and μ M Ang II + 20nM of the TRPC6
20 inhibitor SAR7334 at (C) Control, (D) T6K and (E) T6K + WT podocytes, demonstrating an effect of
21 the inhibitor on cells expressing TRPC6. Baseline current was normalised to 12000 fluorescence
22 intensity units. (F) Both Angiotensin II (1 μ M) and 1-oleoyl-2-acetyl-glycerol (OAG, 100 μ M) are
23 known TRPC6 activators. Neither agonist had a significant effect on calpain activity when cells were
24 pre-incubated with the drug.
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28 **Figure S3 Full length blots from all immunoprecipitation figures.**

29 **Figure S4 Densitometry on protein cleavage and phosphorylation of WT and mutant podocytes**
30 Densitometry is reported as percentage intensity compared to control cells. * P < 0.05, ** P < 0.01,
31 *** P < 0.001.
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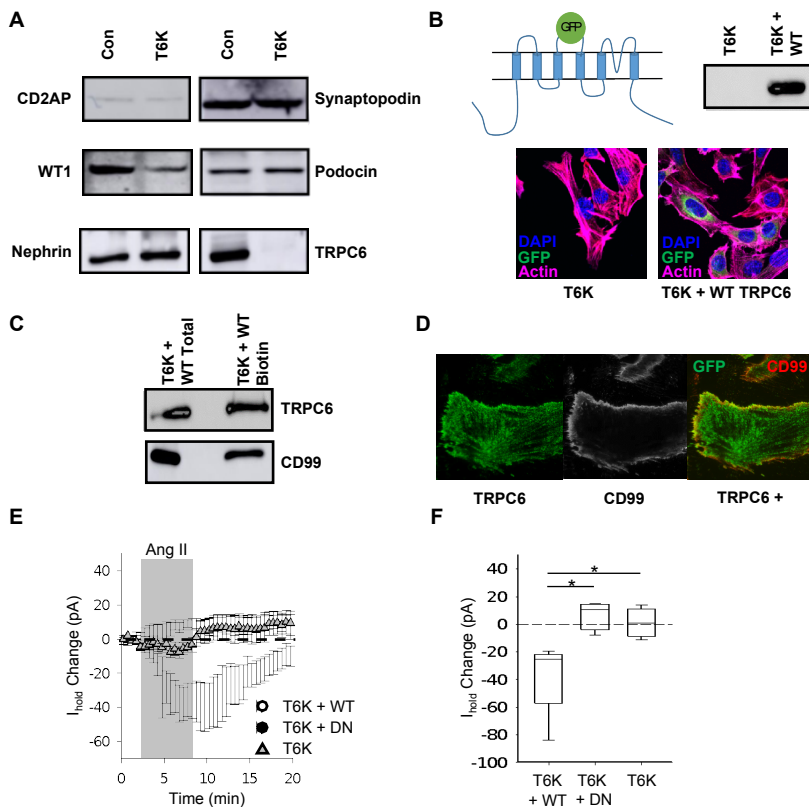


Figure 1 Generation of a TRPC6 KO cell line and functional GFP tagged TRPC6 construct

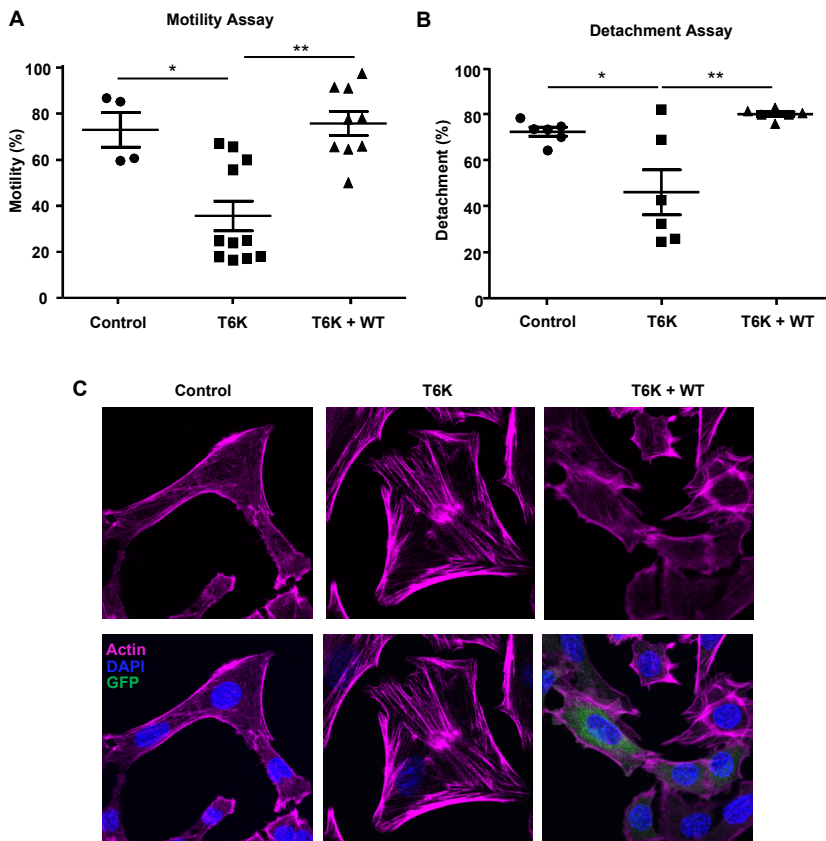


Figure 2 Altered Motility and Detachment of T6K Cells Compared to Controls

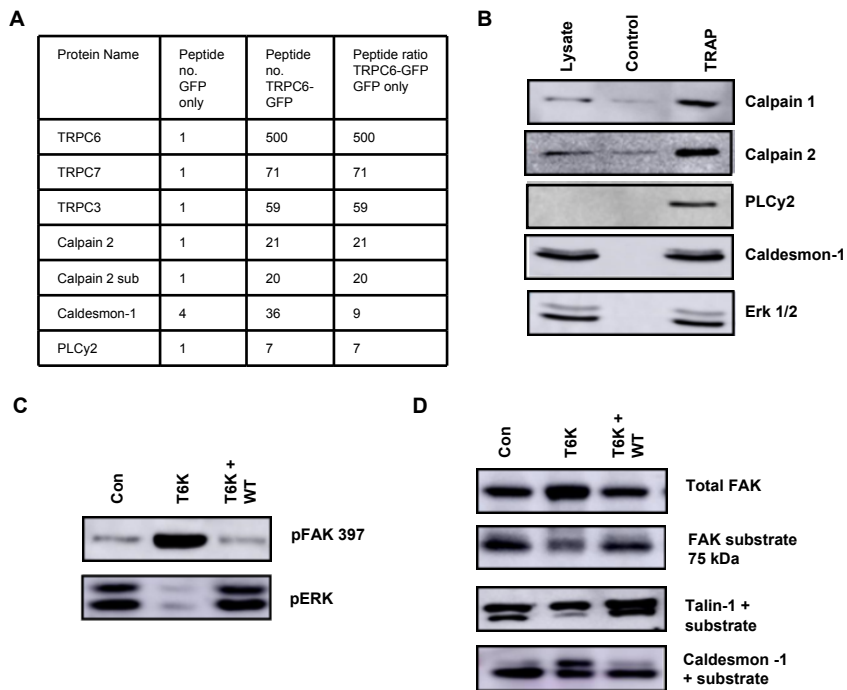


Figure 3 Identification of novel TRPC6 binding partners

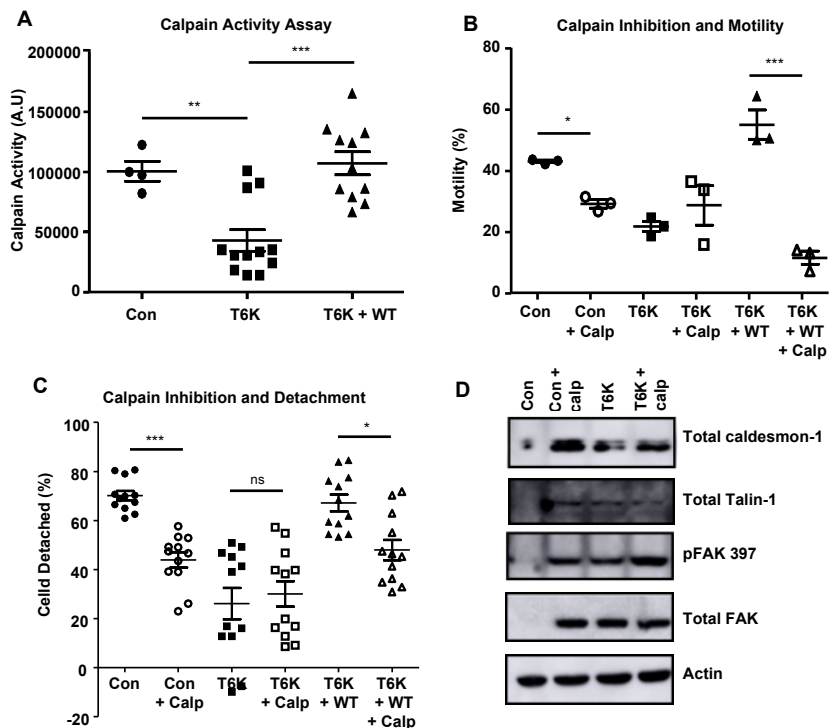


Figure 4 Calpain activity is decreased in T6K cells and calpain inhibition mimics the TRPC6 KO phenotype

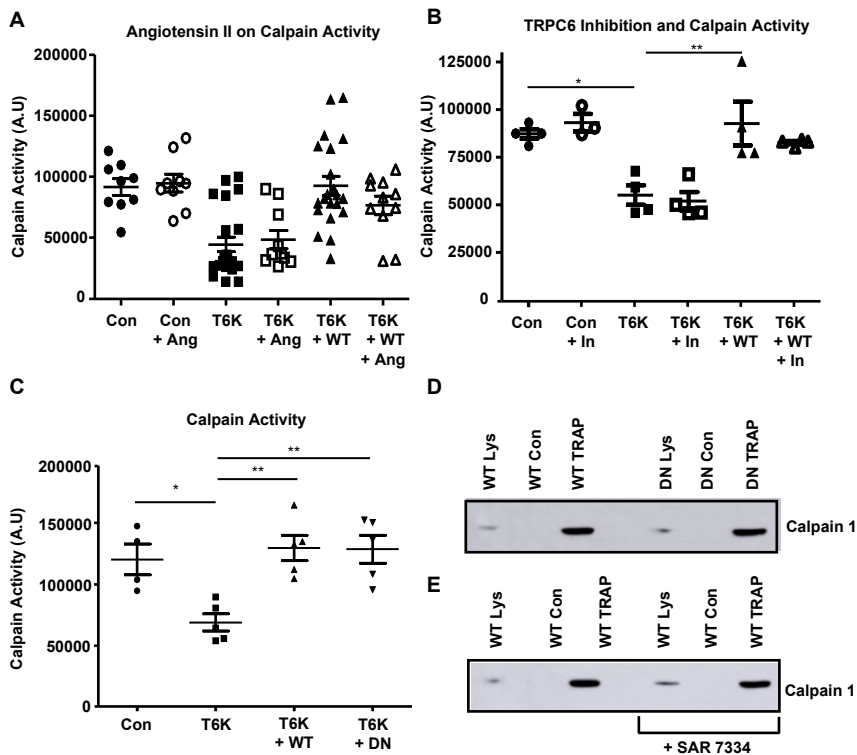


Figure 5 Decreased calpain activity is independent of calcium influx through TRPC6 activation

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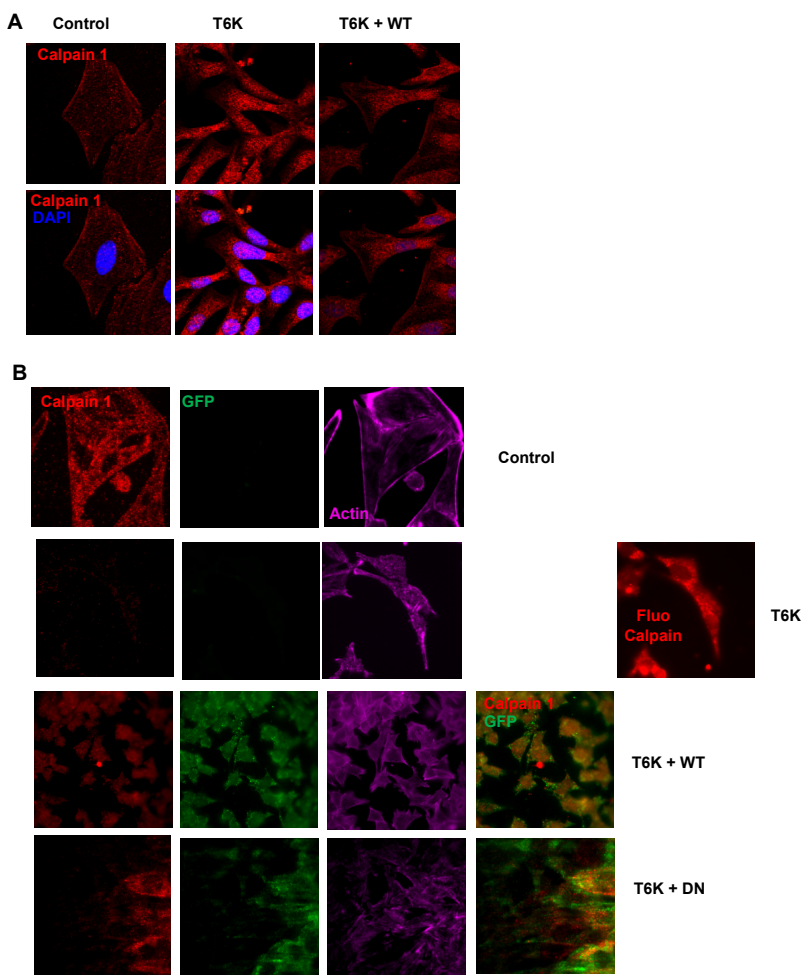


Figure Calpain localisation is altered in TRPC6 KO podocytes

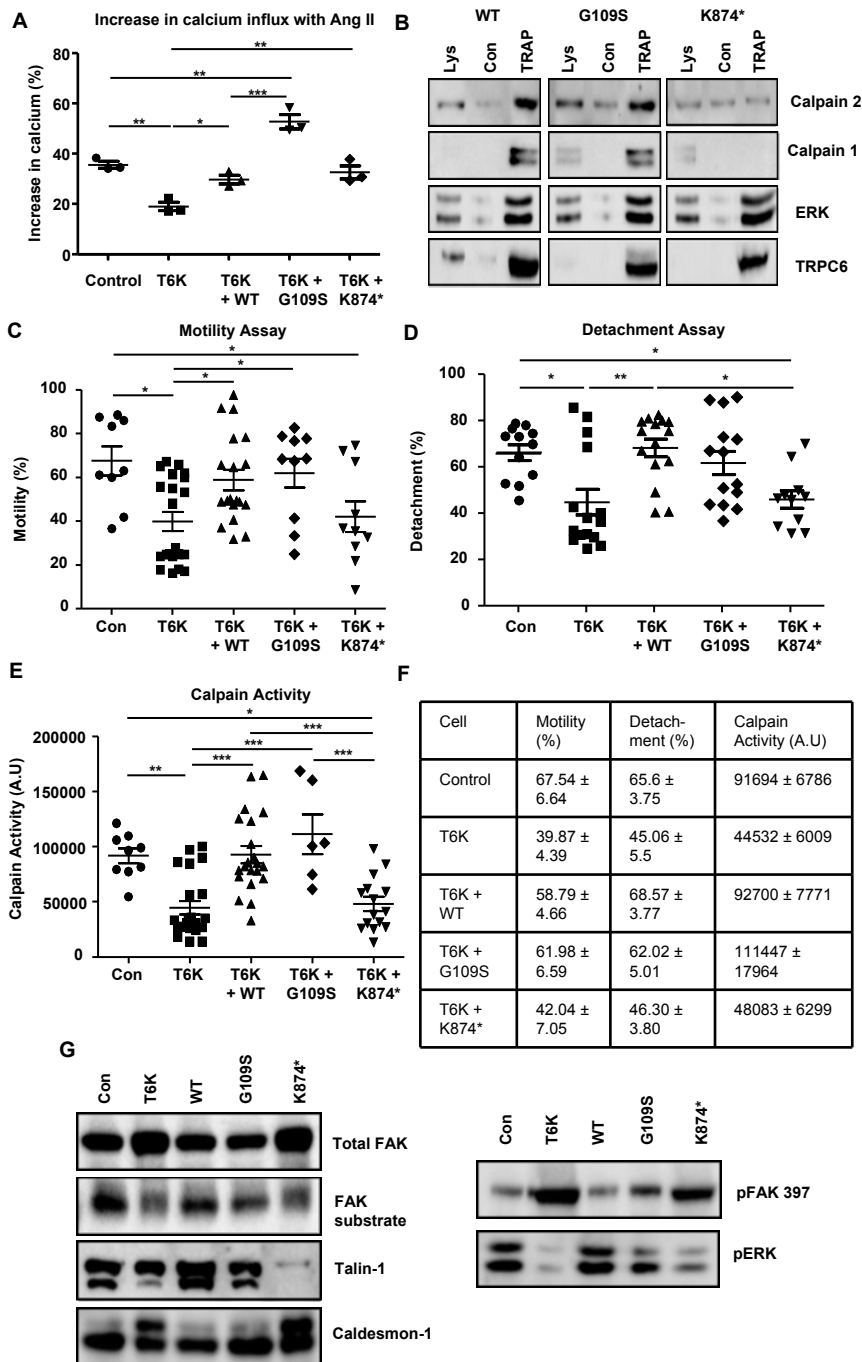


Figure 6 The TRPC6 mutant K874* does not bind to calpain and podocytes expressing this mutant have a similar phenotype to T6K cells

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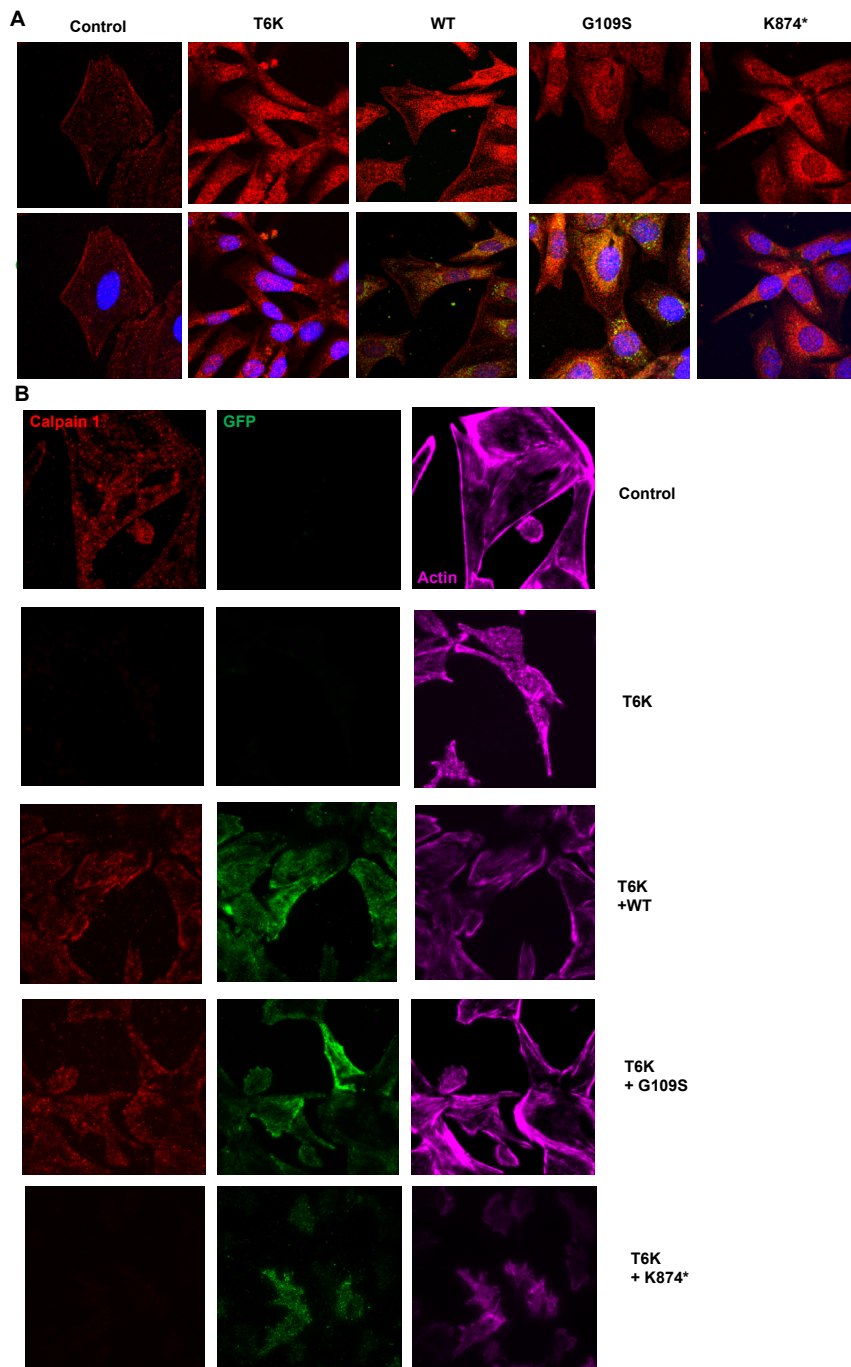


Figure 8 K874* podocytes do not bind to calpain and have a similar phenotype to T6K cells

Figure S1 TRPC channel expression in control, T6K and T6K + WT podocytes.

1
2 Figure S2 Effect of Angiotensin II and the TRPC6 inhibitor SAR 7334 on calcium influx to podocytes and of
3 TRPC6 agonists on calpain activity

4 Figure S3 Full length blots from all immunoprecipitation experiments

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6 Figure S4 Densitometry on protein cleavage and phosphorylation of WT and mutant podocytes.

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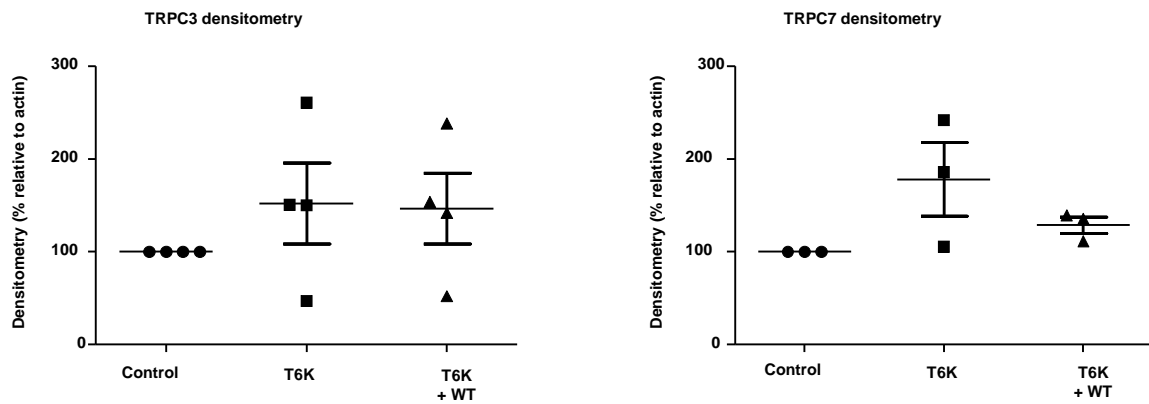


Figure S1 TRPC channel expression in control, T6K and T6K + WT podocytes.

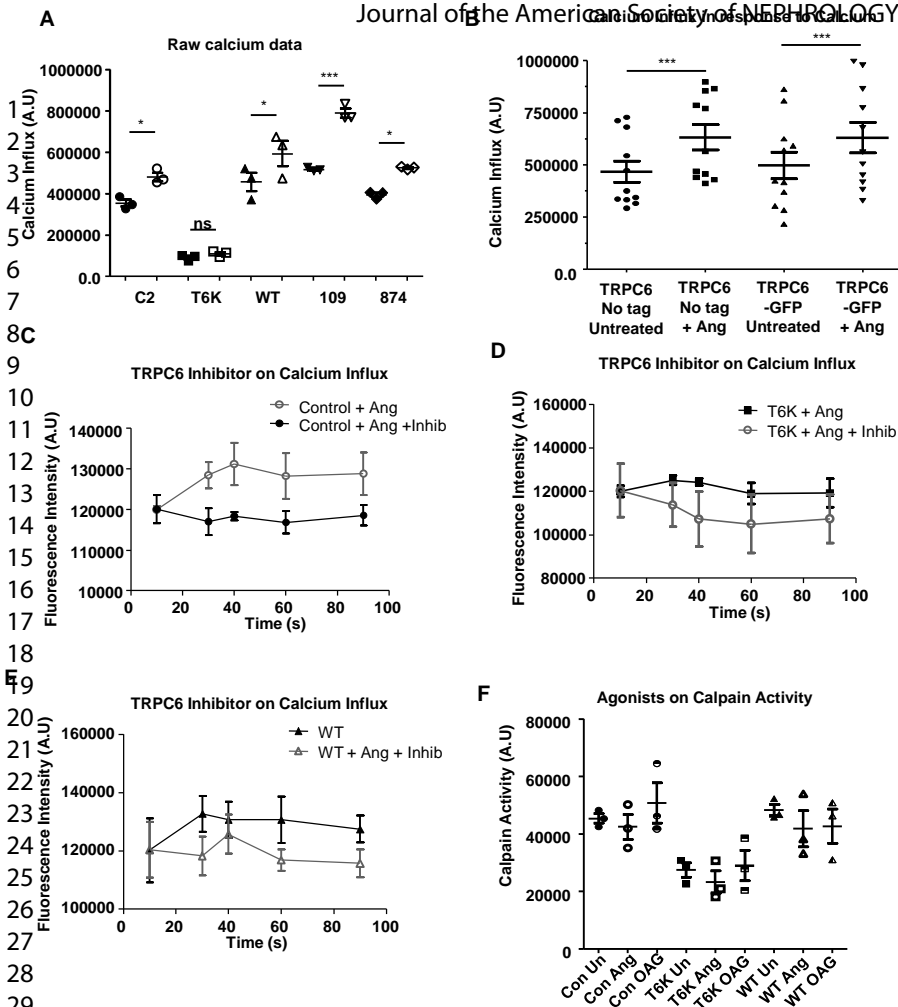
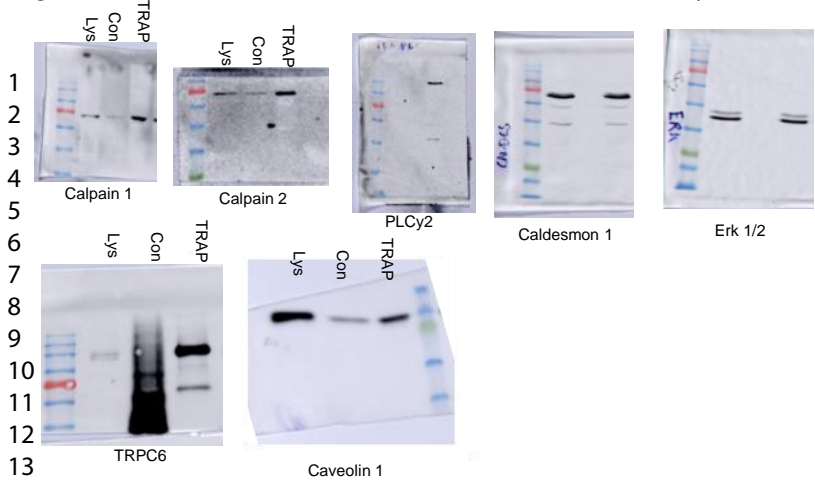
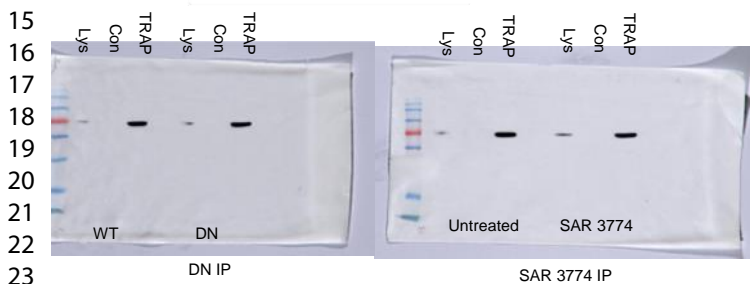


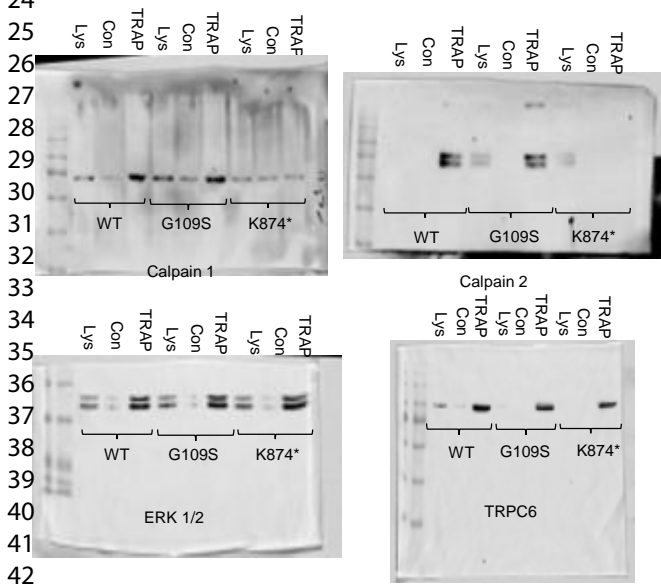
Figure S2 Effect of Angiotensin II and the TRPC6 inhibitor SAR 7334 on calcium influx to podocytes and of TRPC6 agonists on calpain activity



14 Blots from Figure 5



24 Blots from Figure 7



43 Figure S3 Full length blots from all Immunoprecipitation experiments

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Total FAK

FAK substrate

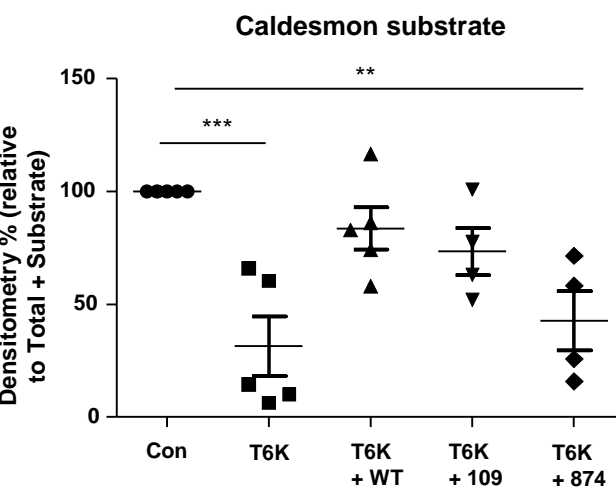
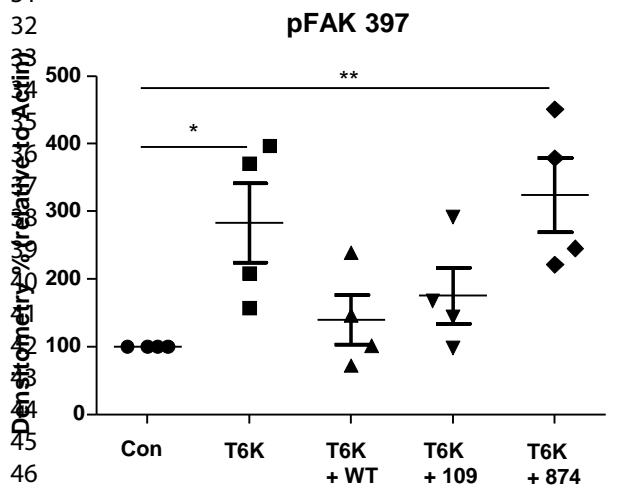
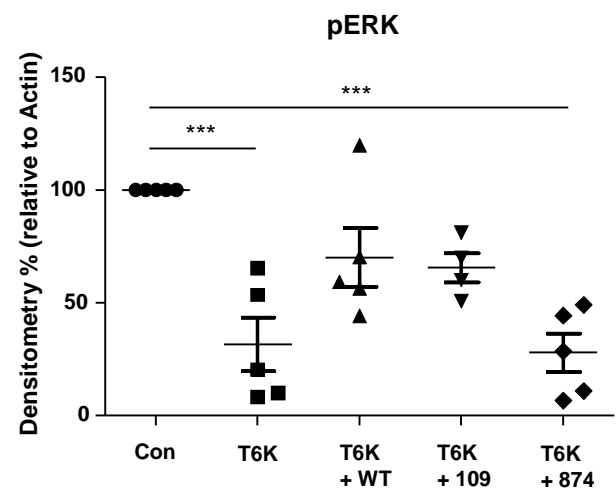
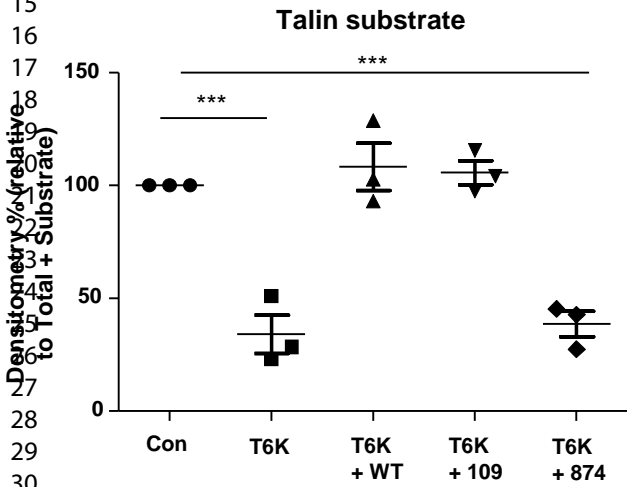
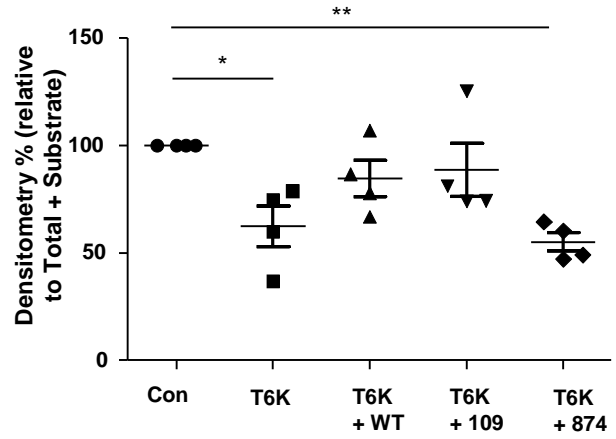
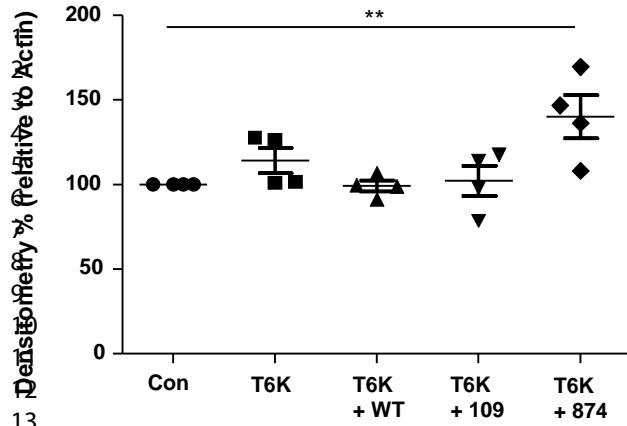
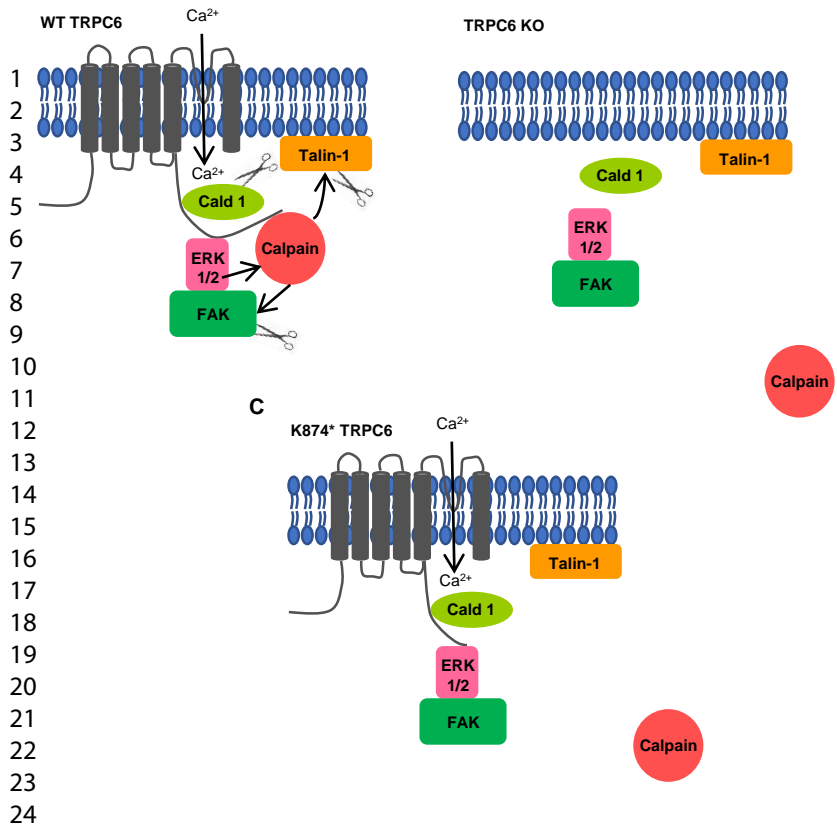


Figure S4 Densitometry on protein cleavage and phosphorylation of WT and mutant podocytes.



25 Summary Schematic. **A** WT TRPC6 binds to, and acts as a structural
 26 scaffold at the membrane for, caldesmon-1 (cald 1), ERK 1/2 and
 27 calpain. This keeps calpain localised just below the membrane where
 28 it can easily cleave its targets talin-1, focal adhesion kinase (FAK), and
 29 caldesmon-1. **B** In the absence of TRPC6 there is no calcium influx to
 30 the cell and calpain is also not localised to the membrane. This means
 31 there is no cleavage of talin-1, FAK or caldesmon-1. **C** The disease
 32 causing mutant TRPC6 K874* has a truncation at its C-terminus.
 33 Calpain no longer binds to this form of TRPC6 and is mis-localised. The
 34 mutant allows the same calcium influx as WT TRPC6. There is no
 35 cleavage of caldesmon-1 talin-1 or FAK. This suggests that the
 36 localisation of calpain to the membrane is important in its function in
 37 the podocyte.