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Enrichment and Separation
At Mitochondrial, Submitochondrial, Protein and Peptide Levels

Isolation of Bovine Heart Mitochondria

High-purity bovine mitochondria, suitable for proteomic analysis, were prepared as reported in Foster *et al.*[1]. Briefly crude mitochondria were obtained essentially as originally described by Smith[2], specifically "Procedure 2" therein. Briefly, bovine hearts were obtained from a local abattoir and packed on ice. In the lab the ventricles were excised, defatted and rinsed. Ventricles (approx. 800g) were rinsed, cubed, minced and allowed to stir for 15 min in isolation buffer (10 mM HEPES, 200 mM mannitol, 50 mM sucrose, 1 mM EGTA; pH 7.2) containing trypsin*, en lieu* of Nagarse used by Smith[2]. The pH of the buffer was periodically readjusted to > 7 with NaOH. Trypsin was removed by squeezing the mince through 2 layers of cheesecloth. The mince was diluted into isolation medium containing pepstatin, leupeptin and PMSF, and homogenized briefly in a heavy-duty blender. The pH of the solution was readjusted to >7 prior to centrifugation of the homogenate at 1000 x g for 10 minutes in a Sorvall GS3 rotor. The supernatant was carefully decanted subsequently centrifuged at 8000 x g for 10 minutes. The supernatant was discarded and the pellet, containing crude mitochondria was kept for density-gradient centrifugation.

Isolation of highly enriched density-purified mitochondria was inspired by Taylor *et al.*[3], who used a method based on protocol of Storrie and Madden[4]. Our variation on the method entailed two rounds of density centrifugation. Briefly, crude mitochondria were resuspended in sucrose buffer (10 mM Tris-HCl pH 7.5, 250 mM sucrose), and layered onto a discontinuous gradient consisting of (from top to bottom) 6% Percoll, 17 % and 35% Histodenz (Sigma), each made up in sucrose buffer. The sample was centrifuged at 50,000 x g for 30 minutes. The layer at the 17%/35% interface was collected and mixed with 12 mL of 35% Histodenz. The mitochondria were placed at the bottom of centrifuge tubes. The 6% Percoll and 17.5% layers were successively layered on top of the mitochondrial layer. Floating density centrifugation was conducted at 50,000 x g for 30 minutes. Again the layer at the 17%/35% interface was collected. Mitochondria were diluted with isolation medium and centrifuged at 10000 x g to remove the Histodenz. This was repeated to remove trace Histodenz. Isolation of the submitochondrial compartments in strategy 1 was conducted on fresh mitochondria. Mitochondrial pellets were frozen at -80°C until used.

Assessment of Mitochondrial Purity

The purity of the bovine mitochondrial preparation has been assessed previously by Foster *et al.*[1], particularly in Figure 3A therein. Briefly, equal amounts of protein from each stage of mitochondrial purification (i.e. homogenate, post-nuclear supernatant, crude mitochondria and gradient pure mitochondria) were probed by immunoblotting with antibodies to Na^+/K^+ ATPase (Santa Cruz), SERCA2a (Thermofisher), cardiac Troponin I (Spectral Diagnostics) and VDAC (Molecular Probes). High-purity mitochondria showed no signal for Na/K ATPase or SERCA2a after 1 min exposure of the blots developed using Supersignal West-Pico Reagent from Thermofisher. Longer exposures (5-10 min) revealed faint signals. The level of contamination in our bovine mitochondrial preparations is often lower than we find in preparations from other species, such

as the rat, where residual contamination can be detected even after further mitochondrial subfractionation. The higher purity in the bovine preparations is likely to stem from the incubation of the heart mince with trypsin for 15 minutes, which is harsher than our other protocols and may lead to degradation of contaminants and weakening of mitochondrial contacts with typical contaminating membranes such as the sarcoplasmic reticulum.

Preparation of Enriched Submitochondrial Compartments

Notwithstanding that our study sought to identify novel channels of the mitochondrial inner membranes, multiple submitochondrial compartments were analyzed. Low abundance proteins tend to be systematically underrepresented in proteomic datasets, since high-abundance peptides dominate the duty cycle of mass spectrometers in data-dependent data acquisition mode[5, 6]. Since mitochondrial subfractionation yields enriched, though not absolutely pure subcompartments, analyzing all mitochondrial subcompartments served to maximize chances of rare IMM protein discovery.

Outer and Inner Membrane-enriched fractions

Mitochondrial subfractions were prepared essentially as described by Maisterrena *et al.* for pig heart mitochondria[7] with variations. Briefly, gradient pure mitochondria (approx 500 mg) were subjected to hypotonic swelling in approximately 1.5 L of 20 mM KH_2PO_4 for 40min, at which point they were centrifuged at 8000 x g to pellet swollen mitoplasts. The supernatant was decanted and kept for isolation of proteins from the intermembrane space (next paragraph). The swollen mitoplasts were resuspended in 250 mM sucrose homogenized with a dounce homogenizer to dislodge the outer mitochondrial membrane. The solution was layered onto a discontinuous sucrose gradient consisting of layers containing of 37.7% 51.7% and 61.5% (w/v). Sucrose gradients were centrifuged at 77,000 x g for 90 min in a SW 32 rotor. Light membranes containing outer membrane markers, were collected from the top of the 37.7% layer and was called the outer mitochondrial membrane (OMM)-enriched fraction. Mitoplasts were collected from the 37.7%/51.7% interface, diluted in 250 mM sucrose, and homogenized in a dounce homogenizer. The suspension was centrifuged at 100,000 x g for 30 min to pellet membranes. The supernatant (containing matrix proteins) was decanted. The pellet was resuspended in 10mM Tris pH 8.0, 250 mM Tris-HCl, sonicated at 20 W for 1 min on ice, and then layered onto a second sucrose density gradient. Again the sample at the 37.7%/51.7% interface was collected, and is hereafter called the IMM-enriched fraction. The OMM and IMM fractions were stored as frozen pellets at -80°C until required

Matrix- and Intermembrane Space-Enriched Fractions.

The supernatant arising from the hypotonic swelling and centrifugation of the mitoplast described in the previous paragraph was collected. Ammonium sulfate was added gradually until the solution was 80% saturated. The solution was stirred for 30 min at 4°C. The solution was centrifuged at 10,000 x g for 20 min a Sorvall GS3. Though there was no compact pellet, a flocculent layer formed on the surface of the supernatant and a thin whitish translucent film adhered to one side of the centrifuge bottle. The bulk of the supernatant was discarded. The flocculent layer and film were recovered using residual 80% ammonium sulfate solution and

concentrated by centrifuging in a 50 mL conical tube at 15,000 x g in a Fiberlite PTi rotor for 15 min.

The matrix-enriched fraction used for proteomic analysis was obtained simply by subjecting mitochondria (approx. 50mg) to hypo-osmotic swelling in a small volume (approx. 4mL) and sonicating the sample at 18-20 W on ice, four times for one minute each. Membranes were removed by centrifugation at 100,000 x g for 30 min. This fraction, though matrix-enriched, would also be expected to contain soluble proteins of the IMS and soluble, weakly-bound membrane associated proteins.

Separation of Macromolecular complexes by Sucrose Density Gradient Centrifugation

Proteins of the IMM-, OMM- and Matrix-enriched fractions were fractionated essentially as described previously[1, 8]. Membranes were solubilized at a concentration of 5mg/mL in 1% lauryl maltoside for 30 min. Insoluble aggregates (minimal) were removed by centrifugation at 100,000 x g for 30min. Soluble protein was then separated on a discontinuous sucrose gradient (10-35% w/v) by centrifugation in a SW 41 rotor at 32,000rpm (approx. 128,000 x g at r_{ave}) for 18 hours at 4°C. Each layer (1.5mL) was collected by making a hole with a 25-gauge needle in the bottom of the polyallomer tube and collecting drop-wise into 2mL microcentrifuge tubes. Each sucrose fraction was dialyzed overnight against 50 mM ammonium bicarbonate, 0.1% lauryl maltoside (3 X 1L) in preparation tryptic digestion.

Separation of Peptides Derived from Sucrose Fractions

By Strong Cation Exchange Chromatography

Dialyzed gradient fractions were digested with trypsin (protein/trypsin approx. 100/1) at 37°C overnight. Ammonium bicarbonate was evaporated in a vacuum centrifuge. Peptides were re-dissolved in de-ionized water and residual ammonium bicarbonate was removed again by evaporation. The combined peptide mixture was fractionated by strong cation exchange (SCX) chromatography on an 1100 HPLC system (Agilent) using a PolySulfoethyl A column (2.1x100mm, 5µm, 300Å, PolyLC, Columbia, U.S.A.). Sample was dissolved in 4mL of SCX loading buffer (25% v/v acetonitrile, 10mM KH_2PO_4, pH 2.8), pH was adjusted to 2.8 by adding 1 N phosphoric acid. The whole sample was loaded and washed isocratically for 30 min at 250µL/min. Peptides were eluted with a gradient of 0-350mM KCl (25% v/v acetonitrile, 10mM KH_2PO_4, pH 2.8) over 40min at a flow rate of 250µL/min. The absorbance at 214 nm was monitored and 8-10 SCX fractions were collected along the gradient.

By Reversed-Phase Liquid Chromatography

The HPLC was driven by an Eksigent nano-2DLC pump. The trap contained C18 (75µm) fused silica fritted with Kasil 1624 and hand packed to 3cm with YMC 5-10 µM irregular C18. The column was C18 75µm column hand packed with YMC ODS-AQ 3.5µm particle size, 120A pore size. The solvent system consisted of 0.1% Formic Acid (Buffer A) and 0.1% Formic Acid /90% Acetonitrile (Buffer B). Samples were injected at buffer composition of 1%B from the autosampler into the nanoflowpath at 8.5%B (flow rate: 300 nL/min). Peptides were eluted by ramping up to 30% B in 15 minutes, then to 60% B by 18 minutes. Buffer B was then quickly ramped to 100% B by 22 minutes and held for 2 minutes before returning to 100 % A ending at 30 minutes.

Proteomic Analysis

Mass Spectrometry

Eluted peptides were injected by electrospray (2.4kV) into an LTQ ion trap mass spectrometer (ThermoFinnigan). Precursor ion scans ranged from 350-1800 m/z. The top eight ions picked for MS/MS scans. Collision Energy was set to 30, (Q=0.250; Activation Time=30) Dynamic Exclusion settings: Repeat count: 2; Exclusion duration: 120 seconds

Database Searching

Mascot and X!Tandem searches conducted against a custom FASTA database of all bovine sequences in NCBI as of 06/11/2008, assuming trypsin as the digestion enzyme. The parent ion mass tolerance was set to 1.5 Da, and fragment mass tolerance was set to 0.8 Da. Oxidation of methionine was specified in Mascot and X!Tandem as a variable modification.

Criteria for Protein Identification

Scaffold (version Scaffold-01_07_00, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at >80.0% probability as specified by the Peptide Prophet algorithm [9]. Protein probabilities were assigned by the Protein Prophet algorithm[10]. Protein identifications were considered for the final list if they scored at >50% protein probability stemming from identification of a high quality peptide. False positive protein identification was minimized by manually evaluating all proteins identified by a single peptide hit.

Manual Evaluation of Single Peptide-Spectrum Matches

Manual curation was carried out essentially as describe in Cammarato *et al.*[11] in the method supplement. In particular, proteins identified on the basis of a single peptide were scrutinized to ensure: 1) that the MS^2 spectra yielded sequential b- and y-ions, 2) that the b- and y-series overlapped, and 3) that spectra were consistent with currently accepted peptide fragmentation biases[12].

Bioinformatic Analysis

Prediction of Mitochondrial Localization

Fully curated protein sequences of K^+-channel isoforms (147 total) were retrieved from UniProtKB. Rat sequences were used, though human sequences were substituted if a rat entry was unavailable in UniProtKB at the time of analysis. The mitochondrial localization probability for the K^+-channel sequences assessed using MitoPred[13] http://bioapps.rit.albany.edu/MITOPRED/.

Consultation of the Maestro database[14], which provides a genome-wide ranking of human and mouse genes according to the likelihood of mitochondrial localization reveals that that the KCNJ1 gene was the highest ranking among inward rectifying potassium channels

Molecular/Cell Biology

Isolation of Neonatal Rat Ventricular Myocytes

NRVMs were enzymatically dissociated from the ventricles of 2-day-old rats with trypsin. Freshly isolated NRVMs were resuspended in M-199 culture medium supplemented with 10% FBS, glucose, and vitamin B12. Two preplating steps were performed to enrich cardiac myocyte content in the culture. The final cell suspension was collected and plated at the desired density for the downstream experiment.

Detection of ROMK Transcripts by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from freshly isolated adult rat heart using the RNeasy Fibrous Tissue Mini Kit (Qiagen) according to manufacturer's instructions. Rat kidney, brain, and liver total RNA were extracted with RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. Isolated Neonatal rat ventricular myocytes were plated into a T75 flask, grown for 2 days into a beating monolayer, then treated with RNAprotect Cell Reagent (Qiagen) to collect and preserve the total RNA content prior to extraction using the RNeasy Mini Kit.

RT-PCR was performed with One step RT-PCR kit (Qiagen) according to manufacturer's instruction, with the specific primers listed below for detecting different ROMK Isoforms. 2µL of 1:100 diluted RT-PCR products were subsequently subjected to another "nested" step with isoform specific primers below to detect the presence of low copy ROMK transcripts using Taq Polymerase (Invitrogen). Resulting PCR product from the nested step were then ran on a 1% agarose gel for visualization (product size shown in the chart below).

ROMK Isoform	RT / Nested	Forward Primer	Reverse Primer	Product Size (bp)
ROMK1	RT	GAACGGAGTGTGTTCAGA	GTCTAGAGATCTTGGCTA	-
	Nested	GAGTGTGTTCAGAGTGCT	GTACCTCCATTTCAGGTCCA	220
ROMK2	RT	GTCTTCACATTTACCCCAGC	GTCTAGAGATCTTGGCTA	-
	Nested	TTTACCCCAGCAATCCATGA	GTACCTCCATTTCAGGTCCA	222
ROMK3	RT	GGCAGTACAGACAATGGTGT	GTACCTCCATTTCAGGTCCA	-
	Nested	GGCAGTACAGACAATGGTGT	GTCTAGAGATCTTGGCTA	271
ROMK6	RT	GAACGGAGTGTGTTCAGA	GTCTAGAGATCTTGGCTA	-
	Nested	AGGGAAGTCATCGTGCATCA	GTACCTCCATTTCAGGTCCA	317

ROMK Fusion Constructs

A plasmid containing the gene encoding for KCNJ1 was obtained from the I.M.A.G.E. consortium via Open Biosystems (Clone ID 30915211). A stretch of nucleotide encoding the first 24 amino acids of the ROMK2 isoform was amplified by PCR and subcloned into the pDONR/zeo vector, then subsequently cloned into the N-terminus of an eGFP fusion expression vector using the Gateway system (Invitrogen). The eGFP fusion expression vector was based on the pEGFP-N1 vector (Clontech), converted into a Gateway destination vector using the Gateway Vector Conversion System kit (Invitrogen).

ROMK2 was amplified by PCR from the original KCNJ1 plasmid by PCR and subsequently cloned into the pENTR/D-TOPO vector (Invitrogen). EF1-α promoter was amplified by PCR from the pEF1alpha-IRES Vector (Clontech), and subcloned into the pENTR5'-TOPO according to manufacturer's instructions (Invitrogen). Gateway LR-plus subcloning was performed to combine

the EF1-α promoter upstream of ROMK2 and into the pLenti6/R4R2/V5-DEST destination vector (Invitrogen). Gateway LR subcloning was also performed between the ROMK2-pENTR/D-TOPO and the converted pEGFP-N1/DEST vector to obtain a CMV-ROMK2-eGFP construct.

Mitochondrial Enrichment from CHO-K1 Cells

CHO-K1 cells were transiently transfected with ROMK2/pEGFP-N1 DEST to overexpress ROMK2 tagged with a eGFP on its C-terminus. After 24 hours, mitochondria were isolated from the cells using a protocol reported by Gottleib and Adachi[15]. Briefly, $2x10^8$ cells were centrifuged and re-suspended in 3 ml of isolation buffer (225mM mannitol, 75mM sucrose, 1mM EGTA @4^oC), then transferred to pre-cooled cavitation chamber of a nitrogen bomb. The cell suspension was subjected to a pressure of 450psi for 15 minutes with stirring, after which it was instantaneously released into atmospheric pressure. Cell homogenate was centrifuged at 1300g to remove nuclear material. The supernatant was then centrifuged at 7500g to collect for crude mitochondria pellet. This pellet was washed twice at 7500g with the isolation buffer, and finally re-suspended in suspension buffer (isolation buffer without EGTA). Samples were subjected to immunoblot analysis as described shortly hereafter.

shRNA Constructs and Knockdown Efficiency Validation

Six validated shRNAs against the core section of KCNJ1 from The RNAi Consortium (TRC) shRNA libraries were obtained from the ChemCORE facility at The Johns Hopkins University (TRCN0000005593, TRCN0000005594, TRCN0000005595, TRCN0000005596, TRCN0000005597, TRCN0000068526). A scrambled shRNA control vector was also obtained from the ChemCORE facility at The Johns Hopkins University. The human U6 promotor, along with the shRNA sequence, was subcloned into the pDONR/zeo vector using Gateway BP subcloning method. The CMV promoter and mCherry fluorophore sequences from the pmCherry-N1 Vector (Clontech) were subcloned into the pENTR5'-TOPO according to manufacturer's instructions (Invitrogen). The shRNA-pDONR/zeo and CMV-mCherry-pENTR5'-TOPO were combined into the pLenti6/R4R2/V5-DEST destination vector (Invitrogen). The resulting constructs were then screened for ROMK knockdown efficiency through transient co-transfection of the shRNA construct and CMV-ROMK2-eGFP in H9C2 cells. Transient transfection was performed using lipofectamine LTX with Plus Reagent (Invitrogen) according to the optimized protocol reported by Vitiello et al[16]. Out of the set, TRCN0000005595 and TRCN0000005596 were individually determined to be most effective in knocking down 50% of GFP signal quantified by flow cytometry in the BD FACScan system (BD Biosciences). When combined, these two shRNAs can knock-down 70% of eGFP signal in flow cytometry. These two shRNA-pLenti6/R4R2/V5-DEST vectors were combined for use in all subsequent experiments.

Lentivirus Production

The following protocol was used to produce lentivirus from ROMK2- or shRNA- pLenti6/R4R2/V5-DEST vectors. 293FT cells were pre-plated onto eight gelatin coated 150mm-plates the day before transfection to ensure 80% confluence in 24 hours. On the day of transfection, for each plate, 30µg of ViraPower Packaging Mix (containing pLP1, pLP2, and pLP/VSVG) and 10µg of pLenti vector were mixed with 82µl Lipofectamine 2000 in Opti-MEM (Invitrogen) at room temperature for 25 minutes, then added dropwise to 293FT cells in Opti-MEM supplemented

with 10% fetal bovine serum and 25µM chloroquine. 9 hours later, this media was replaced by Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10µM sodium butyrate to facilitate virus production[17]. Lentivirus was collected from the media at 24 and 48 hours after transfection, sterilized by filtration through 0.45µm Durapore Membrane Filter (Millipore) and subsequently concentrated by ultrafiltration using Centricon Plus70 (100K) (Millipore) at 720rcf. Lentivirus concentrate was aliquoted and stored at -80^{o}C. Lentivirus was titered with 293FT cells by assessing fluorescence expression level in limiting dilution with 8mg/ml Polybrene (Sigma). In the case where no fluorescent tags are available, concentration level was assessed by 5mg/ml Blasticidin (Invitrogen) selection of transduced 293FT colonies.

Establishment of Stable Cell Lines

H9C2 cells in low passage were transduced with (1) ROMK2-V5 lentivirus (overexpressors); (2) scrambled controlled shRNA lentivirus (negative control); or (3) combined ROMK shRNA lentiviruses (ROMK knockdown), all at MOI of 50. Day 3 after transduction, the cells were subjected to 5mg/ml Blasticidin selection in DMEM supplemented with 10% FBS and Anti-Anti. After 14 days of selection, ROMK levels of these cell lines were assessed by quantitative RT-PCR (detection primer sets are shown in the chart below). Expression levels were quantified using the standard curve absolute quantitation method, normalized to 18s rRNA. For the stable overexpressors, the human primer sets were used to detect ROMK levels, whereas for detecting native expression of ROMK in H9C2 cells, the rat primer sets were used. Stable ROMK2 overexpressors were determined to be expressing at 1.3ng ROMK/ng 18s rRNA, compared to 0.003ng ROMK/ng 18s rRNA in untransduced cells.

Species	**Forward**	**Reverse**	**Probe (6FAM-TAMRA)**
Human	TGGACATCTGGACAACGGTA	CGGGAGGTCTTTGTGAATGT	TCACAGCCTTCTTGGGGAGTTGGT
Rat	GCTTGGAGCCAGGTTGAT	TTTCTGTCAGTGCCCTGAT	ATATCAATGACCACCCTCATTCT

Detection of ROMK, ROMK-GFP, ROMK-V5 by Immunoblot Analysis

Immunoblot analysis was conducted using the same methods for each experiment and differed only with respect to the identity and concentration of primary antibodies, as well as the type of chemiluminescent reagent used. Briefly, protein samples were concentrated by quantitative methanol chloroform precipitation[18] and resolubilized in 2% (w/v) sodium dodecyl sulfate for protein determination using the BioRad DC assay, which is based on the method of Lowry *et al.*[19]. Samples were mixed with 2x LDS sample buffer under reducing conditions, heated and 20 µg were loaded onto a 4-12% NuPAGE Bis-Tris precast gel. Electrophoresis was conducted with MOPS running buffer at 150 V for 50 min. Protein was transferred to nitrocellulose membrane at 20 V, for 9 min, using an iBlot apparatus (Invitrogen). Blots were incubated with 5% milk powder (BioRad) in Tris-buffered saline with 0.1% (v/v) Tween 20 (TBS-T) for 1 hour. They were subsequently incubated with primary antibody diluted in according to the manufacturer, in 2.5% milk in TBS-T overnight at 4°C. Rabbit secondary antibody conjugated to horseradish peroxidase (HRP; Amersham) was used at a dilution of 1/20000. HRP activity was developed by chemiluminescence using Supersignal West-Femto Reagent (Thermo Fisher) and visualized by exposure to Amersham Hyperfilm ECL (GE Biosciences) for 20 min. MagicMark protein ladders (Invitrogen) provided molecular weight standards.

Specific Experiments

Detection of Overexpressed ROMK-GFP Enriched from CHO Cells (Figure 2B)

20 μg from each step of purification (Lysate, Post nuclear supernatant, Crude Mitochondria, Washed Mitochondria) subjected to immunoblot analysis as describe above. Blots were probed with primary antibodies against eGFP, Porin, GFP78 BiP, or Connexin 37 (Abcam) diluted according to manufacturers' instructions. SuperSignal West Pico Substrate Kit (Thermo Scientific) was used to develop the chemiluminescence.

Detection of Overexpressed ROMK and ROMK-V5 in H9C2 Cells (Supplemental Figure)

H9C2 cells (untransduced, transduced with ROMK-V5, ROMK-V5 with negative control shRNA, ROMK-V5 with ROMK-specific shRNA) were lysed with RIPA buffer (Sigma-Aldrich), concentrated and 20 μg of protein from each samples was analyzed as describe above. Primary antibodies to ROMK (Sigma) and V5-tag (Sigma) were diluted according the manufacturer. SuperSignal West Femto Substrate Kit (Thermo Scientific) was used to develop the chemiluminescence.

Detection of Endogenous ROMK and its Ablation in H9C2 Cells

H9C2 cells, transduced with either a scrambled shRNA or a ROMK-specific knockdown shRNA construct, were lysed, concentrated and 20 μg were analyzed as described above. Primary antibody to ROMK (Sigma) was diluted according to the manufacturer. SuperSignal West Femto Substrate Kit (Thermo Scientific) was used to develop the chemiluminescence.

Colocalization/Imaging Studies

Fixed H9C2 Cell Imaging

H9C2 cells in low passage pre-plated on laminin coated #1.5 glass coverslips (Fisherbrand) were transiently transfected with the ROMK2-pLenti6/R4R2/V5-DEST construct with Lipofectamine LTX and Plus Reagent (Invitrogen). 16 hours after transfection, cells were fixed with 4% paraformaldehyde (Sigma), permeabilized with 0.25% Triton X-100 (Sigma), and blocked with 10% goat serum before primary antibodies were applied overnight at 4^{o}C. Polyclonal anti-V5 antibody (Sigma) was used to probe for ROMK2, and ATP synthase subunit beta monoclonal antibody (Invitrogen) was used to probe for mitochondria. Secondary antibodies were applied separately after washing and blocking with 10% goat serum in-between. Cells were incubated with goat anti-mouse IgG Pacific Orange 458 (Invitrogen; ATP synthase staining) overnight at 4^{o}C, and goat anti-rabbit IgG Alexa Fluor 488 (Invitrogen; V5 staining) for 1 hour at room temperature. Cells were rinsed and mounted in Prolong Gold with antifade and cured for 48 hours prior to imaging using two-color stimulated emission depletion (STED) microscopy (Leica TCS STED CW; 531nm depletion beam wavelength).

Live Neonatal Rat Ventriclar Myocytes Imaging

Freshly isolated neonatal rat ventricular myocytes (NRVMs) were transfected with the expression construct via electroporation with the AMAXA system (Lonza) by electroporation according to the manufacturer's instructions. Transfected cells were allowed to recover for 3 days prior to live cell imaging using 2-photon laser scanning fluorescence microscopy (Bio-Rad MP1024; excitation 900nm, emission at 525nm for eGFP, 605nm for tetramethylrhodamine methyl ester). Prior to imaging, cells were incubated with 50nM tetramethylrhodamine methyl ester (TMRM) at 37^{o}C to label polarized mitochondria.

Mitochondrial Swelling Assay for MitoK_{ATP} Activity.

Volume changes secondary to respiration-driven mitochondrial uptake of K+ salts and water were followed by light scattering (1/A) [20]. Mitochondria were isolated from hearts of male Sprague-Dawley rats and assayed at 0.1 mg/ml in medium containing K^+ salts of Cl^- (120 mM), HEPES (10 mM), EGTA (0.1 mM), succinate (10 mM), MgCl2 (0.5 mM), ATP (200 μM), rotenone (2.5 μM) oligomycin (1 μg/ml), and phosphate (10 mM), pH 7.2. MitoKATP was opened with diazoxide (30 μM) in the presence of TPNQ at concentrations given in Fig. 3B, and steady state values of 1/A were measured at 120 s. 100% "MitoKATP Activity" is the diazoxide-activated activity without TPNQ and 0% is the value without diazoxide or TPNQ.

Thallium Uptake Assay

Stable H9C2 cells were plated on laminin-coated circular glass coverslips such that they will be at 80% confluency on the day of the assay. Prior to the assay, cells were loaded with a fluorescent indicator, either benzothiazole coumarin acetyoxymethyl ester (BTC-AM; 20μM) or Fluozin-2 AM (5μM) (Invitrogen). BTC was shown to be effective in measuring mitochondrial K_{ATP} activity in isolated mitochondria[21], while Fluozin-2 has previously been applied in high throughput screening of small molecule inhibitors for plasma membrane localized ROMK channels[22]; both dyes are sensitive to thallium, which is used to substitute for potassium. Dyes were loaded in their acetoxymethyl ester (AM) form mixed with 0.2% (w/v) Pluronic F-127 prior to addition to incubating media. Cells were incubated with BTC-AM for 10 minutes, or in the case of Fluozin-2 AM, incubated for 20 minutes. All incubation steps were carried out at 37°C, followed by two PBS wash steps to remove membrane bound dyes. Just prior to the Tl^+ assay, cells were treated with 150μM digitonin for 30s to permeabilize the plasma membrane, as evidenced by rapid loss of the cytosolic component of dye, leaving only the mitochondrial compartment loaded with the indicator. This protocol has previously been used by Ljubkovic *et al.*[23] to preferentially localize potassium sensitive indicators to the mitochondria.

Images were collected by exciting at 480nm and recording the fluorescence emission at 525nm using a cooled CCD camera (Cascade II 512, Photometrics). Bath Tl+ was rapidly switched from 0 to 2mM Tl^+ at 100s by means of a custom-built flow switching device positioned directly over the field of interest to minimize bath exchange times. The chloride-free, 0 Tl^+ assay bath solution contained (in mM): 195 mannitol, 10 HEPES, 2 $MgSO_4$, 2 Na_2HPO_4, 2 succinate, 0.6 CaOH, 1 EGTA and 1ug/ml oligomycin (pH 7.2) and the 2mM Tl^+ solution was identical except 2mM $TlSO_4$ was added. Images were collected every second, and the fluorescence intensities of the dyes within the mitochondria were determined offline by ImageJ. F/F0 was calculated for each time point (F0 was the basal fluorescence in the 0 Tl^+ condition) and the initial rates were determined by averaging the first derivatives of F/F0 ratios at 106-125s for BTC-AM loaded cells (Figs. 3C and 3D), and at 102-119s for Fluozin-2 AM loaded cells (Figs. 3E, 3F, and S1).

To study thallium uptake in NRVMs, freshly isolated NRVMs were plated on fibronectin-coated glass coverslips which allowed for the formation of monolayers prior to the assay. Cells were pre-loaded with Fluozin-2 AM. Buffer solutions were the same except that 100μM cyclopiazonic acid was added to specifically inhibit the SERCA pumps. Data collection was performed as described above. Statistical analysis was done with Microcal Origin by 2-way ANOVA with post hoc Tukey test and $p<0.05$ was the criterion of statistical significance.

Cell Death Assay

Stable H9C2 cell lines with various expression levels of ROMK2 were plated on 6 well plates at equal densities. The cells were subjected to 0, 50μM, or 100μM tert-butyl hydroperoxide (tBHP) treatment for 22 hours to induce oxidative stress. After incubation with tBHP, cells were labeled with Vybrant apoptosis assay kit #2 (Invitrogen) for annexin-V-FITC and propidium iodide (PI) according to the manufacturer's instructions for flow cytometry. Cells were immediately subjected to flow cytometry analysis in the BD FACScan system (BD Biosciences), using FL-1 channel for detecting annexin-V and FL-3 channel for PI. Unlabeled cells were considered live cells, cells positively stained only with annexin-V were considered apoptotic cells, and cells that were labeled with PI were considered to be dead cells. All experiments were performed with the appropriate controls. Statistical analysis was done with Microcal Origin by 2-way ANOVA with post hoc Tukey test and $p<0.05$ was the criterion of statistical significance.

All experimental protocols complied with the Guiding Principles in the Use and Care of Animals published by the National Institutes of Health and were approved by IACUC at The Johns Hopkins University or at Portland State University (data in Figures 3A and 3B).

Author Contributions

Bovine heart proteomic discovery work and bioinformatic analyses were performed by DBF. Molecular cloning, lentivirus production, stable cell line establishment, colocalization studies, thallium assays, and cell death assays were designed, performed, and analyzed by ASH. Mitochondrial light scattering assays were performed and analyzed by AG in the laboratory of KG. All authors took part in writing and editing the manuscript.

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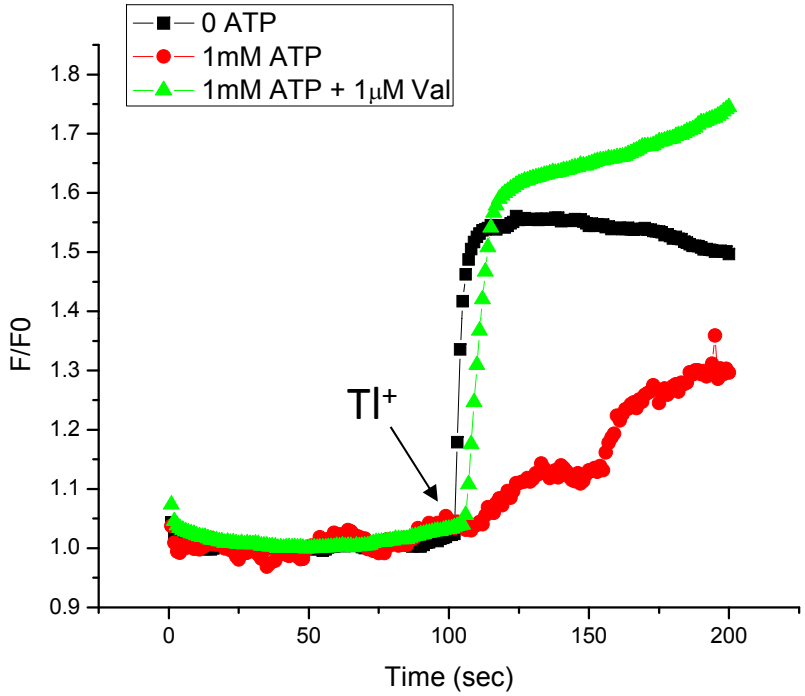
SUPPLEMENTAL MATERIAL

Online Figure I



Online Figure II

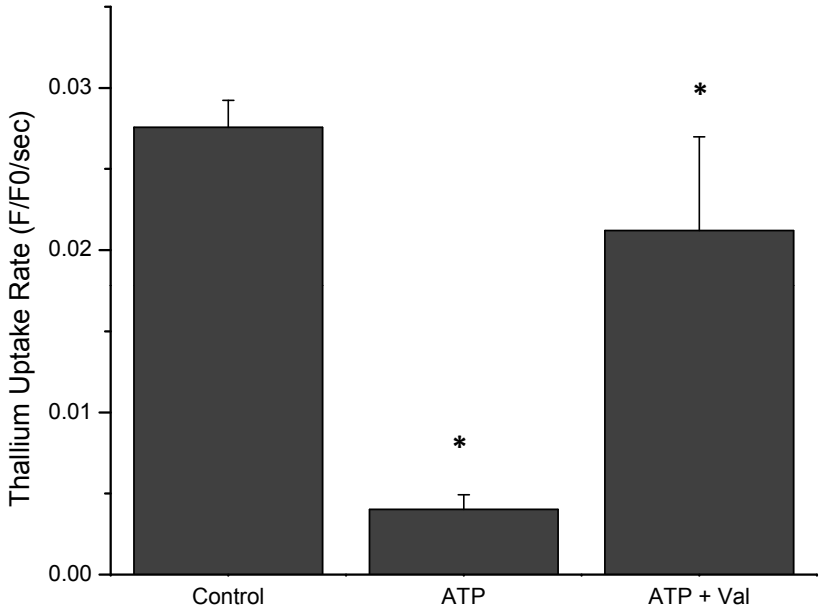


Figure I) Thallium uptake in the absence of ATP (black) and in the presence of 1mM ATP (red) or 1mM ATP plus 1μM valinomycin (green) in scrambled control H9C2 cells.

Figure II) Summary of initial thallium uptake rates for each group (n=3 for each group). * denotes statistically significant differences compared to control cells alone ($p<0.05$).

Online Figure III
Knockdown of endogenous ROMK

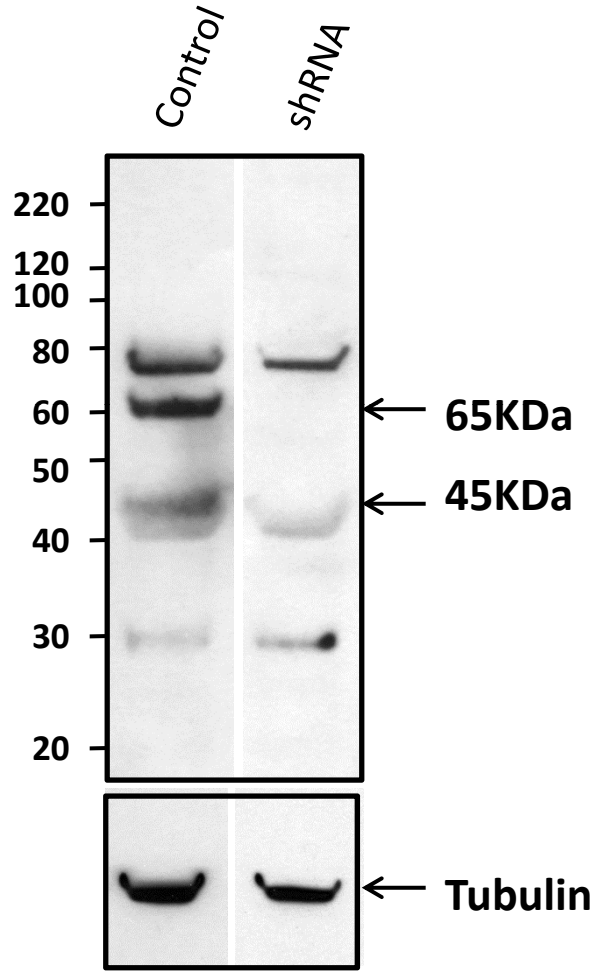


Figure III. H9C2 cells stably expressing scrambled shRNA (control), or a combination of 2 ROMK shRNAs (shRNA), were harvested for immunoblot analysis (see methods supplement). Cell Lysates (20 µg) were probed with an antibody to ROMK (Sigma Prestige, anti-KCNJ1 Cat #: HPA026962). The control blot showed 5 bands of differing intensity. One band, around 45kDa, consistent with the known molecular weight of either ROMK1 or ROMK2, is absent from the shRNA lane. A second band, around 65 kDa was also knocked down in the shRNA lane, though its origin is unknown.

Online Figure IV
ROMK2-V5 overexpression and its knockdown by ROMK shRNA

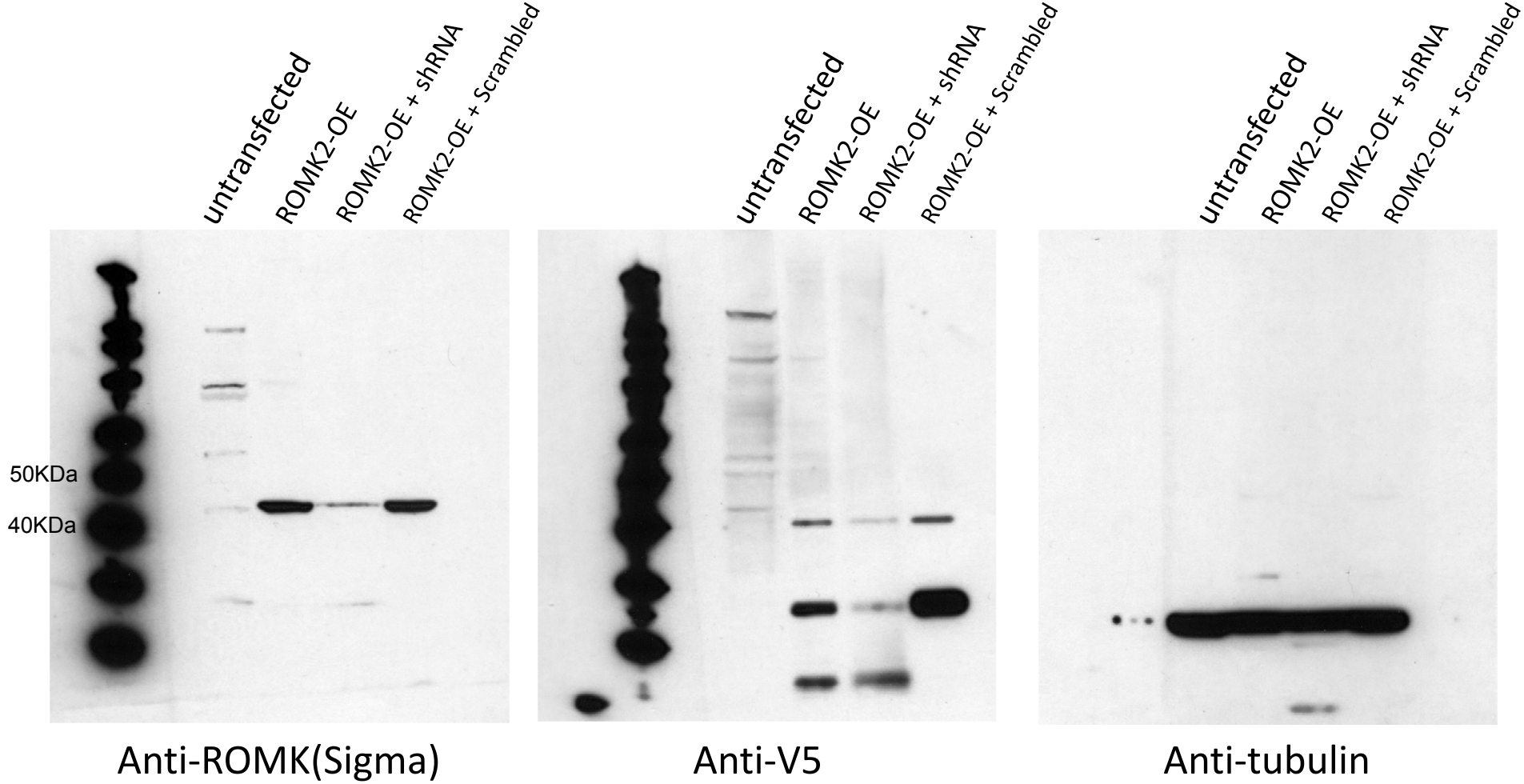


Figure IV. Untransfected H9C2 cells, cells stably expressing an epitope-tagged ROMK2-V5 (ROMK2-OE), or cells expressing both ROMK2-V5 and a ROMK shRNA were harvested for immunoblot analysis (see methods supplement). Cell Lysates (20 μg) were probed with an antibody to ROMK (Sigma; leftmost blot), Anti-V5 (center) or a tubulin loading control (right). Both the anti-ROMK antibody and the V5 antibody detected a band between 40 and 50 Kda that increased with ROMK overexpression and decreased with concomitant ROMK shRNA expression, while a scrambled control shRNA had no effect on ROMK expression.