Direct interaction between the actin-binding protein filamin-A and the inwardly rectifying potassium channel, Kir2.1

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Running title: Filamin binds to inward rectifier K⁺ channels

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Summary
The role of filamins in actin cross-linking and membrane stabilization is well established, but recently their ability to interact with a variety of transmembrane receptors and signalling proteins has led to speculation of additional roles in scaffolding and signal transduction. Here we report a direct interaction between filamin-A and Kir2.1, an isoform of inwardly rectifying potassium channel expressed in vascular smooth muscle and an important regulator of vascular tone. Yeast two-hybrid screening of a porcine coronary artery cDNA library using the carboxyl terminus of Kir2.1 as bait yielded cDNA encoding a fragment of filamin–A (residues 2481-2647). Interaction between filamin-A and Kir2.1 was confirmed by in vitro overlay assay of membrane-bound Kir2.1 with glutathione S-transferase fusion protein of the isolated filamin clone. Additionally, antibodies directed against Kir2.1 coimmunoprecipitated filamin-A from arterial smooth muscle cell lysates, and immunocytochemical analysis of individual arterial smooth muscle cells showed that Kir2.1 and filamin co-localise in ‘hotspots’ at the cell membrane. Interaction with filamin-A was found to have no effect on Kir2.1 channel behaviour, but rather increased the number of functional channels resident within the membrane. We conclude that filamin-A is potentially an important regulator of Kir2.1 surface expression and location within vascular smooth muscle.

Introduction
Filamins are large actin-binding phosphoproteins that cross-link actin filaments, stiffening or ‘gelling’ the dispersed microfilament net immediately beneath the cell membrane (1,2). They also establish critical links between the submembrane actin gel and integral membrane proteins, which stabilize the membrane particularly during changes in cell shape associated with cell motility and migration (3,4). Indeed to date, over twenty different protein partners have been identified for filamin. These include not only transmembrane proteins such as β-integrins (5,6), D2 and D3 dopamine receptors (7) and Kv4.2 potassium channels (8), but also intracellular signalling
molecules such as the Rho family of GTPases (9), the stress-activated kinase SEK-1 (also known as MKK-4; 10) and protein kinase Cα (11). This ability to aggregate cytoskeletal elements, transmembrane receptors and cytoplasmic signalling proteins is potentially important not only in the stabilization of receptors at the cell surface, but also in cell signal integration (1,2).

The role of filamin within the vasculature is incompletely understood. It almost certainly plays an important role in cellular migration during vascular development since mutations of the filamin-A gene that block filamin expression and cause human periventricular heterotopia, an X-linked disease characterized by recurrent epileptic seizures, also lead to unusually high incidences of congenital vascular abnormalities (12). Unlike the brain, where filamin-A is concentrated during periods of active neuronal migration then downregulated in the adult, filamin-A persists at high levels in the adult vasculature (13). Within vascular smooth muscle cells, it is known to be involved in bundling actin into stress-fibres within the body of the cell and to associate with the plasma membrane at specialised adhesion sites known as membrane associated dense plaques (14). These belong to a family of adherens type junctions that include the focal adhesions of cultured cells (15,16) and are the sites at which the cytoskeleton and contractile apparatus of smooth muscle cells anchor to the plasma membrane (17). In common with other focal adhesions, the dense plaque regions act as assemblage sites for a number of signalling molecules and are potentially important loci for the integration of signal transduction pathways controlling processes such as actin-remodelling and contractility (18). Whether filamin plays any role in this signal integration and whether it binds to proteins other than actin within smooth muscle cells has not been fully investigated.

Here we report the results of a yeast two-hybrid screen of a porcine coronary artery cDNA library that identify filamin-A (also known as actin-binding protein 280 and filamin-1) as a binding partner of the inwardly rectifying potassium channel, Kir2.1. Inwardly rectifying potassium (Kir) channels
constitute a functionally diverse family of channel proteins that allow $K^+$ to move into the cell more easily than out (19,20). This asymmetry in the current-voltage relation results from the channel’s susceptibility to voltage-dependent block by intracellular polyamines and magnesium ions and allows for modulation of the electrical properties of cells without excessive $K^+$ loss. Consequently, Kir channels are involved in setting and maintaining the resting membrane potential, modulation of prolonged action potentials in electrically excitable cells and potassium homeostasis and secretion (19,20). Kir2.1 is the only isoform of the classical strong (Kir2) inward rectifier subfamily expressed in smooth muscle cells (21) and, crucially, has been shown to be an important regulator of smooth muscle membrane potential, and hence vascular tone, under conditions of metabolic stress (22).

We investigate the potential functional consequences of filamin-A binding to Kir2.1 and show, through the use of human melanoma cells that lack filamin-A, that the interaction does not affect the way the channel behaves, but instead is important for its functional surface expression. We propose that filamin-A acts as a cytoskeletal anchoring protein for Kir2.1 ensuring its stable surface expression and potentially controlling its location and proximity to other proteins at the cell membrane. In smooth muscle cells this interaction may function to recruit Kir2.1 to signalling complexes within membrane specializations such as the dense plaque region.

**Experimental Procedures**

*Antibodies, Polyacrylamide Gel Electrophoresis and Immunoblotting*

The following primary antibodies were used: mouse monoclonal anti-filamin-A (Santa Cruz Biotechnology, Inc); rabbit polyclonal anti-GST (Sigma-Aldrich); mouse monoclonal anti-GFP (Abcam Ltd); rat monoclonal high-affinity anti-HA (Roche Molecular Biochemicals); rabbit polyclonal anti-Kir2.1 (a kind gift from Dr. R. Norman, Dept. Medicine, University of Leicester; 23). Horseradish peroxidase (HRP), Texas Red and fluorescein isothiocyanate (FITC)- conjugated
anti-rabbit, anti-mouse and anti-rat secondary antibodies were from Jackson ImmunoResearch Laboratories, Inc. Protein extracts were resolved by SDS-polyacrylamide gel electrophoresis on 12% polyacrylamide-Tris gels and transferred electrophoretically onto nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech). Membranes were blocked overnight at 4°C in blocking solution containing 5% (w/v) skim milk powder and 0.1% Tween 20 in Tris-buffered saline. Primary antibodies were diluted in blocking solution containing 1% skim milk powder and 0.1% Tween 20 in Tris-buffered saline and incubated with the membranes for 2-3 hours at room temperature. Membranes were washed in Tris-buffered saline then incubated with horseradish peroxidase-conjugated secondary antibodies for a further hour at room temperature. Labelled bands were visualized using enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech) according to manufacturer’s protocol.

**Plasmid Constructs**

Plasmid construct filamin-A (repeats 19-24) fused to the NH2-terminal HA epitope tag in pcDNA-HANII was a generous gift from Prof. Sonnenburg (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Wild-type and eGFP-Kir2.1 in pcDNA3 were as described previously (24). Dual tagged HA- and eGFP-Kir2.1 in pcDNA3 was generated by digestion of an internal cassette flanked by BsmB1 and Xho1 restriction sites, and containing the HA extracellular epitope, from HA-Kir2.1 pcDNA3 (25) and subsequent ligation of the cassette into eGFP-Kir2.1 pcDNA3.

**Cells and Cell Transfection**

Human melanoma cell line, filamin+ (lacking expression of filamin-A) and isogenic cell line, filamin+ (stably transfected with full length filamin-A) were a kind gift from Dr. J. Hartwig (Harvard Medical School, Boston, U.S.A.). Cells were grown in α-minimal essential medium supplemented with 8% new born calf serum, 2% foetal calf serum. Filamin+ cells were cultured in G418 (0.5 mg/ml) to maintain selection of cells harbouring the transfected plasmid. HEK293 were
grown in minimal essential medium supplemented with 1% non-essential amino acids and 10% (v/v) fetal bovine serum. All media and reagents were from Life Technologies, Inc. Cells were transiently transfected using Lipofectamine transfection reagent (Invitrogen Ltd) according to the manufacturer’s protocol. Transfections were performed in six-well culture plates with cells at 80% confluence.

**Yeast two-hybrid library screen**

Yeast two hybrid analysis was performed according to the Matchmaker library construction and screening kit (BD Biosciences Clontech). Bait construct, comprising amino acids 307-428 of mouse Kir2.1, was amplified by PCR (Pfu polymerase) using the wild-type Kir2.1 expression construct in pcDNA3 as template. PCR product was A-tailed and cloned into the pGEM-T vector (Promega) before subcloning into BamHI and EcoRI sites of the GAL4 binding domain plasmid, pGBK7. Plasmid was transformed into yeast strain, Y187, harbouring the LacZ reporter gene under control of GAL4-binding sites.

Total RNA was isolated (SV total RNA isolation kit, Promega) from porcine coronary arteries dissected from hearts donated by a local abattoir (Joseph Morris & Sons, Leicestershire, U.K.) and reversed transcribed using SMART (switching mechanism at 5’ end of RNA transcript) technology. cDNA was co-transformed with SmaI linearized GAL-4 activation domain vector, pGADT7, into yeast strain AH109 harbouring reporter genes, HIS3 and ADE2, under the control of GAL4-binding sites. Library carrying AH109 were mated with Y187 carrying bait plasmid, pGBK7-2.1C, and plated onto synthetic quadruple drop-out medium (SD/-Ade/-His/-Trp/-Leu). Positive colonies were screened for β-galactosidase activity using filter-lift assay. Activation domain plasmids, pGADT7, were isolated from yeast colonies displaying positive phenotype and transformed into bacteria to obtain plasmids suitable for sequencing reactions. Plasmid inserts were sequenced (PNACL,
University of Leicester, Leicester, U.K.) and compared against the GenBank database by BLAST search analysis (National Institute of Health).

*Generation of truncation constructs and two-hybrid mapping studies*

Mapping studies of protein-protein interactions between the C-terminal filamin clone and Kir2.1 truncation constructs were analysed by filter-lift and liquid culture β-galactosidase assay in yeast strain, Y187. Truncation constructs of the Kir2.1 carboxyl-terminus (amino acids: 307-366, 367-428, 307-326, 325-346, 347-366) were amplified by PCR (Pfu polymerase) using the yeast two-hybrid vector, pGBKKT7-Kir2.1C, as template and unique primers incorporating EcoRI and BamHI sites at 5’ and 3’ ends, respectively. PCR fragments were A-tailed and cloned into pGEM-T (Promega) before subcloning into BamHI/EcoRI restriction sites of the GAL4-binding domain vector, pGBKKT7. Mutations of pGBKKT7-Kir2.1 (aa 307-326 and 307-428) corresponding to Anderson’s syndrome (Δ314-315) were generated by QuikChange mutagenesis (Stratagene). All inserts were verified by automated sequencing (PNACL facility, University of Leicester). Plasmids were incorporated into yeast by standard lithium acetate-mediated transformation (BD Biosciences Clontech) and transformed colonies selected for by growth on double drop-out medium (SD-Trp/-Leu). Standard methodology provided by BD Clontech was used to perform β-galactosidase filter-lift and liquid culture assays using 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal) and o-nitrophenyl β-D-galactopyranoside (ONPG) as substrate, respectively.

*Recombinant protein expression and in vitro binding assay*

Filamin-A carboxyl-terminal fragment (αα 2481-2647), isolated from the yeast two-hybrid screen, was excised from EcoRI and XhoI sites of the GAL4 activation domain vector, pGADT7, and subcloned in-frame into the GST fusion protein vector, pGEX-6P-1 (Amersham Pharmacia Biotech). Kir2.1C (aa 307-428) construct was PCR amplified using the Kir2.1 pcDNA3 construct as
template and cloned into NdeI and BamHI sites of the N-terminal His-tagged vector, pET15b.

DNA constructs cloned into pGEX-6P-1 and pET-15b were transformed into DH5α and BL21(DE3)pLysS competent cells, respectively. Overnight cultures were diluted (1:100) into fresh Luria Bertani broth, grown to O.D.≈0.5 and induced with iso-propyl-β-D-thiogalactopyranoside (1 mM, 3-4 hr, 37 °C). DH5α pellets, harbouring the GST-filamin (aa 2481-2647) fusion protein, were resuspended in ice-cold PBS, sonicated (×2 10 second bursts) and incubated with Triton X-100. Supernatants were incubated with glutathione-sepharose beads and bound proteins washed with PBS (×3). GST-fusion protein was released with glutathione elution buffer (20mM glutathione, 100mM Tris-HCl (pH 8.0), 120mM NaCl). BL21(DE3)pLysS pellets, harbouring insoluble His-tagged Kir2.1C (aa 307-428), were resuspended in buffer A (20 mM Tris, 80mM NaCl, pH 8.0) containing 8M urea and insoluble material cleared by centrifugation (13 000 rpm, 5 min, 4°C). Supernatants were incubated with Talon metal affinity resin (BD Biosciences Clontech) and bound His-tagged Kir 2.1C refolded by washing through serial dilutions of urea (8 to 0 M in buffer A).

Renatured His-tagged Kir 2.1C (aa 307-428) was resolved by SDS-PAGE (12 %) and blotted onto nitrocellulose. Membranes were incubated overnight with block buffer and overlaid with equal quantities of GST and GST-tagged filamin (aa 2481-2647) fusion protein (1 hr, 20 °C). Following extensive washing in blot buffer, membranes were exposed to anti-GST antibody (1:1000 in blot buffer, 1 hr, 20 °C), washed and incubated with HRP-conjugated anti-rabbit antibody (1:10 000 in blot buffer, 1 hr, 20 °C).

**Coimmunoprecipitation**

Mammalian HEK293 cells were transiently transfected with plasmid cDNA encoding HA-tagged filamin (carboxyl-terminal repeats, 19-24) and eGFP-tagged Kir2.1. Transfected cells were lysed in lysis buffer (20mM Tris-HCl; 250mM NaCl; 3mM EDTA; 3mM EGTA; pH7.6) containing 1%
Triton X100 and protease inhibitors (1:100 dilution, Sigma Protease Inhibitor Cocktail containing AEBSF, aprotinin, bestatin, leupeptin, pepstatin A) and insoluble material cleared by centrifugation. Soluble fractions were incubated with monoclonal anti-GFP antibody (5 µg) or mouse non-immune control serum (5 µg) overnight at 4°C. Rat arterial smooth muscle lysates were prepared by homogenization of tissue in ice-cold lysis buffer in a hand-held homogeniser. Insoluble material was cleared by centrifugation and soluble fractions incubated overnight with rabbit polyclonal anti-Kir2.1 or rabbit non-immune control serum as above. Antigen-antibody complexes were captured with protein-A sepharose (Amersham Pharmacia; 4°C, 2 hr). Beads were washed extensively prior to removal of bound proteins by boiling in SDS-sample buffer. Samples were resolved by SDS-PAGE, transferred onto nitrocellulose membrane and analysed by immunoblotting.

**Cell Imaging**

Cells were grown to 80% confluence on poly-L-lysine coated glass coverslips before transfection with Lipofectamine (Invitrogen). Prior to imaging of live cells, coverslips were sealed to the base of an imaging chamber with vacuum grease and maintained in PBS at room temperature. Alternatively, filamin+ and filamin- cells were fixed and permeabilised in ice-cold methanol. Fixed cells were incubated with anti-filamin (1:200) primary antibody diluted in PBS containing 10 % (v/v) goat serum overnight at 4 °C. Excess antibody was removed by extensive washing in PBS prior to incubation with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit secondary antibody (1:200 in PBS containing 10 % v/v goat serum) for 2 hours at room temperature under dark conditions. After repeated washing (PBS), coverslips were mounted onto microscope slides using fluorescent mounting medium (Dako Ltd). Live and fixed cells were viewed using an inverted PerkinElmer Ultraview™ confocal laser scanning microscope, equipped with a krypon/argon laser and ×60 oil immersion lens with numerical aperture of 1.0. Images of cells expressing the eGFP-HA-Kir2.1 construct were analysed using a method similar to that described previously (26).
Tagged image file format (TIFF) images were imported into NIH ImageJ, the cells outlined and the mean pixel values obtained. Pixel values were on an 8-bit scale ($2^8 = 256; 0-255$). Laser levels were kept constant between 488nm and 568nm channels.

**Smooth muscle immunocytochemistry**

Smooth muscle cells were isolated enzymatically using a modification of methodology described previously (27), whereby secondary enzymatic digestion of arterial segments with collagenase and hyluronidase was omitted. Cells were plated onto poly-L-lysine coated coverslips prior to fixation and permeabilisation in paraformaldehyde and Triton X-100 (0.1%), respectively. Antibody staining and cell imaging were as described for heterologous cells. Cells were incubated overnight at 4°C with mouse monoclonal anti-filamin (diluted 1:1000 in PBS with 10% v/v goat serum) and rabbit polyclonal anti-Kir2.1 (1:500) and visualised with anti mouse Texas red- and anti rabbit FITC – conjugated secondary antibodies, respectively.

**Electrophysiology**

Whole-cell currents were recorded from either filamin$^+$ or filamin$^-$ cells typically 24-48 hours post-transfection using an Axopatch 200B amplifier (Axon Instruments). Currents recorded in response to voltage steps were filtered at 5kHz (-3dB, 8-pole Bessel), digitized at 10kHz using a DigiData 1320A interface (Axon Instruments), and analysed using pCLAMP software. Electrodes were pulled from borosilicate glass (outer diameter 1.5mm, inner diameter 1.17mm; Clarke Electromedical, Pangbourne, UK) and fire polished to give a final resistance of 5 MΩ when filled. The pipette-filling solution contained (mM): KCl, 140; MgCl$_2$, 1; EGTA, 10; HEPES, 10; pH 7.2. The external solution contained (mM): KCl, 70; NaCl, 70; MgCl$_2$, 2, CaCl$_2$, 2; HEPES; 10; pH 7.25. The junction potential between pipette and external solutions was sufficiently small (<1.5 mV) to be neglected. As far as possible, analogue means were used to correct capacity transients. Up to 90% compensation was routinely used to correct for series resistance. Single channel
currents recorded from either filamin\(^+\) or filamin\(^-\) cells were filtered at 2kHz and digitized at 10kHz. Single channel all-point amplitude histograms and single channel open probability were calculated using pClamp software. Electrodes for single channel recordings were pulled from borosilicate glass (outer diameter 1.5mm, inner diameter 0.86mm; Clarke Electromedical) and fire polished to give a final resistance of 15-20M\(\Omega\) when filled. The pipette-filling solution for single channel experiments contained (mM): KCl, 200; MgCl\(_2\), 2, CaCl\(_2\), 2; HEPES; 10; pH 7.25. The external solution contained (mM): KCl, 140; MgCl\(_2\), 2, CaCl\(_2\), 2; HEPES; 10; pH 7.25. All experiments were performed at room temperature (18-22\(^\circ\)C), and the results are expressed as mean ± SEM. Statistical significance was evaluated using the Student’s unpaired t-test, level of significance was *\(p<0.05\).

Results

**Identification of filamin-A as a binding partner for Kir2.1 by yeast two-hybrid screening**

To isolate binding partners of Kir2.1, a carboxyl-terminal segment of the channel (aa 307-428) was subcloned into the GAL4-binding domain bait vector, pGBKT7. This was used as bait to screen a porcine coronary artery cDNA library incorporated into the GAL4-activation domain vector, pGADT7, by yeast two-hybrid analysis. A screen of 1x10\(^6\) yeast colonies resulted in a large number (1738) exhibiting positive phenotype (growth on synthetic quadruple drop-out medium SD/-Ade/-His/-Trp/-Leu). These colonies were screened by \(\beta\)-galactosidase filter assay, but only 10 demonstrated a positive reaction (development of blue colour at approximately 40 minutes). One of the positive clones encoded a partial cDNA representing part of repeat 23, the hinge 2 region and repeat 24 of filamin-A (actin-binding protein-280, aa 2481-2647; Fig. 1A). Translation of the sequence revealed that the isolated porcine clone exhibited a 94% homology with its human homologue (156 out of 166 amino acids; Fig. 1B).
Mapping of the region on Kir2.1 responsible for interacting with filamin

To further define the carboxyl-terminal Kir2.1 amino acid motif/motives responsible for interaction with filamin, successive deletion constructs of Kir2.1 (aa 307-428) were tested by yeast two-hybrid analysis for interaction with the isolated filamin clone. Figure 2Ai shows the truncation constructs of Kir2.1 that were cloned into the bait vector, pGBKKT7. These were cotransformed into yeast strain, Y187 along with the filamin clone in prey vector, pGADT7, for quantitative β-galactosidase assays. Activation of the reporter gene occurred with amino acids 307-367 of Kir2.1 as bait, but not amino acids 367-428. The strength of interaction increased with successive deletions of the 307-367 amino acid channel segment such that amino acids 307-326 demonstrated the strongest interaction tested (Fig. 2Bi). A similar observation has been reported for the interaction of glycoprotein GPIbα with filamin-A, where stronger yeast two-hybrid signals were obtained with smaller protein fragments (28). Transfection of the Kir2.1 truncation constructs with empty prey plasmid, pGADT7, prevented the positive interactions observed in the β-galactosidase filter-lift and liquid assays.

Mutations in Kir2.1 can give rise to the development of Andersen’s syndrome; a rare condition characterised by dysmorphic features, periodic paralysis and cardiac arrhythmias (29). One genetic variant of the disease has been documented as an in-frame deletion of amino acids 314-315. Since our results suggest that amino acids in the region 307-326 are involved in the interaction of Kir2.1 with filamin, we investigated the importance of amino acids 314-315 to filamin binding. We constructed 314-315 deletion mutants within the yeast two-hybrid bait vectors, pGBKKT7-Kir2.1 aa 307-428 (Δ314-315) and pGBKKT7-Kir2.1 aa 307-326 (Δ314-315) (Fig. 2Aii). Figure 2Aii and Bii shows that deletion of the Andersen’s syndrome amino acids 314-315 had no effect on the ability of filamin to interact with Kir2.1 amino acids 307-326, as assayed by filter-lift and liquid β-galactosidase experiments, respectively. Transformation of the latter Kir2.1 aa 307-326 (Δ314-
deletion constructs together with an empty prey domain vector prevented the positive result observed for the β−galactosidase assays, demonstrating the specificity of the interactions.

**Filamin interacts in vitro with Kir2.1**

The interaction between filamin and the carboxyl terminus of Kir2.1 (Kir2.1C) was confirmed using an *in vitro* GST overlay assay. The filamin cDNA clone (aa 2482-2647) was excised from pGADT7 and subcloned into pGEX-6P-1 for expression as a GST-fusion protein. Using a Far-Western overlay assay, GST-filamin was shown to interact with membrane bound His-tagged Kir2.1C, whilst GST alone failed to show any interaction (Fig. 3A).

**Filamin-A coimmunoprecipitates with Kir2.1**

To establish an interaction between Kir2.1 and filamin within intact mammalian cells, co-immunoprecipitation experiments were performed. HEK293 cells were co-transfected with cDNA constructs corresponding to eGFP-tagged Kir2.1 (entire protein) and HA-tagged filamin-A (C-terminal truncation of repeats 19-24). Antibodies directed against the eGFP epitope on Kir2.1 were able to coimmunoprecipitate the HA-filamin C-terminal construct (Fig. 3B), suggesting that these two proteins form complexes within HEK293 cells. Both filamin and Kir2.1 are expressed within arterial smooth muscle (30, 21, 22) and we were interested in establishing whether such an interaction was likely to occur within native tissue. Figure 4A shows that antibodies directed against Kir2.1 coimmunoprecipitate full-length (280kDa) filamin-A from arterial smooth muscle lysates – consistent with a physical interaction between these two proteins within smooth muscle cells.

**Kir2.1 and filamin colocalize in arterial smooth muscle cells**

Since Kir2.1 is an integral membrane protein and filamin organises the actin cytoskeleton immediately beneath the membrane, these proteins would be expected to be in close proximity...
within cells. Figure 4B,C shows confocal images of an isolated arterial smooth muscle stained with antibodies directed against filamin-A and Kir2.1, and visualized with secondary antibodies conjugated with the non-overlapping fluoroprobes Texas Red (Fig. 4B) and FITC (Fig. 4C), respectively. The images show both proteins localized predominantly to the plasma membrane and more specifically a slightly punctate distribution was observed for both filamin and Kir2.1, suggesting that both proteins are concentrated in certain areas on the membrane. Kir2.1 and filamin colocalise to some, but not all puncta (Fig. 4B and C inset) indicating the potential existence of membrane complexes made up of Kir2.1 and filamin within smooth muscle cells.

**Kir2.1 is targeted to the plasma membrane in the absence of filamin-A**

Interaction between Kir2.1 and filamin within cells could fulfil a number of different roles, including regulation of the surface expression and location of Kir2.1. Studies into the physiological roles of filamin have been aided greatly by the development of tumor cell lines from human malignant melanomas that lack filamin-A (filamin⁻ cells; also called M2 cells; 4). These cells show extensive and continuous blebbing of the plasma membrane and, consistent with the role of filamin in membrane stabilization and cell motility, remain rounded and unable to extend membrane projections and spread normally (Fig. 5A). Normal cell morphology and motility can be restored by stable transfection with cDNA encoding filamin (filamin⁺ cells; also called M2A7 cells, Fig. 5B). Filamin can be seen in the rescued filamin⁺ cells concentrated at the cell periphery in close apposition to the membrane (Fig. 5D), no filamin-A immunoreactivity is observed in the filamin-deficient cells (Fig. 5C). Levels of actin and other actin-associated proteins such as gelsolin, profilin and α-actinin are comparable between filamin⁺ and filamin⁻ cells (4).

Filamin-deficient cells have been reported to have a reduced surface expression of many receptors and ion channels, suggesting that organisation of the submembranous cytoskeleton may be important for the appropriate insertion or removal of proteins from the membrane. As an extreme
example of this, expression of the D2 dopamine receptor in filamin-deficient cells results in an almost total loss of receptor expression in the membrane (7). To test if filamin had a similar influence on the trafficking of Kir2.1 we expressed eGFP-tagged Kir2.1 (24) in filamin-deficient and filamin-replenished cell lines, filamin⁺ and filamin⁻, respectively. Figures 5E and F show confocal images of filamin⁻ and filamin⁺ transiently transfected with eGFP-tagged Kir2.1. The expressed eGFP-Kir2.1 protein is clearly visible at or near the plasma membrane of both cell types. eGFP-Kir2.1 is also observed throughout the cell body of both cell types, harboured within intracellular compartments.

**Effect of Kir2.1-filamin association on whole cell Kir2.1 currents**

To establish whether the Kir2.1 channels observed at the cell periphery in Figures 5 E,F are inserted into the membrane we used the conventional whole-cell clamp technique to record membrane currents from single filamin⁺ (Fig. 6A) and filamin⁻ cells (Fig. 6B) that had been transiently transfected with cDNA encoding eGFP-Kir2.1. Currents were recorded in response to voltage steps from a holding potential of −17mV (the equilibrium potential for K⁺ (E_K) under these recording conditions) to test potentials ranging from +60mV to −100mV in 10mV increments. Voltage steps positive to E_K elicited only small outward currents, whereas steps negative to E_K produced substantial inward currents consistent with the membrane expression of Kir2.1. No significant whole cell currents were recorded from non-transfected filamin⁺ or filamin⁻ cells under these conditions. In the absence of filamin, whole-cell Kir2.1 currents were significantly reduced (Fig. 6C and D). At −100mV inward current in filamin⁺ cells was 1015 ± 145 pA/pF (n=8) whilst it was only 598 ± 122 pA/pF in filamin-deficient filamin⁻ cells (n=8; p<0.05).

**Kir2.1 single channel recordings from filamin⁺ and filamin⁻ cells**

The reduction in whole-cell Kir2.1 current in filamin-deficient cells may attributed to several factors: 1) a reduction in the number of functional channels at the cell surface; 2) a reduction in
Kir2.1 single channel conductance; 3) a change in the probability of single channel opening; 4) reduced Kir2.1 expression levels in filamin cells. To help distinguish between these possibilities we recorded single channel currents from filamin and filamin cells transiently transfected with eGFP-Kir2.1. To ensure that we did not disrupt Kir2.1-filamin interactions by pulling excised patches, we used the cell-attached configuration of the patch-clamp technique. Typical Kir2.1 single channel recordings from filamin-deficient cells are shown in Figure 7A, consistent with the expression of Kir2.1 we were only able to record significant single channel currents at potentials negative to the equilibrium potential for $K^+$ (+10mV, under these recording conditions). The single channel conductance of Kir2.1 was identical in filamin and filamin cells (Fig. 7B). Unitary conductance at −100mV was 25.5 ± 0.4 pS in filamin cells and 23.9 ± 1.1 pS in filamin cells (n=3 each). Open probabilities for Kir2.1 were also unaffected by the presence of filamin. At −100mV $P_{\text{open}}$ was 0.65 ± 0.07 for filamin cells and 0.68 ± 0.05 for filamin cells (n=3 each; Fig. 7C).

These data suggest that the reduction in whole-cell Kir2.1 currents in filamin-deficient cells stems from either a reduction in the number of functional channels inserted into the cell membrane or a difference in the overall expression levels of Kir2.1 between filamin and filamin cells.

**Total expression versus surface expression**

Only a percentage of the total number of channels expressed will be inserted in the membrane at any one time. To determine how many channels are in the membrane for a given level of total expression, we constructed a Kir2.1 channel with intracellular and extracellular epitope tags. This construct (eGFP-HA-Kir2.1) produces a Kir2.1 channel that has eGFP fused to the intracellular amino-terminus and an HA epitope inserted into the extracellular loop between the first transmembrane segment (M1) and the pore-forming H5 region. Figure 8A-D shows confocal images of non-permeabilized filamin and filamin cells that have been transiently transfected with the eGFP-HA-Kir2.1. The cells have been stained with antibodies against the extracellular HA-epitope and distribution of the HA-epitope has been visualized with secondary antibodies.
conjugated with the fluorophore Texas Red. No antibodies are required to visualize the distribution of the eGFP tag. When excited at 488nm, the intensity of intracellular eGFP fluorescence gives an indication of the total level of eGFP-HA-Kir2.1 expression within filamin\textsuperscript{+} and filamin\textsuperscript{-} cells (Fig. 8A,C) while, the intensity of the extracellular Texas Red signal at 568nm gives an indication of the number of channels inserted into the membrane (Fig. 8B,D). We found that the fraction of expressed eGFP-HA-Kir2.1 channels that were inserted into the membrane was significantly less in filamin-deficient cells as compared to filamin-containing cells (Fig. 8E), highlighting a potential role for filamin in regulating Kir2.1 surface expression.

**Discussion**

Our findings suggest that the actin-binding protein filamin-A acts as a cytoskeletal anchoring protein for Kir2.1 within cells, stabilizing its surface expression and potentially recruiting the ion channel to signalling complexes within membrane specializations in arterial smooth muscle cells.

Kir2.1 is the predominant isoform of the classical strong inward rectifier to be expressed in smooth muscle (21). Targeted disruption of the Kir2.1 gene produces arteries that fail to dilate in response to the modest elevations in extracellular K\textsuperscript{+} that are typically associated with periods of hypoxia and ischaemia, indicating the essential role for Kir2.1 in the regulation of vascular tone under conditions of metabolic stress (22). Little is known however about the mechanisms involved in regulating the surface expression or location of Kir2.1 in smooth muscle cells, or indeed whether the channel complexes with other proteins that modulate its activity. To identify potential proteins that interact with Kir2.1 within the vasculature we screened a porcine coronary artery cDNA library using the yeast two-hybrid system and a carboxyl-terminal construct of Kir2.1 as bait. The screen yielded cDNA encoding a carboxyl-terminal fragment (residues 2481-2647 corresponding to part of repeat 23, hinge region 2 and repeat 24) of filamin–A, a cytoskeletal protein known to be involved in actin cross-linking and tethering of cell surface receptors to the actin cytoskeleton (1,2). The interaction
between filamin-A and Kir2.1 was confirmed by overlay of His-tagged Kir2.1 with a GST-fusion protein of the isolated filamin clone. The ability for Kir2.1 and filamin-A to form a complex within intact cells was verified by using antibodies directed against an epitope tag on Kir2.1 to coimmunoprecipitate filamin-A (C-terminal repeats 19-24) from heterologous cells transfected with cDNAs encoding both proteins. Furthermore, antibodies directed against Kir2.1 were able to isolate full-length filamin-A from lysates of arterial smooth muscle, indicating a physical interaction between these proteins in native cells. Indeed, immunocytochemical analysis of individual vascular smooth muscle cells showed that Kir2.1 and filamin are co-localised in ‘hotspots’ on the membrane, consistent with them existing together at membrane specializations. The nature of these specialized sites is speculative but they may represent dense plaque regions on the smooth muscle. These are points of attachment between the cytoskeleton, the contractile apparatus and the plasma membrane and are structurally similar to the focal adhesion sites of cultured cells (14,15,16). In common with other focal adhesions, dense plaque regions act as assembly sites for a number of different signalling proteins and cytoskeletal elements, including filamin, integrins, focal adhesion kinase (FAK), paxillin, and non-receptor tyrosine kinases (18). Any potential functional significance of recruiting Kir2.1 to these regions remains to be determined.

Members of the Kir2 subfamily of inward rectifying potassium channels have previously been shown to interact with the PDZ domains of selected members of the membrane associated guanylate kinase (MAGUK) protein family (31, 32, 33, 34). Interaction with MAGUKs is believed to facilitate the subcellular targeting of ion channels and the formation of functional ion channel signalling complexes. Kir2.1 and Kir2.3 both interact with PSD-95/SAP90, a cytoskeletal component of postsynaptic densities, via a type I PDZ binding motif (T/S-X-V/I) located at the channel’s extreme carboxyl terminus (31). Kir2.3 colocalizes with PSD-95 at post-synaptic membranes in the rat forebrain (31, 35). A likely binding partner for the strong inward rectifiers in non-neuronal tissues has been shown to be the ubiquitously expressed MAGUK, SAP-97/hDlg,
which again interacts with Kir2.1, 2.2 and 2.3 via the carboxyl terminal PDZ binding motif, and colocalizes with Kir2.2 in T-tubules of cardiac ventricular myocytes (34). Recent evidence also suggests that SAP97 may mediate formation of large multi-protein complexes centred around Kir2 isoforms in both the heart and the brain (36).

The region of the carboxyl terminus of filamin that interacts with Kir2.1 is particularly rich in binding sites for transmembrane and signalling proteins and may also function as a protein scaffold. Filamins are composed of two identical rodlike subunits (each of 240-280kDa) that associate head to head via a carboxyl terminal self-association site to form dimers that appear by electron microscopy as elongated V-shaped strands (37, 38, 39). The free amino terminal region of each subunit contains an actin-binding motif common to other actin interacting proteins such as β-spectrin, dystrophin and α-actinin. The rest of the filamin chain forms a semi-rigid rod composed of 24 repeated sequences interrupted by two short flexible hinge regions. The majority of filamin-interacting proteins bind to a region just upstream of the dimerization site. The calcitonin receptor (40); the voltage-gated K\(^+\) channel Kv4.2 (8); SEK-1 (also known as MKK-4, a member of the stress-activated Jun kinase cascade; 10); TRAF-2 (Tumour necrosis factor (TNF) receptor-associated factor-2; 41); the small GTPases Rac, Rho, CDC42 and RalA (9), protein kinase C\(\alpha\) (11) and now Kir2.1, all bind in the region between repeats 20-24 that includes the second hinge region.

We identified the reciprocal binding site of filamin on Kir2.1 as being primarily a stretch of 19 amino-acids (307-326) on the channel’s carboxyl terminus. The much weaker interaction observed with filamin and amino acids 325-346 of Kir2.1 suggests that other regions of Kir2.1 may also contribute to binding in the final association complex. For the potassium channel Kv4.2, the binding site for filamin was mapped to a proline-rich PTPP motif (8). This motif does not appear in the filamin-binding regions on Kir2.1 and suggests that different mechanisms might be employed for scaffolding different ion channels. Indeed, no obvious sequence homology exists between any
of the protein domains that associate with the filamin-A repeats, indicating that secondary or
tertiary structures may be important in defining binding specificity.

Interactions with the actin cytoskeleton through filamin-A may regulate not only the location of
Kir2.1 on cell membranes and its vicinity to other proteins, but also the channel’s stability at the
cell surface. We found whole-cell Kir2.1 currents recorded from human melanoma cells that lack
filamin-A to be significantly smaller than Kir2.1 currents recorded from stable transformants of
these cells expressing filamin-A. Kinetic analysis of single Kir2.1 channel currents showed that the
absence of filamin has no significant effect upon single channel amplitude or open probability, but
most likely diminishes the number of functional channels in the cell membrane. We confirmed this
finding by use of a double-tagged Kir2.1 construct that allows us to monitor surface expression as a
fraction of total expression. A number of other filamin-interacting proteins including β-integrins
(28); Kv4.2 voltage-gated potassium channels (8); D2 dopamine receptors (7) and calcitonin
receptors (40) show markedly decreased levels of functional cell surface expression in the absence
of filamin. The reason for this is unclear, although the presence of a correctly organised
submembranous actin cytoskeleton may be essential for the insertion or removal of proteins into or
out of the plasma membrane. In support of this, binding of filamin to the carboxyl tail of the
calcitonin receptor has been shown to be important in the recycling of the receptors back to the
membrane following internalization (40).

To conclude, the role of filamin within the vasculature is incompletely understood, but elsewhere its
ability to create peripheral actin gel networks by orthogonal crosslinking of actin filaments is
essential for membrane elasticity and cell motility, particularly during development (4,12).
Additionally, its ability to link integral membrane proteins to the cytoskeleton and intracellular
signalling molecules point to potential roles in scaffolding and the stabilization of receptor/ion
channel surface expression. We identify Kir2.1 as belonging to an ever-increasing number of
transmembrane proteins interacting with cytoskeletal protein filamin-A and suggest that this interaction may be a method of targeting and regulating surface expression of Kir2.1 in vascular smooth muscle.

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Footnotes

1 The abbreviations used are: Kir2.1, inward rectifier potassium channel 2.1; HRP, Horseradish peroxidase; FITC, fluorescein isothiocyanate; PCR, polymerase chain reaction; C-terminal, carboxyl terminal; N-terminal, amino-terminal; HA, haemagglutinin; PBS, phosphate-buffered saline; HEK-293, human embryonic kidney 293; eGFP, enhanced green fluorescent protein; GST, glutathione S-transferase; ONPG, p-nitrophenyl β-D-galactopyranoside; X-Gal, 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside; IPTG, iso-propyl-β-D-thiogalactopyranoside; MKK-1, MAP kinase kinase.

Figure Legends

Figure 1. Yeast two-hybrid screening of a porcine coronary cDNA library identifies filamin-A as a binding partner of Kir2.1. A. Representation of the domain structure of filamin-A, showing the region between residues 2481 and 2647 (corresponding to part of repeat 23, the hinge 2 region and repeat 24) isolated from the yeast two-hybrid screen. Filamins are composed of two identical rodlike subunits that associate head to head via a carboxyl terminal self-association site to form dimers. The free amino terminal region of each subunit contains an actin-binding motif. The rest of the filamin chain forms a semi-rigid rod composed of 24 repeated sequences interrupted by two short flexible hinge regions (H1 and H2). B. Aligned sequences of human filamin-A and cloned porcine protein isolated from yeast two-hybrid screen. Highlighted amino acids correspond to differences between the porcine protein and its human homologue.

Figure 2. Residues 307-326 on the carboxyl tail of Kir2.1 bind to filamin-A and the binding is not attenuated by an Andersen’s syndrome mutation: deletion of amino acids 314-315. A. Schematic diagram illustrating strength of interaction between Kir2.1 carboxyl terminal constructs (amino acids indicated in left hand of table; bait vector), with (ii) and without (i) Andersen’s syndrome mutation (∆ amino acids 314-315) and filamin-A (amino acids 2481-2647; prey vector).
in yeast two-hybrid filter lift assay (development of blue colour after 30-40 minutes, ++; development of blue colour 60-80 minutes, +; no development of blue colour after 12 hrs, -). B. Interactions of Kir2.1 truncation constructs (amino acids indicated above individual bars), with (ii) and without (i) Andersen’s syndrome mutation and filamin-A (filled bars) or empty prey vectors (hatched bars) were assessed by liquid beta-galactosidase assay. Data are presented as the average of 3 experiments performed in triplicate.

**Figure 3** Kir2.1 and filamin interact in vitro and in vivo. A. Renatured His-tagged Kir2.1 (amino acids 307-428) protein was resolved by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane. Membranes were overlaid with equal quantities of GST or GST-filamin (amino acids 2481-2647) and immunoblotted with anti-GST. GST-filamin bound to Kir2.1 (307-428) whereas GST alone failed to interact. B. HEK293 cells were transiently transfected with cDNAs encoding HA-filamin (carboxyl terminal repeats 19-24) and eGFP-Kir2.1 (full length protein). Soluble cell fractions were incubated with monoclonal anti-GFP antibody (5µg, lane 3) or mouse non-immune control serum (5µg, lane 2) for 12 hours at 4°C and antigen-antibody complexes captured with protein-A sepharose (2 hours, 4°C). Samples were resolved by SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membrane, and immunoblotted with anti-HA. 10% of cell lysate was run in the extract lane (lane 1).

**Figure 4.** Kir2.1 and filamin-A coimmunoprecipitate from arterial smooth muscle lysates and colocalize in regions on the plasma membrane of arterial smooth muscle cells. A. Immunoprecipitation of proteins from rat arterial smooth muscle lysates was performed using rabbit polyclonal antibodies directed against Kir2.1, or rabbit non-immune control serum as described in ‘Experimental Procedures’. Precipitated proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membrane, and immunoblotted with anti-filamin-A. 25% of smooth muscle lysate was run in the extract lane. Full length filamin-A runs as a single
band of molecular weight 280kDa. 

B, C. Confocal images of rat arterial smooth muscle cell stained with mouse anti-filamin-A and rabbit anti-Kir2.1. The top end of the cell is out of the focal plane. The subcellular distribution of filamin-A (B) was visualized by addition of a Texas Red-conjugated anti-mouse secondary antibody, and Kir2.1 by addition of a FITC-conjugated anti-rabbit secondary (C). In control experiments no cross-reactivity was observed between the secondary antibodies and no staining was observed if the secondaries were applied alone. Both proteins were found predominantly in the plasma membrane and colocalized in discrete punctate regions (indicated inset and with arrows). Scale bar indicates 50 µm.

**Figure 5. Kir2.1 is trafficked to the membrane in filamin-deficient cells**  
A, B. Bright field images of human melanoma cell lines lacking expression of filamin-A (A), and an isogenic cell line stably transfected with cDNA encoding full-length filamin-A (B). Consistent with the role of filamin in membrane stabilization and cell motility, filamin-deficient cells remain rounded and unable to extend membrane projections and spread normally. C, D. Confocal images of filamin\(^+\) and filamin\(^-\) stained with anti-filamin-A and visualized with a FITC-conjugated secondary antibody. Filamin-A is concentrated at the cell periphery in rescued filamin\(^+\) cells (D) no filamin-A immunoreactivity is observed in the filamin-deficient cells (C). E, F. confocal images of filamin\(^-\) and filamin\(^+\) cells transiently transfected with eGFP-tagged Kir2.1. The expressed eGFP-Kir2.1 protein was clearly visible at or near the plasma membrane of both cell types.

**Figure 6. In the absence of filamin-A, whole-cell Kir2.1 currents are significantly reduced**  
Membrane currents recorded from single filamin\(^+\) (A) and filamin\(^-\) (B) cells expressing eGFP-Kir2.1 in response to voltage steps from a holding potential of \(-17\) mV to test potentials ranging from +60 to -100 mV in 10 mV increments. Extracellular \([K^+]\) was 70 mM; intracellular \([K^+]\) was 140 mM. C. Mean current-voltage relation for filamin\(^+\) cells expressing Kir2.1 (filled circles), or filamin\(^-\) cells expressing Kir2.1 (open circles), \(n=8\) each. Untransfected filamin\(^+\) cells are shown in filled
triangles; untransfected filamin− cells in open diamonds. D. Histogram summarizing the effect of filamin on whole-cell Kir2.1 currents. In the absence of filamin, whole cell Kir2.1 currents were significantly reduced. At −100mV inward current in filamin+ cells was 1015 ± 145 pA/pF (n=8) whilst it exhibited a significantly reduced value of 598 ± 122 pA/pF in filamin− cells (n=8; p<0.05).

**Figure 7. The reduction in whole-cell Kir2.1 current in the absence of filamin-A is not due to changes in single channel amplitude or open probability** A. Cell-attached recordings at different holding potentials of single Kir2.1 channels in filamin-deficient cells. Extracellular [K+] was 140mM; [K+] in the pipette-filling solution was 200mM. B. Mean single channel current-voltage relation for filamin+ cells (filled symbols), or filamin− cells (open symbols) both expressing Kir2.1 (n=3 each). Unitary conductance at −100mV was 25.5 ± 0.4 pS in filamin+ cells and 23.9 ± 1.1 pS in filamin− cells. C. Histogram summarizing open probabilities for Kir2.1 in the presence or absence of filamin. At −100mV Popen was 0.65 ± 0.07 for filamin+ cells and 0.68 ± 0.05 for filamin− cells (n=3).

**Figure 8. Filamin-A affects the number of Kir2.1 channels that are inserted in the membrane** A. B. Confocal images of a filamin+ cell transiently transfected with cDNA encoding double-tagged eGFP-HA-Kir2.1. Cells are non-permeabilized and have been stained with an antibody directed against the extracellular HA-tag on eGFP-HA-Kir2.1. The distribution of the extracellular HA-tag was visualized by incubation with a Texas Red-conjugated secondary antibody. Excitation at 488nm (A) reveals the subcellular distribution of the intracellular eGFP-tag fused to Kir2.1 and gives an indication of levels of total channel expression throughout the cell. Excitation of the same cell at 568nm (B) allows visualization of the Texas Red-conjugated secondary antibody and gives an indication of the levels of the extracellular HA-tag and thus the number of expressed eGFP-HA-Kir2.1 channels that are inserted in the membrane. Laser levels were the same for both images. C. D. Identical experiment to A, B, but with a filamin− cell transfected with eGFP-HA-Kir2.1. E.
Histogram showing that the fraction of expressed Kir2.1 channels inserted into the membrane is significantly less in filamin-deficient cells (0.49 ± 0.02) compared to filamin-containing cells (0.59 ± 0.02; p<0.05; n=8 each).
Fig. 1

A. FILAMIN-A

Actin-binding domain

N

H1

H2

Dimerization domain

Hinge 2

Repeat 23

Repeat 24

aa 2481

aa 2647

B.

Human 2481: EGYRVTYTPMAPGSYLISIKYGGPYH
Porcine clone: EGYRVTYTPMAPGSYLISIKYGGPYH

2507: IGGSPFKAKVTGPRLVSNHSLLHETSSVFVDSL
IGGSPFKARVTGHRVSNHSLLHETSSVFVDSL

2539: TKATCAPQHAPGPGPADAASKVAKGLGLSK
TKTASAPQHAPGPGAADASKVLAKGLGLSK

2570: AYVGQKSSFTVDCSKAGNNMLLVGVPRT
AYVGQKGSTVDCSKAGNNMLLVGVPRT

2601: PCEEILVKHVGSRLYSVSYLLKDGEYTLVVK
PCEEILVKHVGSRLYSVSYLLKDGEYTLVVK

2632: WGHEHIPGSPYRVVVP
WGDEHIPGSPYRVLVP
### Fig. 2

#### Ai.

| Bait vector | Prey vector |
|-------------|-------------|
| **C-terminal fragments of Kir2.1** | Filamin-A (aa 2481-2647) | Empty |
| aa 307-326 | β-gal | β-gal |
| aa 307-366 | ++ | - |
| aa 367-428 | ++ | ND |
| aa 307-326 | ++ | - |
| aa 325-346 | + | - |
| aa 347-366 | - | - |

#### Bi.

| Bait vector | Prey vector |
|-------------|-------------|
| **C-terminal fragments of Kir2.1** | Filamin-A (aa 2481-2647) | Empty |
| 313-316 (Δ314-315) | β-gal | β-gal |
| aa 307-428 | ++ | - |
| 313-316 (Δ314-315) | + | - |
| aa 307-326 | ++ | - |
Fig. 3

A.  

kDa  |  GST  |  GST-Filamin (aa 2481-2647) 
---  |  ---  |  ---  
20   |  ---  |  ---  

B.  

kDa  |  Extract  |  IP: Non-immune  |  IP: GFP(Kr2.1) 
---  |  ---  |  ---  |  ---  
84   |  ---  |  ---  |  ---  
58   |  ---  |  ---  |  ---  

IB: HA (Filamin-A)
Fig. 4

A. 

| kDa | Extract | IP: Non-immune | IP: Kir2.1 |
|-----|---------|----------------|------------|
| 280 |         |                |            |

IB: Filamin-A

B. Anti-filamin-A

C. Anti-Kir2.1
Fig. 5

Filamin⁻ (M2) cells    Filamin⁺ (M2A7) cells

A. Bright field    B. Bright field

C. Anti-filamin-A    D. Anti-filamin-A

E. eGFP-Kir2.1    F. eGFP-Kir2.1
Fig. 8

Filamin+ eGFP (Total) HA/Texas red (Surface)
A. B.

Filamin- C. D.

E.

Surface fluores./total fluores.

Filamin+ Filamin-
Direct interaction between the actin-binding protein filamin-A and the inwardly rectifying potassium channel, Kir2.1
Laura J. Sampson, Mark L. Leyland and Caroline Dart

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