SUPPLEMENTAL MATERIAL

EXPANDED METHODS

All animal protocols were reviewed and approved by the University of Tennessee Health Science Center Animal Care and Use Committee. Male Sprague-Dawley rats (8 weeks) were euthanized with an intraperitoneal injection of sodium pentobarbital (150 mg/kg). The brain was removed and placed in an ice-cold (4°C) physiological saline solution (PSS) consisting of (in mmol/L): 6 KCl, 112 NaCl, 24 $NaHCO_3$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 1.8 $CaCl_2$, and 10 glucose, which was gassed with 21% O_2-5% CO_2-74% N_2 to pH 7.4. Resistance-size arteries were carefully dissected away from the brain and the connective tissue removed. Where appropriate, cerebral artery myocytes were enzymatically dissociated, as previously described.[1]

PCR

Total RNA was extracted from either whole arteries or ~ 200-300 isolated selected arterial myocytes using TRIzol (Life Technologies) or the Absolutely RNA Nanoprep kit (Stratagene), respectively. First-strand cDNA was generated from 1-5 ng of total RNA using Protoscript M-MULV (New England Biolabs). PCR was performed on first-strand cDNA using primers sequences shown in Supplemental Table 1. PCR was performed as follows: 93°C for 3 minutes then 40 cycles of 93°C for 30 seconds, 50°C for 30 seconds, and 72°C for 1 minute with a final extension step of 72°C for 7 minutes. PCR products were separated on 1.5% agarose gels.

Protein Analysis

Arteries were homogenized in lysis buffer, centrifuged for 8 minutes at 6,000 *g*, and the supernatant stored at -20°C. Laemmli buffer was added and the samples boiled for 3 minutes prior to loading onto the gel. Samples (40 µg) were separated on a SDS-polyacrylamide gel and transferred onto nitrocellulose membranes. Membranes were incubated with either goat polyclonal anti-LRRC26 (1:100, Santa Cruz Biotechnology), rabbit polyclonal anti-BK β1 (1:500, Abcam), mouse monoclonal anti-BKα (1:500, Neuromab, UC Davis), or mouse monoclonal anti-actin (1:10000, Millipore). For antigenic peptide experiments, membranes were incubated with goat polyclonal anti-LRRC26 (1:100) and antigenic peptide (20 µg/ml). Following incubation with their respective secondary antibodies, membranes were developed using a chemiluminescent detection kit (Pierce) and imaged with a Kodak In Vivo F Pro Imaging System (Carestream Molecular Imaging). Band densitometry was analyzed using Quantity One software (Bio-Rad). LRRC26, β1, and BKα band densities were normalized to actin.

Surface Biotinylation

Arteries were incubated on a rocker for 1 hour in a solution consisting of phosphate buffered saline (PBS) with 1 mg/ml each of EZ-Link Sulfo-NHS-LC-LC-Biotin and EZ-Link Maleimide-PEG2-Biotin (Pierce). Free biotin was quenched by washing the arteries in PBS with 100 mmol/L glycine. Biotinylated arteries were homogenized in a lysis buffer (50 mmol/L tris HCl, 150 mmol/L NaCl, 5 mmol/L EDTA, 1% triton X, 0.1% SDS), centrifuged for 8 minutes at 6,000 *g*, and the supernatant collected. Following protein estimation, the sample (1 µl beads / µg protein) was incubated with avidin beads (Monomeric Avidin Agarose, Pierce) for 1 hour on a rocker. The sample was centrifuged at 13,000 *g* and the supernatant (nonbiotin-bound proteins) set aside. The beads were washed 3 times, and then Laemmli buffer (2.5% SDS, 10% glycerol, 0.01% bromphenol blue, and 5% β-mercaptoethanol in 100 mmol/L Tris·HCl; pH 6.8) was added to both the beads and the nonbiotin-bound protein lysate. Samples were then boiled for 3 minutes to elute the biotinylated proteins from the avidin beads and to denature the proteins for analysis. The biotinylated sample was centrifuged at 13,000 *g* and the supernatant removed

and transferred to a new tube. Western blotting was used to determine the relative distribution of surface (biotinylated) and intracellular (nonbiotinylated) fractions.

Immunofluorescence and ImmunoFRET Microscopy

Isolated myocytes were plated on poly-L-lysine-coated coverslips, fixed with 3.7% paraformaldehyde and permeabilized with 0.1% Triton X-100. For colocalization experiments, cells were blocked with PBS containing 5% bovine serum albumin (BSA) and incubated with goat polyclonal anti-LRRC26 (1:100, Santa Cruz Biotechnology) overnight at 4°C. For antigenic peptide experiments, cells were incubated with goat polyclonal anti-LRRC26 (1:100) and antigenic peptide (20 µg/ml). Cells were washed with PBS and incubated with anti-goat Alexa 488 secondary antibody for 1 hour. Myocytes were incubated with Alexa 546-tagged wheat germ agglutinin (1:100, Life Technologies). After washing, coverslips were secured to slides with mounting media (1:1 glycerol:PBS) and images acquired using a laser scanning confocal microscope (LSM Pascal, Carl Zeiss). Alexa 488 and 546 secondary antibodies were excited at 488 and 543 nm with emission detected at 505-530 and ≥ 560 nm, respectively. Weighted colocalization was determined with the LSM FRET Macro tool (v2.5, Carl Zeiss).

For immunoFRET, myocytes were fixed and incubated with one of the following primary antibodies: goat polyclonal anti-LRRC26 (1:100, Santa Cruz Biotechnology), mouse monoclonal anti-BKα (1:100, Neuromab, UC Davis) or rabbit polyclonal TRPM4 (1:100, Thermo Fisher Scientific) overnight at 4°C. Cells were then incubated for 1 hour with the following secondary antibodies: anti-goat Alexa 488 or anti-goat Alexa 546 (LRRC26), anti-mouse Alexa 546 (BKα), or anti-rabbit Alexa 488 (TRPM4). After washing, coverslips were dried and mounted onto glass slides. Fluorescence images were acquired using a laser-scanning confocal microscope. Alexa 488 and 546 secondary antibodies were excited at 488 and 543 nm with emission detected at 505-530 and ≥ 560 nm, respectively. Images were background-subtracted, and N-FRET calculated on a pixel-by-pixel basis for the entire image and in regions of interest (within the boundaries of the cell) using the Xia method [2] and LSM FRET Macro tool (v2.5, Carl Zeiss). The Förster co-efficient for the Alexa Fluor pair is ~ 6.3 nm.

Co-Immunoprecipitation

For each experiment, lysate was harvested from cerebral arteries pooled from 6 rats using ice-cold Radio-Immunoprecipitation (RIPA) buffer, giving ~700 µg total protein. Co-immunoprecipitation was performed using the Catch and Release V2.0 Co-immunoprecipitation kit (Millipore) as per the manufacturer's protocol. Briefly, arterial lysate was incubated with control mouse IgG or BKα mouse monoclonal antibody (1 µg/µL) with 10 µL of antibody affinity ligand and 0.5 mL of the capture resin in the column provided at 4 °C overnight. The column was then centrifuged at 5000 rpm for 30 sec and the flow through discarded. The capture resin was washed twice with the wash buffer provided and bound proteins released with 70 µL of denaturing buffer. The eluate was boiled for 3 min and run on a SDS-PAGE gel. Protein samples were analyzed by Western blotting using mouse monoclonal anti-BKα (NeuroMab) or goat polyclonal anti-LRRC26 (Santa Cruz) and horseradish peroxidase-conjugated secondary antibodies, as previously described.[3]

LRRC26 Knockdown

Cerebral arteries were placed in an electroporation chamber (Bex) containing 200 µl of PBS with either control or LRRC26-specific siRNAs (Life Technologies) for 5 minutes. The arteries were transfected using an electroporator (CUY21Vivo-SQ electroporator, Bex Co. Ltd.) and stored at 37°C in DMEM-F12 50/50 (HEPES-free) culture medium supplemented with 1% penicillin-streptomycin for 48-72 hours prior to use. Arteries permeabilized using electroporation or reverse permeabilization develop similar levels of myogenic tone.[4, 5]

Electrophysiology

Single BK channel or transient BK currents were recorded in isolated myocytes using the inside-out or perforated cell patch-clamp configurations, respectively. An Axopatch 200B amplifier and Clampex 8.2 (Molecular Devices) were used to record currents. For inside-out patch-clamp, the pipette and bath solutions both contained (in mmol/L): 130 KCl, 10 HEPES, 5 EGTA, 1.6 HEDTA, 1 $MgCl_2$, and 10 µmol/L free Ca^{2+} (pH 7.2). Free Ca^{2+} was adjusted to between 1 and 300 µmol/L and free Mg^{2+} concentration maintained at 1 mmol/L with $CaCl_2$ and $MgCl_2$, respectively. Free Ca^{2+} concentration was calculated using WEBMAXC Standard (http://www.stanford.edu/ ~cpatton/webmaxcS.htm) and measured using Ca^{2+}-sensitive (no. 476041; Corning) and reference (no. 476370; Corning) electrodes. To measure channel voltage-sensitivity, 300 ms voltage pulses between -100 and +100 mV were applied in 20 mV increments using a holding potential of -40 mV. BK channel apparent Ca^{2+}-sensitivity was measured at a steady voltage of -40 mV. For perforated-patch experiments, the bath solution contained (in mmol/L): 134 NaCl, 6 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 10 glucose (pH 7.4). The pipette solution contained (in mmol/L): 140 KCl, 1.9 $MgCl_2$, 0.037 $CaCl_2$, 10 HEPES, 0.1 EGTA, and 2 Na_2ATP (pH 7.2). For all patch-clamp experiments, data were digitized at 5 kHz and filtered at 1 kHz. Analyses for voltage- and apparent Ca^{2+}-sensitivity experiments were performed offline using Clampfit 9.2 (MDS Analytical Technologies). BK channel activity (NP_o) was calculated using the following equation: $NP_o = \Sigma\ (t_1 + t_2 \ldots t_i)$, where t_i is the relative open time (time open / total time) for each channel level. Open probability (P_o) was calculated by dividing NP_o by the total number of channels. The total number of channels in a patch was determined by applying a voltage of +100 mV with 1 mmol/L free Ca^{2+} in the bath solution. Voltage- and apparent Ca^{2+}-sensitivity data were fit with the Boltzmann function: $Y = P_o\text{min} + [(P_o\text{max} - P_o\text{min})/(1+\exp[(K_d\text{-}X)/\text{slope}])]$, where Y is the open probability, $P_o$min and $P_o$max represent the minimum and maximum open probability, respectively, K_d is the half-maximal voltage of activation or the dissociation constant for Ca^{2+}, X represents voltage or Ca^{2+}, and slope represents the steepness of the curve. Transient BK currents were analyzed offline using a custom software program provided by Drs. M.T. Nelson and A.D. Bonev (University of Vermont). The criterion for a transient BK current was defined as a current equal to or greater than three times the single channel amplitude.

Confocal Ca^{2+} imaging

Cerebral artery segments were cannulated and incubated in the dark with fluo-4 AM (10 µmol/L) (Molecular Probes, Invitrogen, Eugene, OR) and 0.05% Pluronic F-127 (Molecular Probes, Invitrogen, Eugene, OR) for 1 h followed by a 30-minute wash. Intracellular Ca^{2+} signals in smooth muscle cells were imaged using a Noran Oz laser-scanning confocal microscope with a 60X water-immersion objective (NA 1.2) by illuminating with 488-nm light and collecting emitted light >500 nm. Sequential images (256 × 240 pixels, 56.3 × 52.8 µm) of each region of the arterial wall containing ~8-10 smooth muscle cells were recorded every 16.6 ms (i.e., at 60 Hz) for 10 seconds. In each artery, 2-3 regions that contained different smooth muscle cells were scanned under each condition. Ca^{2+} spark frequency and amplitude was analyzed offline using custom software written with IDL 5.3 kindly provided by Dr. M. T. Nelson (University of Vermont). The full 10 second acquisition period of each image stack was analyzed to measure Ca^{2+} sparks which were detected by dividing fluorescence (F) in an area 1.54 µm × 1.54 µm (7 × 7 pixels, 2.37 μm^2) in each image by a baseline fluorescence (F_0) that was determined by averaging 10 images without Ca^{2+} spark activity. A Ca^{2+} spark was defined as a local rapid increase in F/F_0>1.2. Ca^{2+} spark frequency was obtained from multiple regions of the same artery and then averaged, giving data for each artery. Multiple arterial Ca^{2+} spark frequency values were then averaged, generating mean data with standard errors. In results, n (experimental number) refers to the number of arteries from which mean data were obtained.

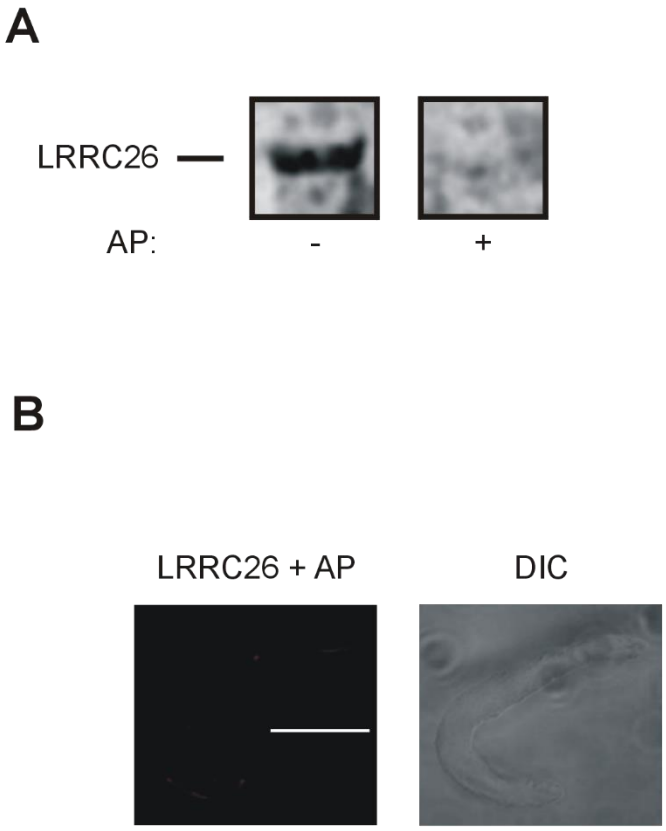
Pressurized Artery Myography

Middle cerebral artery segments were cannulated in a perfusion chamber (Living Systems Instrumentation) maintained at 37°C and continuously perfused with PSS. Intravascular pressure was controlled through a reservoir system and monitored with a pressure transducer. Wall diameter was measured using a charge-coupled device camera and the edge detection function of IonWizard (Ionoptix) by acquiring data at 1 Hz. Myogenic tone (%) was calculated as: $100 \times (1 - D_{active} / D_{passive})$, where D_{active} is active arterial diameter and $D_{passive}$ is the passive arterial diameter determined by the application of Ca^{2+}-free PSS supplemented with 5 mmol/L EGTA. Myogenic tone was assessed over a range of intravascular pressures (20-100 mmHg) with D_{active} and $D_{passive}$ obtained at each pressure to generate a pressure-response curve.

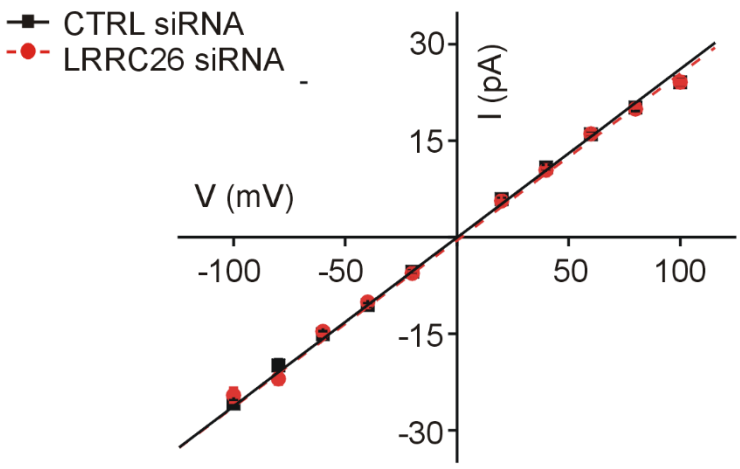
Statistical analysis

Data are expressed as mean ± SE. An independent samples t-test was used to determine if a significant difference existed between group means. The criterion for statistical significance was the same for all tests ($\alpha = 0.05$).

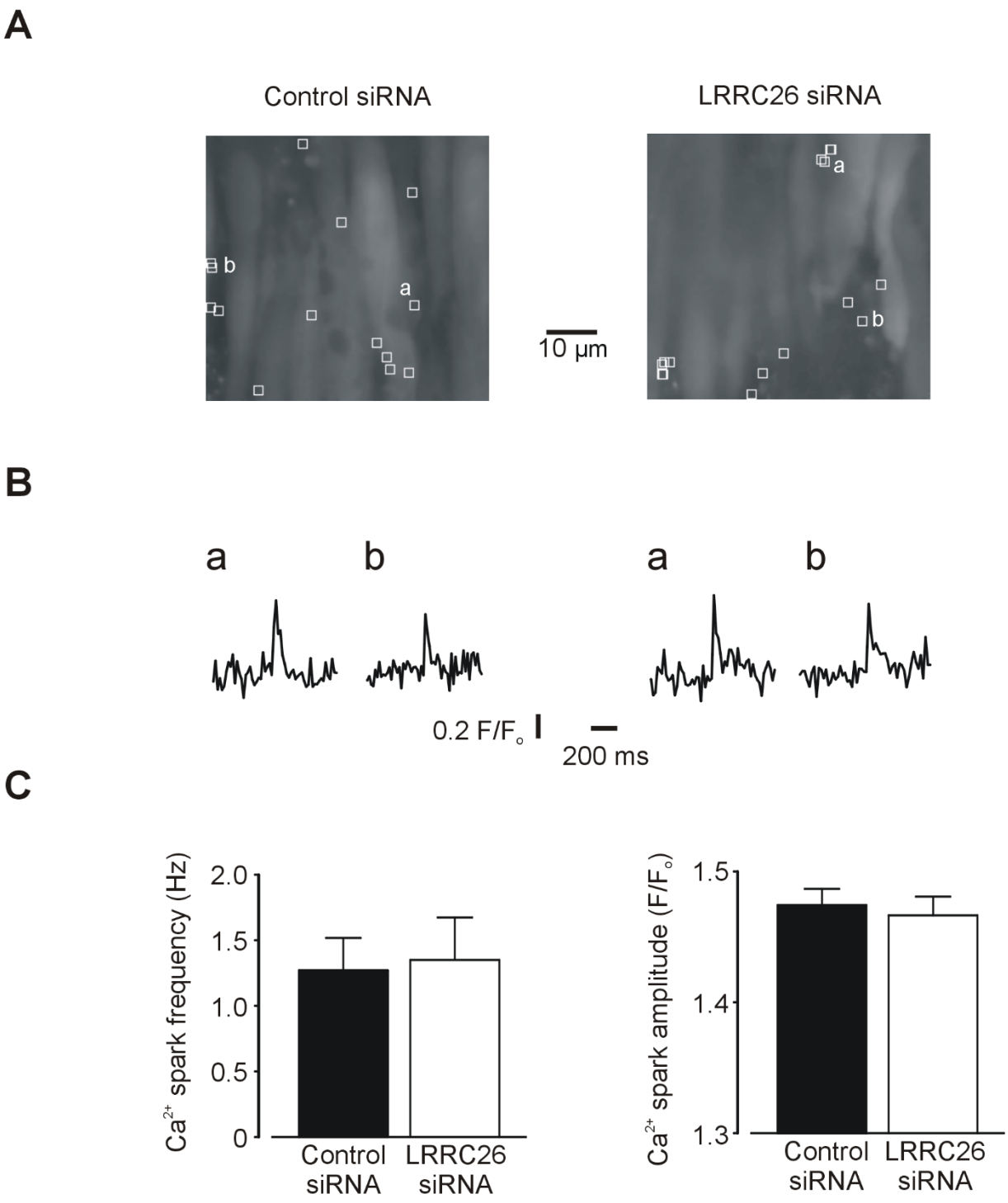
SUPPLEMENTAL FIGURES AND FIGURE LEGENDS



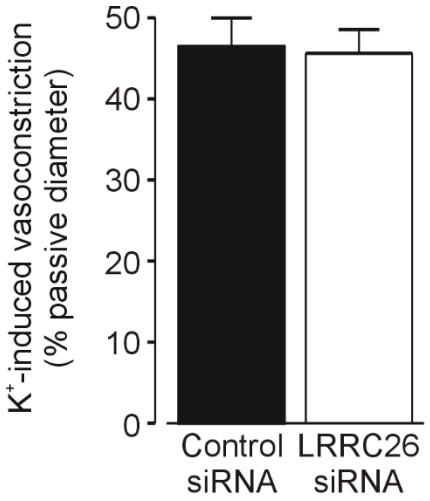
Online Figure I. LRRC26 antigenic peptide specifically blocks Western blot and immunofluorescence detection of LRRC26. A-B: Western blot (A) and confocal images (B) illustrating the effect of the antigenic peptide to block LRRC26 detection using a LRRC26-specific antibody. Scale Bar=10 μm. AP (antigenic peptide), DIC (differential interference contrast)



Online Figure II. LRRC26 knockdown did not affect single BK channel conductance. Mean data for control (262±5 pS, n=10) and LRRC26 (260±5 pS, n=7) siRNA-treated myocytes.



Online Figure III. LRRC26 knockdown did not alter Ca^{2+} sparks in arterial myocytes. A: confocal images demonstrating average fluo-4 AM fluorescence in control siRNA- and LRRC26 siRNA-treated arterial myocytes. B: representative traces of Ca^{2+} sparks that occurred at labeled areas in panel A. C: mean data of Ca^{2+} spark frequency and amplitude (control siRNA: n=6, LRRC26 siRNA: n=6).



Online Figure IV. LRRC26 knockdown did not affect 60 mmol/L K^+-induced constriction at 60 mmHg (control siRNA: n=6, LRRC26 siRNA: n=7).

SUPPLEMENTAL TABLES

Online Table I

Primer sequences used for RT-PCR

Transcripts	Primers
LRRC26	
Forward	5'-CTGCTATACCTAGTCCTGCG-3’
Reverse	5'-CTTACGCAGCCAGGTGCAAA-3’
Myosin heavy chain 11	
Forward	5'-AGGAACTGGAGGCGCTCAAGACA-3’
Reverse	5'-TTGTCACTTCCTGTTCCCTC-3’
Platelet-endothelial cell adhesion molecule-1	
Forward	5’-TCTTTCAGGATTCAGCTGAG-3'
Reverse	5'-GCCGACTTTCCATATGGATG-3’
Aquaporin-4	
Forward	5'-GATCCTCTACCTGGTCACA-3’
Reverse	5'-CACAGCTGGCAAAAATGGTA-3’

SUPPLEMENTAL REFERENCES

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4. Bannister JP, Adebiyi A, Zhao G, Narayanan D, Thomas CM, Feng JY, Jaggar JH. Smooth muscle cell $\alpha_2\delta$-1 subunits are essential for vasoregulation by $Ca_V1.2$ channels. *Circ Res.* 2009;105:948-55

5. Bannister JP, Thomas-Gatewood CM, Neeb ZP, Adebiyi A, Cheng X, Jaggar JH. $Ca_V1.2$ channel N-terminal splice variants modulate functional surface expression in resistance size artery smooth muscle cells. *J Biol Chem.* 2011;286:15058-66