SUPPLEMENTAL

MATERIAL

Data S1.

Supplemental Methods

Patients

Human lung parenchymal samples were obtained from 10 control patients with healthy lungs (failed donor lungs); seven were female, three were male. Samples from patients with familial pulmonary arterial hypertension (FPAH) were obtained; three were female, two were male. Samples from patients with congenital cardiac defect -associated pulmonary arterial hypertension (APAH) were obtained; five patients were female. cDNA samples were provided by the Pulmonary Hypertension Breakthrough Initiative (PHBI).[1] The protocol, "Studying Gene Expression in Pulmonary Arterial and Lung Tissue in Healthy and Diseased Samples," (#AAAQ2454) was approved by the Institutional Review Board at Columbia University Medical Center. *KCNK3* mutations in PAH patients were identified as previously reported.[2]

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The TaqMan gene expression system was used to quantify mRNA expression (Applied Biosystems). RNA and cDNA from lung samples were prepared for gene expression analysis using a previously described protocol.[1] No-template controls lacking cDNA were included, to verify the specificity of the assay at recognizing cDNA templates of interest. Experiments were performed in duplicate for each sample. Data are expressed as means of the average cycle threshold (Ct) value of duplicates, and as fold changes in expression (2^{ddCt} method). Commercially available assays from Applied Biosystems were used for priming/probing of cDNA samples, including:

1) KCNK3 (Assay ID= Hs00605529_m1)
2) KCNK9 (Assay ID= Hs00363153_m1)
3) GAPDH (Assay ID= Hs02758991_g1)

Expression of KCNK3 and KCNK9 in PAH and control lungs was normalized to GAPDH expression. Average cycle threshold (Ct) values at which signals for each gene appeared were calculated based on 40-cycle assays. For samples in which no amplification signal was produced, a Ct value of "40" was assigned for the purpose of calculating mean Ct values.

Molecular Biology

Mutations were engineered into human *KCNK3* cDNA in a pcDNA3.1+ expression vector by site-directed mutagenesis using QuickChange (Stratagene).[2] Human *KCNK9* cDNA in a pIRES-GFP and pcDNA3.1+ vector was used. Where noted, *KCNK3* constructs were tagged with a C-terminal green fluorescent protein (GFP). Tandem-linked *KCNK3-KCNK3* and *KCNK9-KCNK3* dimer constructs were engineered by joining two KCNK subunits with a glycine-rich linker, and subcloned into a pcDNA3.1+ vector.

Materials

ONO-RS-082 (Enzo Life Sciences), ML365 (MedChem Express), and ruthenium red and DMSO (Life Technologies) were purchased commercially. ONO-RS-082 and ML365 were dissolved in DMSO and ruthenium red in water in 100mM stock solutions and stored at -20°C. Drugs were diluted to 10μM in drug-containing external solutions, and DMSO 10μM

was added where appropriate to control (drug-free) solutions. Lipofectamine, Lipofectamine LTX, and Plus Reagent (Invitrogen) were used for transfection. 0.25% Trypsin/EDTA and Trypsin Neutralizer (Gibco) were used for splitting cell cultures. Human pulmonary artery smooth muscle cells (Gibco) were grown in Smooth Muscle Growth Medium-2 with supplements (SmGM-2 bulletkit, Lonza). COS7 cells (American Type Culture Collection) were cultured in medium containing DMEM 1X + GlutaMAX-1 with 4.5g/L D-Glucose and 110mg/L Sodium Pyruvate (Gibco), and supplemented with 10% Fetal Bovine Serum (Gibco) and 1% Penicillin/Streptomycin (Gibco).

Cell Culture and Heterologous Channel Expression

KCNK3 and *KCNK9* channel constructs were expressed in cultured hPASMC and COS7 cell lines. GFP (in a pcDNA3.1+ vector) was co-expressed or tagged to the C-terminus of the channel as a marker of transfection. A previously established transfection protocol using Lipofectamine reagents was employed in COS7 cells,[2] with modifications to optimize efficiency in hPASMCs.

COS7 cells:

On day 0, a T25 flask ~40-50% confluent was transfected with *KCNK3* or *KCNK9* cDNA using the following protocol: 2μg of *KCNK3* or *KCNK9* cDNA (20μl from a stock solution of 0.1μg/μl cDNA) + 1μg GFP if the *KCNK3* construct was not GFP-tagged + 20μl PLUS reagent + OptiMEM medium supplemented with L-glutamine up to 200μl were mixed and incubated at room temperature for 20 minutes. Of note, for co-expression of two *KCNK3* (or *KCNK3* + *KCNK9*) constructs, 1.5μg of each cDNA was transfected.

Next, 20μl Lipofectamine + 180μl OptiMEM medium supplemented with L-glutamine were added to the cocktail and incubated for 20 additional minutes at room temperature. Next, 1.6ml of OptiMEM supplemented with L-glutamine was added to the cocktail and mixed, and the 2ml transfection cocktail was pipetted into the COS7 cell flask after removal of the culture medium. The flask was returned to the 37°C cell culture incubator (conventional growth conditions, 5% CO_2) for approximately 4-6 hours. After incubation, the transfection cocktail was replaced by COS7 culture medium.

On day 1 in the afternoon, cells transfected on day 0 were split into 10cm dishes in the culture hood as follows: culture medium was removed from the T25 flask, and 5ml of 1X PBS was quickly added and removed, before 2ml 0.25% Trypsin/EDTA was added to the flask. The flask was then placed back in the 37°C incubator for approximately 5 minutes. After cells were dislodged from the flask bottom, 5ml of cell medium was added to the flask and pipetted to dislodge more cells. The 7ml cell solution was pipetted out of the flask and transferred into a 15ml conical tube, and centrifuged for 1.5 minutes at 1xg. The conical tube was returned to the culture hood, medium removed, and new cell medium added for cell suspension. Cells were then pipetted into 10cm dishes, and placed back in the 37°C incubator. On day 2 (and day 3 if cells were still healthy), the 10cm dishes were used for patch clamp experiments.

Human Pulmonary Artery Smooth Muscle Cell Line:

In hPASMCs, transfection of *KCNK3* cDNA required the following protocol: On day 0, hPASMCs in a T25 flask were split into 10cm dishes suitable for patch clamp experiments. Cells were split using the manufacturer's protocol (Gibco), which required 0.25% Trypsin/EDTA for cell detachment from the flask, and Trypsin Neutralizer to resuspend cells before plating in 10cm dishes. Cells were grown in supplemented Smooth Muscle Growth Medium-2 (Lonza).

On day 1, cell were transfected under the culture hood, as follows: per 10cm dish, 0.6μg *KCNK3* cDNA tagged with GFP at the C-terminus (from a cDNA stock solution of 0.1μg/μl) + 0.6μl PLUS reagent + 50μl OptiMEM medium supplemented with L-glutamine, were mixed and incubated at room temperature for 10 minutes. Next, 1.5μl Lipofectamine LTX per dish was added to the cocktail and incubated for an additional 30 minutes at room temperature. Equal proportions (58.1μl) of the cocktail were then pipetted into each 10cm dish, and dishes were returned to the 37°C incubator. On day 2 (and day 3 if cells were still healthy), 10cm dishes were used for patch clamp experiments.

Electrophysiology

KCNK3 channel current and membrane potential changes were recorded by whole-cell patch clamp in hPASMCs and COS7 cells. An Axopatch 200B amplifier (Axon Instruments), Digidata 1440A model, and pClamp 10 software were used for recording and analysis (Molecular Devices, CA). Pipette resistances generally ranged from 1-4 MOhm. For all voltage clamp experiments, cells were held at -80mV and a 500ms voltage

ramp was applied once every 3 seconds, with voltage increasing linearly from -120mV to +60mV before returning to holding. Expressed *KCNK3*, *KCNK9*, and tandem dimer constructs were recorded. For current clamp experiments, changes in membrane potential were recorded over time after first applying the voltage ramp to verify stability of the patch and expression of KCNK3 channels.

Perfusion of extracellular solutions containing pharmacological agents or different pH values occurred at recorded intervals during patch clamp experiments, at room temperature under normoxic conditions. All experiments with pharmacological agents were conducted at extracellular pH 7.4.

For experiments in COS7 cells, solutions were prepared as previously reported [2]: pipette (internal) solution (in mmol/L) contained: 150 KCl, 3 MgCl2, 5 EGTA, 10 HEPES, adjusted to pH 7.2 with KOH. Bath (extracellular) solution (in mmol/L) contained: 150 NaCl, 5 KCl, 1 MgCl2, 1.8 CaCl2, 10 HEPES adjusted to pH 6.4, 7.4, or 8.4 with NaOH.

For experiments in hPASMCs, solutions were adapted from a previous study:[3] pipette (internal) solution (in mmol/L) contained: 135 K-methanesulphonate, 20 KCl, 2 Na2ATP, 1 MgCl2, 1 EGTA, 20 HEPES, adjusted to pH 7.2 with KOH. Bath (extracellular) solution (in mmol/L) contained: 140.5 NaCl, 5.5 KCl, 1.5 CaCl2, 1 MgCl2, 10 glucose, 0.5 Na2HPO4, 0.5 KH2PO4, 10 HEPES, adjusted to pH 6.4, 7.4, or 8.4 with NaOH.

In COS7 cells and hPASMCs, solutions at pH 5.0 contained 10 mmol/L 2-(N-morpholino)ethane sulfonic acid instead of HEPES. Solutions at pH 10.4 contained 10 mmol/L Tris-Base instead of HEPES.

Voltage Clamp Analysis

Cell capacitance values were recorded for individual cell recordings, and whole cell currents were normalized to cell capacitance (pA/pF) where indicated. Measurements of current for drug analysis (ONO-RS-082, ML365, and ruthenium red) were taken at -50mV to minimize contributions from background cellular ionic currents. This was performed by taking the mean current at -50mV over an approximately 5 millisecond range centered around -50mV, from the average of 2 to 6 consecutive traces based on stability of the recording. Measurements of current were taken at +60mV for pH experiments (as previously shown in [2]), by calculating the mean current during the last ~5 milliseconds of the trace, based on the average of 2 to 6 consecutive traces depending on stability of the recording at each pH value.

Leak subtraction was manually performed on all recordings in this study. Based on the Nernst equilibrium potential for potassium (E_K) close to -80mV, negative current values recorded at the holding potential of -80mV were designated as leak current, and a proportional amount of leak current was extrapolated across the voltage ramp range; an assumption applied for analysis given the virtually voltage-independent properties of the KCNK3 and KCNK9 channels. Hence, analysis of mean current in any drug condition at -50mV required adding 5/8 of the value of the leak current at -80mV to the mean current

value recorded at -50mV, and analysis of mean current at +60mV required subtracting 6/8 of the value of the leak current at -80mV from the mean current value recorded at +60mV.

Current Clamp Analysis

Measurements of membrane potential were taken as the mean from the last ~10 seconds in any given pH or drug condition. To standardize drug analysis, all ONO membrane potential measurements were taken from the last ~10 seconds of five minutes of ONO application, and all ONO+ML365 membrane potential measurements were taken from the last ~10 seconds of two minutes of ONO+ML365 co-application. Using pClamp software, a 50X data reduction of recordings was performed for data transfer compatibility to Origin for figure purposes. Data analysis was performed on raw data only.

Statistical Analyses

Graphic analysis was performed with Origin 7.0 and 9.0 (Microcal Software, Northampton, MA). pClamp 10 software was used for analysis of raw electrophysiological recordings. Data are reported as means ± SEM, based on *n* observations. Student's *t* tests and one-way ANOVA with post-hoc Tukey tests were applied as indicated, and significant differences were determined based on $p < 0.05$. Statistical tests were performed using Origin and Excel (Microsoft, Bellevue, WA) software.

Supplemental Figure Legends:

Figure S 1. KCNK3-GFP expression and activity in hPASMCs. A, Summary of current clamp results of KCNK3-GFP expressed in hPASMCs, with mean membrane potentials (mV) measured at pH 6.4 (blue), pH 7.4 (black), and pH 10.4 (red) shown (n=2 to 4 cells per pH value). **B**, Comparison of the ML365-sensitive current (pA/pF at -50mV, pH 7.4), in hPASMCs expressing KCNK3-GFP (n=3 cells) versus no transfection (n=6 cells). **C**, Gradient of current expression (pA/pF at 60mV, pH 7.4) in hPASMCs expressing KCNK3-GFP (n=20 cells), V221L KCNK3-GFP (n=7 cells), and GFP only (n=3 cells). **D**, Sample current trace of V221L KCNK3-GFP in hPASMC at pH 7.4 (black) and pH 8.4 (red). **E**, Summary of V221L KCNK3 expression in COS7 cells versus V221L KCNK3-GFP expression in hPASMCs (n=4 to 7 cells per lane), showing greater current activity of expressed channels in hPASMCs (pA/pF at 60mV, pH 7.4 in black, pH 8.4 in red). Data are represented as means ± SEM. * indicates $p<0.05$ by the unpaired Student's *t* test.

Figure S 2. ONO-RS-082's effect on homomeric and heterodimeric mutant KCNK3 channels associated with PAH. Voltage clamp recordings in COS7 cells are depicted. **A-D**, The effect of ONO-RS-082 10 μM (red traces) at extracellular pH 7.4 on currents from cells expressing T8K KCNK3 (**A**); V221L KCNK3 (**B**); E182K KCNK3 (**C**); and heterodimeric WT-E182K KCNK3 (**D**). Control (pre-drug) conditions at extracellular pH 7.4 (black traces) are shown for each recording.

Figure S3. The impact of KCNK9 expression and current activity on KCNK3 function. A, The effect of ruthenium red (RR) 10 μM (red trace) on KCNK3 channels in COS7 cells is shown. Control trace (pre-drug, pH 7.4) is depicted in grey. **B**, Sample RR time course of action on KCNK3 in control and drug conditions, measured at -50mV, from a starting current amplitude of 196 pA indicated by the arrow. **C**, RR's effect on KCNK9, KCNK3, and KCNK9-KCNK3 channels is summarized, showing fold change in current at -50mV (n=5 to 8 cells per condition). **D**, Quantitative real-time PCR analysis of human lung samples from healthy (Control) and congenital cardiac defect-associated PAH (APAH) patient lungs. Expression of KCNK3 (black bars), and KCNK9 (grey bars) are compared, based on mean cycle threshold (Ct) values observed for each gene; Ct > 35 indicates no quantifiable gene expression (n=5 patient lungs for each lane). Bars shown mean ± SEM. **E**, Fold difference in KCNK3 gene expression, calculated by the 2^{-ddCt} method, in FPAH and APAH versus Control patient lungs. No significant (N.S.) fold changes were observed compared to control. **F-G**, Co-expression of KCNK9 with WT KCNK3 channels (panel F), and KCNK9 with V221L KCNK3 channels (panel G) with sample voltage clamp recordings shown for each condition at pH 6.4 (blue), pH 7.4 (black) and pH 10.4 (red). **H**, Summary of current activity for co-expression of KCNK9 with WT KCNK3 (left) versus V221L KCNK3 (right). Current at pH 6.4 (blue) and pH 7.4 (black) is normalized to max current at pH 10.4 (n=5 to 8 cells per lane). Experiments were performed in COS7 cells. Bar graphs show mean ± SEM. * indicates $p<0.05$ by the paired Student's *t* test for the comparison of control versus ruthenium red conditions in panel C; * indicates $p<0.05$ by the unpaired Student's *t* test in panel H.

Figure S4. KCNK3 heterodimeric GFP fusion dimer confirms the more severe loss of function in G203D versus V221L-containing KCNK3 channels. A, A WT-G203D KCNK3-GFP fusion heterodimer was engineered by interconnecting two KCNK3 subunits with a glycine-rich linker, and inserting a C-terminal GFP tag. Given the relative severity of G203D KCNK3 dysfunction, the GFP fusion construct ensured channel expression in fluorescent cells studied. Sample traces from a voltage clamp recording are shown at extracellular pH 5 (blue), pH 7.4 (black), and pH 10.4 (red), revealing small currents across the pH range. **B**, Summary of current densities (pA/pF at 60mV) at pH 5 (blue), pH 7.4 (black), and pH 10.4 (red) for the WT-V221L KCNK3 heterodimer versus the WT-G203D KCNK3-GFP heterodimer (n= 6 to 16 cells per pH bar). Bars show mean ± SEM. * indicates $p<0.05$ at pH 7.4 and pH 10.4, by the unpaired Student's *t* test.

Supplemental Figures

Figure S1.

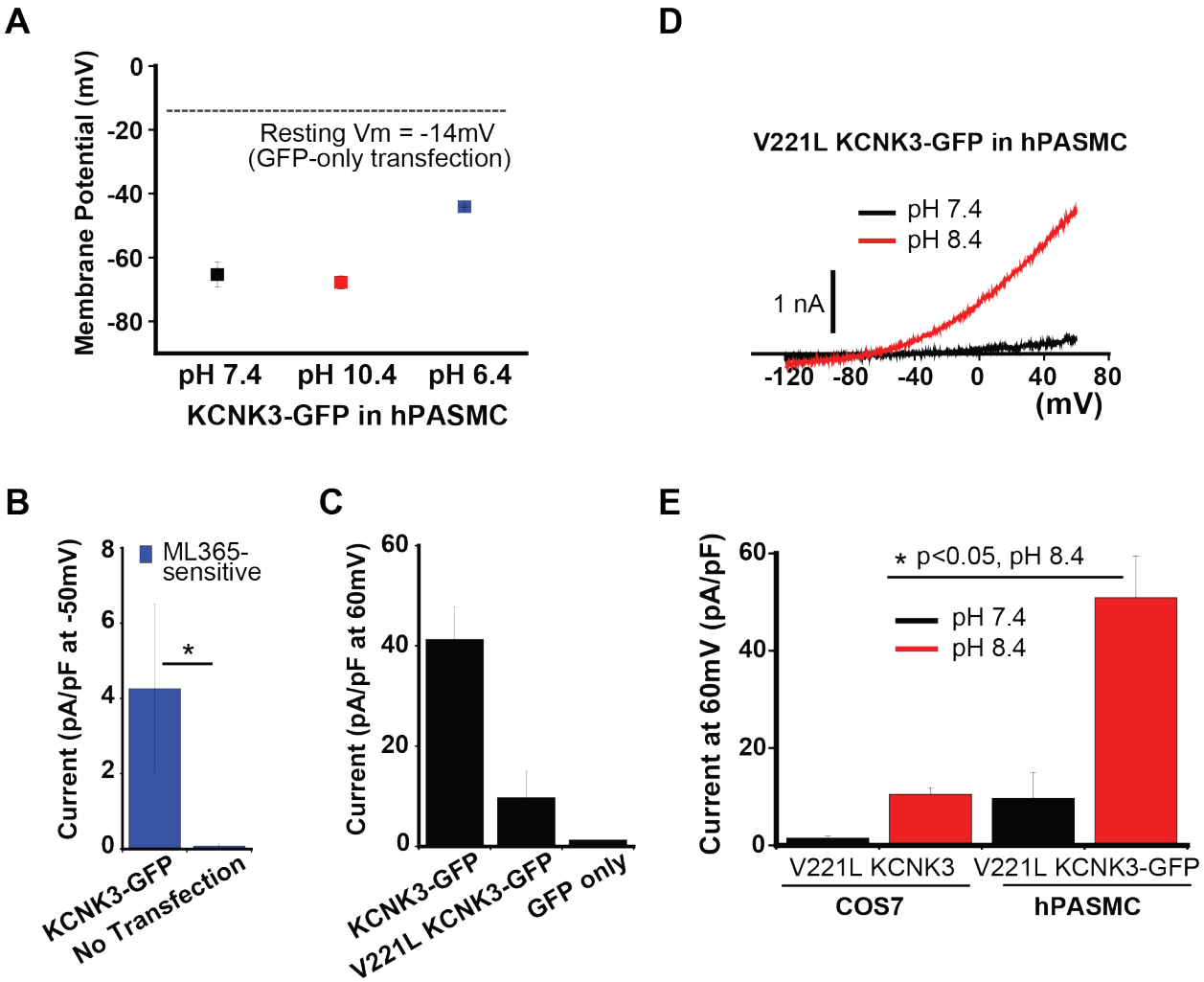
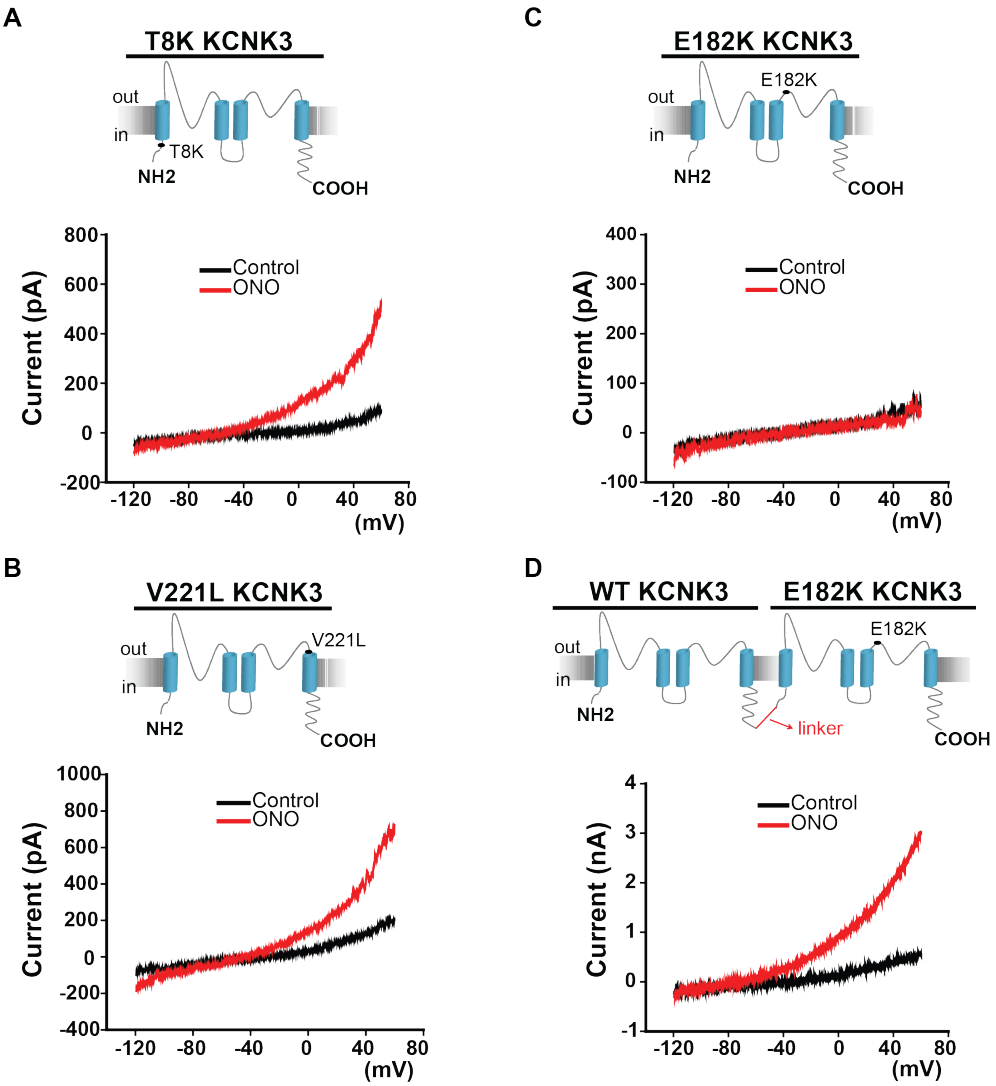


Figure S2.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out
in
T8K
NH2
COOH
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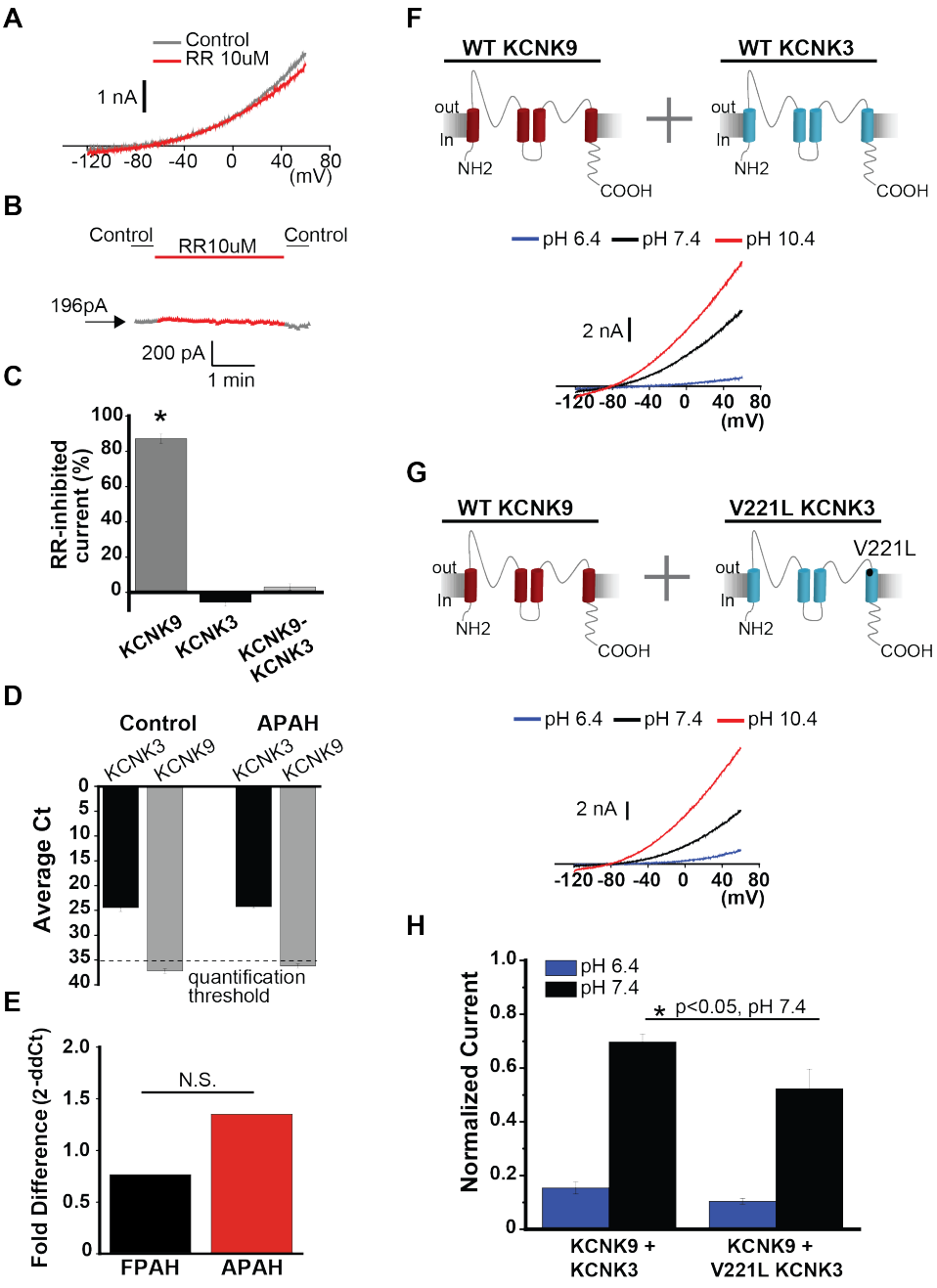
Control
ONO
(mV)
C
E182K KCNK3
E182K
B
V221L KCNK3
V221L
D
WT KCNK3
E182K KCNK3
linker
Current (nA)

Figure S3.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(mV)
B
Control
RR10uM
Control
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200 pA
1 min
C
RR-inhibited current (%)
100
80
60
40
20
0
*
KCNK9
KCNK3
KCNK9-KCNK3
D
Control
APAH
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KCNK3
KCNK9
Average Ct
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5
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15
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25
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35
40
quantification threshold
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Fold Difference (2-ddCt)
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1.5
1.0
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0
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FPAH
APAH
F
WT KCNK9
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out
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NH2
COOH
pH 6.4
pH 7.4
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2 nA
-120 -80 -40 0 40 80
(mV)
G
WT KCNK9
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V221L
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pH 10.4
2 nA
-120 -80 -40 0 40 80
(mV)
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Normalized Current
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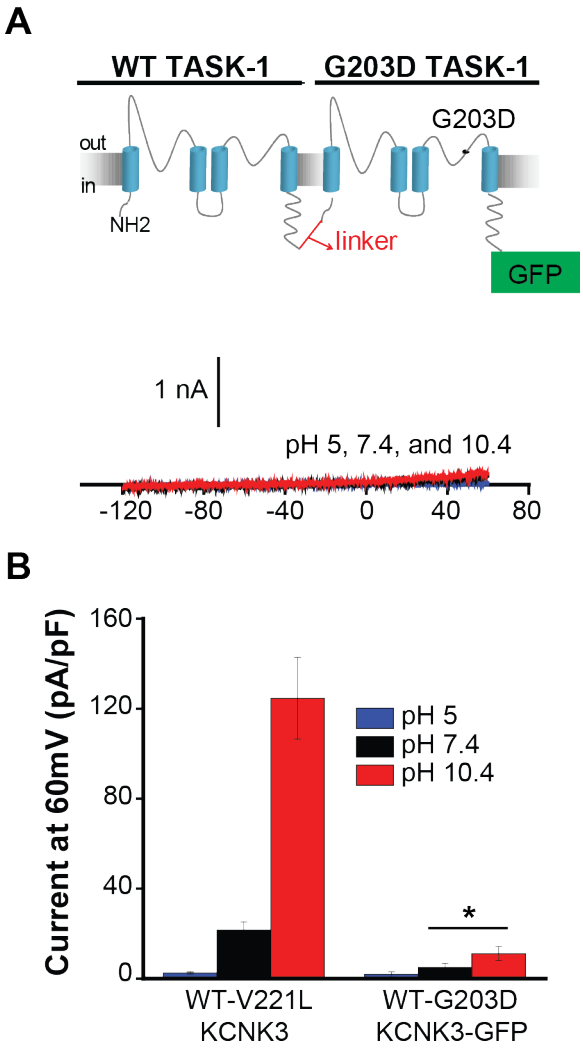
* p<0.05, pH 7.4
KCNK9 + KCNK3
KCNK9 + V221L KCNK3

Figure S4.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GFP
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pH 5, 7.4, and 10.4
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-80
-40
0
40
80
B
Current at 60mV (pA/pF)
160
120
80
40
0
pH 5
pH 7.4
pH 10.4
*
WT-V221L
KCNK3
WT-G203D
KCNK3-GFP

Supplemental References:

1. Stearman RS, Cornelius AR, Lu X, Conklin DS, Del Rosario MJ, Lowe AM, Elos MT, Fettig LM, Wong RE, Hara N, Cogan JD, Phillips JA, 3rd, Taylor MR, Graham BB, Tuder RM, Loyd JE, Geraci MW. Functional prostacyclin synthase promoter polymorphisms. Impact in pulmonary arterial hypertension. *Am J Respir Crit Care Med*. 2014;189:1110-20.

2. Ma L, Roman-Campos D, Austin ED, Eyries M, Sampson KS, Soubrier F, Germain M, Tregouet DA, Borczuk A, Rosenzweig EB, Girerd B, Montani D, Humbert M, Loyd JE, Kass RS, Chung WK. A novel channelopathy in pulmonary arterial hypertension. *N Engl J Med*. 2013;369:351-61.

3. Olschewski A, Li Y, Tang B, Hanze J, Eul B, Bohle RM, Wilhelm J, Morty RE, Brau ME, Weir EK, Kwapiszewska G, Klepetko W, Seeger W, Olschewski H. Impact of TASK-1 in human pulmonary artery smooth muscle cells. *Circ Res*. 2006;98:1072-80.