Online Appendix

Mutation E169K in junctophilin-2 causes atrial fibrillation due to impaired RyR2 stabilization

AUTHORS: David Beavers, BS, Wei Wang, PhD, Sameer Ather, MD, PhD, Niels Voigt, MD, Alejandro Garbino, MD, PhD, Sayali S. Dixit, PhD, Andrew P. Landstrom, MD, PhD, Na Li, PhD, Iacopo Olivotto, MD, Dobromir Dobrev, MD, Michael J. Ackerman, MD, PhD & Xander H.T. Wehrens, MD, PhD

Supplemental Methods

Hypertrophic Cardiomyopathy Genetic Analysis

Between January 1, 2002 and December 31, 2003, 203 consecutive, unrelated index cases with a confirmed clinical diagnosis of HCM were enrolled with local IRB approval at the Azienda Ospedaliera-Universitaria Careggi in Florence, Italy, and Ospedale San Camillo, in Rome, Italy. All cases were genotyped for mutations in nine sarcomeric HCM-associated genes (*MYH7, MYL2, MYL3, MYBPC, TNNI3, TNNT2, TNNC1, TPM1,* and *ACTC*).

Generation of pseudo-knockin mice

Cardiac-specific E169K and A399S mutant junctophilin-2 (JPH2) transgenic (Tg) mice (E169K-Tg, A399S-Tg) were generated by subcloning mutant mouse JPH2 cDNA into an alpha myosin heavy chain (αMHC) Tg vector (kindly provided by Dr. Thomas Cooper, Baylor College of Medicine). A previously generated HA-tagged wild-type JPH2 pcDNA3.1 construct was used for primer-based site-directed mutagenesis to introduce the E169K and A399S mutations (1). The αMHC Tg vector, E169K, and A399S JPH2 inserts were digested with HindIII and NotI restriction enzymes and

ligated. The Tg vectors were linearized with BsiWI and AseI and then injected into the pronuclei of fertilized C57/BL6 oocytes, and transferred to pseudopregnant recipients (2). The generation of mice with cardiac-specific inducible short hairpin RNA (shRNA)-mediated knockdown of JPH2 (sh-JPH2) and Tg mice overexpressing WT JPH2 has been described previously (1).

Pseudo-knockin (PKI) mice were generated by crossing the JPH2 Tg (WT, E169K, and A399S) mice with sh-JPH2 mice. The double Tg mice were then crossed to αMHC-MerCreMer (MCM) mice (Jackson Laboratory, Bar Harbor, ME). The triple Tg mice were dosed with tamoxifen (30 mg/kg intraperitoneally; Sigma-Aldrich Co., St. Louis, MO) to induce shRNA-mediated knockdown of total JPH2 levels to protein levels similar to those in non-transgenic (NTg) or wild-type (WT) mouse hearts. Because the tamoxifen-treated triple Tg mice were very similar to heterozygous knock-in mice in terms of the total JPH2 levels, about 50% of which was transgenic, were named these mice 'pseudo-knockin' (PKI) mice. To obtain equal total JPH2 levels, WT-PKI, E169K-PKI, and A399S mice were generated by injection of tamoxifen for 3, 2, and 3 days, respectively. The PKI mice were studied between 2 and 3 weeks after tamoxifen injection, when total JPH2 levels were stable. Mouse models used for differential JPH2 protein levels were dosed with tamoxifen and used as described previously (1).

Transthoracic echocardiography

Mice were anesthetized using 1.5-2% isoflurane in 100% O_2. Body temperature was maintained between 36.5-37.5°C on a heated platform, and ECG and temperature were continuously monitored. Cardiac function was assessed using a VisualSonics VeVo 770 Imaging System (VisualSonics, Toronto, Canada) equipped with high-frequency 30 MHz probe, as described (3).

Myocyte isolation

The heart was removed following isoflurane anesthesia and rinsed in 0 Ca^{2+} Tyrode solution (137 mm NaCl, 5.4 mm KCl, 1 mm $MgCl_2$, 5 mm HEPES, 10 mm glucose, 3 mM NaOH, pH 7.4). The

heart was cannulated through the aorta and perfused on a Langendorf apparatus with 0 Ca^{2+} Tyrode (3 ~ 5 minutes, 37 °C), then 0 Ca^{2+} Tyrode containing 20 μg/ml (0.104 a.u./ml) Liberase TH Research Grade (Roche Applied Science) for 10 ~ 15 minutes at 37 °C. After digestion, the heart was perfused with 5 ml Kreb's buffer (KB) solution (90 mm KCl, 30 mm K_2HPO_4, 5 mm $MgSO_4$, 5 mm pyruvic acid, 5 mm β-hydroxybutyric acid, 5 mm creatine, 20 mm taurine, 10 mm glucose, 0.5 mm EGTA, 5 mm HEPES, pH 7.2) to wash out collagenase. Both atria were minced in KB solution and gently agitated, then filtered through 210 μm polyethylene mesh. After settling, atrial myocytes were washed once with KB solution, and stored in KB solution at room temperature before use.

Confocal Ca^{2+} imaging

Ventricular myocytes were incubated with 2 μM Fluo-4-acetoxymethyl ester (Fluo-4 AM, Invitrogen) in normal Tyrode solution containing 1.8 mM Ca^{2+} for 60 minutes at room temperature. The cells were then washed with dye-free normal Tyrode solution (1.8 mM Ca^{2+}) for 15 minutes for de-esterification. Then cells were transferred to a chamber equipped with a pair of parallel platinum electrode on a laser scanning confocal microscope (LSM 510, Carl Zeiss) with a x40 oil immersion objective. Fluo-4 was excited at the 488 nm with emission collected through a 515 nm long pass filter. Fluorescence images were recorded in line-scan mode with 1024 pixels per line at 500 Hz. After being paced at 1 Hz (5 ms at 10 V) for 2 minutes, only myocytes showing clear striation and normal contractility were selected for further experiments. Once steady state calcium transient was observed, pacing was paused for 30 seconds to record spontaneous Ca^{2+} release events (Ca^{2+} waves and Ca^{2+} sparks). Steady state SR Ca^{2+} was estimated by rapid application of 10 mM caffeine after pacing.

Ca^{2+} spark parameters were analyzed using the SparkMaster program (4). The threshold for detection of Ca^{2+} spark events was 3.8 times the standard deviation of the background noise over the mean value of the background. Spontaneous Ca^{2+} waves were defined as unstimulated increases in intracellular Ca^{2+} in 2 or more adjacent 3-μm sections (5,6). The fractional SR Ca^{2+}

release was calculated by normalizing Ca^{2+} transient amplitude to SR Ca^{2+} load for individual cells.

Cell permeabilization

For certain experiments, myocytes were permeabilized by brief exposure (30-60 s) to the saponin β-escin (20 μM) in intrecellular solution that contains 110 mM K-aspartate, 1mM EGTA, 10 mM phosphocreatine, 5 U/ml creatine phosphokinase, 4% dextran (MW 40,000), 5 mM Mg-ATP, 1 mM $MgCl_2$ and 20 μM Fluo-4 pentapotassium salt and 200 nM free Ca^{2+} (pH to 7.2 with KOH). Once the cell membrane was permeabilized, experiments were conducted in the same internal solution without saponin.

Small peptide design

A 33 amino acid oligopeptide was synthesized (Genscript, Piscataway, NJ) that included an N-terminal FLAG tag and the 25 amino acid sequence centered on residue E169 in JPH2 (Sequence: DYKDDDDK-SPLRTSLSSLRS**E**HSNGTVAPDSPA). The peptide had a purity of 76.8% and was resuspended in Milli-Q water prior to being applied to isolated, permeabilized atrial myocytes. The scrambled control peptide (purity 83.6%) consisted of the same amino acids with the 25 amino acid sequence split in center and the N and C-terminal residues inverted (Sequence: DYKDDDDK-EHSNGTVAPDSPASPLRTSLSSLRS).

Single-channel RyR2 kinetics using planar lipid bilayer

Single channel recordings of RyR2 from MCM and MCM-shJPH2 mice were performed as described (7). Briefly, The *trans* chamber representing the luminal side of SR contained 250 mM Hepes, 50 mM KCl, and 53 mM $Ca(OH)_2$, and the *cis* chamber representing cytosolic side of SR contained 250 mM HEPES, 125 mM Tris-base, 50 mM KCl, 1 mM EGTA, 0.5 mM $CaCl_2$, pH 7.35. After baseline recordings were completed, 10 mM JPH2 peptide was added into the *cis* chamber. After 15 min, the open probability was measured again in the presence of the peptide.

Simultaneous intracellular [Ca^{2+}] and membrane current measurements in atrial human myocytes

Human atrial tissue was either used for myocyte isolation or flash-frozen in liquid nitrogen for biochemical studies. All measurements in isolated human atrial myocytes were made at 37°C in whole-cell patch clamp configuration with simultaneous intracellular [Ca^{2+}] measurement as described (8). pClamp-Software (V10.2, Molecular Devices, Sunnyvale, CA) was used for data acquisition and analysis. Intracellular [Ca^{2+}] was quantified using Fluo-3-acetoxymethyl ester (Fluo-3 AM; Invitrogen, Carlsbad, CA; 10 μmol/L, 10 min loading and 30 min de-esterification). In addition Fluo-3 was included into the electrode solution containing (in mmol/L): EGTA 0.02, Fluo-3 0.1 (Invitrogen), GTP-Tris 0.1, HEPES 10, K-aspartate 92, KCl 48, Mg-ATP 1, Na_2-ATP 4; pH=7.2. Borosilicate glass microelectrodes had tip resistances of 2-5 MΩ when filled with pipette solution. Seal-resistances were 4-8 GΩ. For voltage-clamp experiments series resistance and cell capacitance were compensated. During experiments myocytes were superfused at 37°C with a bath solution containing (in mmol/L): $CaCl_2$ 2, glucose 10, HEPES 10, KCl 4, $MgCl_2$ 1, NaCl 140, probenecid 2; pH=7.4. For voltage-clamp experiments, K^+ currents were blocked by adding 4-aminopyridine (5 mmol/L) and $BaCl_2$ (0.1 mmol/L) to the bath solution.

Western Blotting and Co-immunoprecipitation

Western blotting and co-immunoprecipitation were performed as described (1,9). Tissue lysates were made from flash frozen hearts by homogenization followed by sonication in Radio-Immunoprecipitation Assay (RIPA) lysis buffer containing 10% CHAPS, 20 mM NaF, 1 mM Na_3VO_4, and 1x protease and phosphatase inhibitor tablets (Roche Diagnostics, Indianapolis, IN). For protein expression western blot experiments 75 μg of total protein was diluted in 2x lithium dodecyl sulfate (LDS) buffer (Invitrogen, Carlsbad, CA) containing 0.5% β-mercaptoethanol, heated to 70°C for 10 minutes and resolved on a 5-12% sodium dodecyl sulfate (SDS)

polyacrylamide electrophoresis gel, followed by electrotransfer to polyvinylidene difluoride (PVDF) membrane at 20V overnight at 4°C.

For co-immunoprecipitation experiments, 500 μg of total protein heart lysate was precleared with washed protein A-sepharose beads (Rockland, Gilbertsville, Pa) for two hours at 4°C. 2 μL of anti-RyR2 antibody (MA3-916, Affinity Bioreagents) or mouse IgG1 isotype control (Sigma Aldrich) was incubated with protein A-sepharose beads for 1 hour at 4°C. Antibody-bound beads were then incubated with precleared heart lysates overnight at 4°C. After incubation, beads were washed with the RIPA buffer solution described above without CHAPS detergent, then resuspended in 2x LDS buffer with β-mercaptoethanol, incubated at RT for 15 min, heated to 70°C for 10 min and loaded on a 6% SDS polyacrilamide gel and transferred overnight.

All membranes were blocked in 5% milk for ~1 hour and then incubated with primary antibody 4 hours at room temperature or overnight at 4°C followed by incubation in 1:5,000 dilution of fluorescent secondary antibody (Alexafluor 680 anti-mouse IgG, Invitrogen or IRDye800 conjugated anti-rabbit IgG, Rockland) for 90 min at room temperature. Primary antibodies used include a custom polyclonal rabbit anti-JPH2 antibody (1:1,000), monoclonal mouse anti-HA antibody (1:5,000, H3663, Sigma), monoclonal mouse anti-RyR2 (1:5,000, MA3-916, Affinity Bioreagents), and monoclonal mouse anti-GAPDH (1:10,000, MAB374, Millipore). Blot imaging was performed with the Licor Odyssey two channel IR fluorescence scanner.

References

1. van Oort RJ, Garbino A, Wang W, et al. Disrupted junctional membrane complexes and hyperactive ryanodine receptors after acute junctophilin knockdown in mice. Circulation 2011;123:979-88.
2. Ladd AN, Taffet G, Hartley C, Kearney DL, Cooper TA. Cardiac tissue-specific repression of CELF activity disrupts alternative splicing and causes cardiomyopathy. Mol Cell Biol 2005;25:6267-78.
3. Respress JL, Wehrens XH. Transthoracic echocardiography in mice. J Vis Exp 2010;39:1738.
4. Picht E, Zima AV, Blatter LA, Bers DM. SparkMaster: automated calcium spark analysis with ImageJ. Am J Physiol Cell Physiol 2007;293:C1073-81.
5. Hove-Madsen L, Llach A, Bayes-Genis A, et al. Atrial fibrillation is associated with increased spontaneous calcium release from the sarcoplasmic reticulum in human atrial myocytes. Circulation 2004;110:1358-63.
6. Liu N, Denegri M, Ruan Y, et al. Short communication: flecainide exerts an antiarrhythmic effect in a mouse model of catecholaminergic polymorphic ventricular tachycardia by increasing the threshold for triggered activity. Circ Res 2011;109:291-5.
7. van Oort RJ, McCauley MD, Dixit SS, et al. Ryanodine receptor phosphorylation by calcium/calmodulin-dependent protein kinase II promotes life-threatening ventricular arrhythmias in mice with heart failure. Circulation 2010;122:2669-79.
8. Voigt N, Li N, Wang Q, et al. Enhanced sarcoplasmic reticulum Ca^{2+} leak and increased Na^{+}-Ca^{2+} exchanger function underlie delayed afterdepolarizations in patients with chronic atrial fibrillation. Circulation 2012;125:2059-70.
9. Chelu MG, Sarma S, Sood S, et al. Calmodulin kinase II-mediated sarcoplasmic reticulum Ca^{2+} leak promotes atrial fibrillation in mice. J Clin Invest 2009;119:1940-51.

Online Table 1: Characteristics of patients

	SR	pAF
Patients, n	25	17
Gender, m/f	22/3	9/8*
Age, y	67.7±1.7	72.3±1.9
Body mass index, kg/m^2	28.2±0.8	27.9±1.2
CAD, n	11	8
MVD/AVD, n	8	5
CAD+MVD/AVD, n	6	4
Hypertension, n	21	14
Diabetes, n	6	4
Hyperlipidemia, n	18	9
LVEF, %	55.0±2.0	49.7±2.9
Digitalis, n	3	3
ACE inhibitors, n	14	6
AT1 blockers, n	3	2
β-Blockers, n	17	11
Dihydropyridines, n	6	4
Diuretics, n	11	10
Nitrates, n	1	4
Lipid-lowering drugs, n	13	9

CAD, coronary artery disease; MVD/AVD, mitral/aortic valve disease; LVEF, left ventricular ejection fraction; ACE, angiotensin-converting enzyme; AT, angiotensin receptor.

*P<0.05 vs. SR from Fisher's exact test, respectively.

Online Figure Legends

Online Figure S1. A. Cardiac hypertrophy and QTc prolongation in patients positive for JPH2 mutation E169K. Representative echocardiographic images showing septal hypertrophy in the proband (~35 mm) and genotype-positive father (~15 mm), but normal septal dimensions in the genotype-negative sister (red arrow). **B**. ECG tracings showing prolonged QTc intervals in the proband and genotype-positive father, but not in the genotype-negative sister.

Online Figure S2. Generation and characterization of JPH2 levels in PKI mice. A, Schematic representation of targeting vectors used to generate cardiac-specific JPH2-WT pseudo-knockin mice (WT-PKI), JPH2-E169K PKI mice (E169K-PKI) and JPH2-A399S PKI mice (A399S-PKI) that express hemagglutinin (HA)-tagged JPH2 in front of α-myosin heavy chain (αMHC) promoter. The respective JPH2 Tg mice were crossed with αMHC-MerCreMer (MCM) and JPH2-shRNA (sh-JPH2) mice to lower total JPH2 levels using cardiac-restricted shRNA against JPH2. The resulting total JPH2 levels in the hearts of the triple Tg mice were similar to those in hearts of non-transgenic NTg mice. Therefore, we refer to these mice as pseudoknockin (PKI) mice. **B,** Western blots demonstrating similar expression of total and HA-tagged JPH2 in WT-PKI, E169K-PKI, and A399S-PKI mice. Total JPH2 levels were similar to those in NTg mice after dosing with tamoxifen to induce cardiac-specific shRNA-mediated JPH2 knockdown in PKI mice.

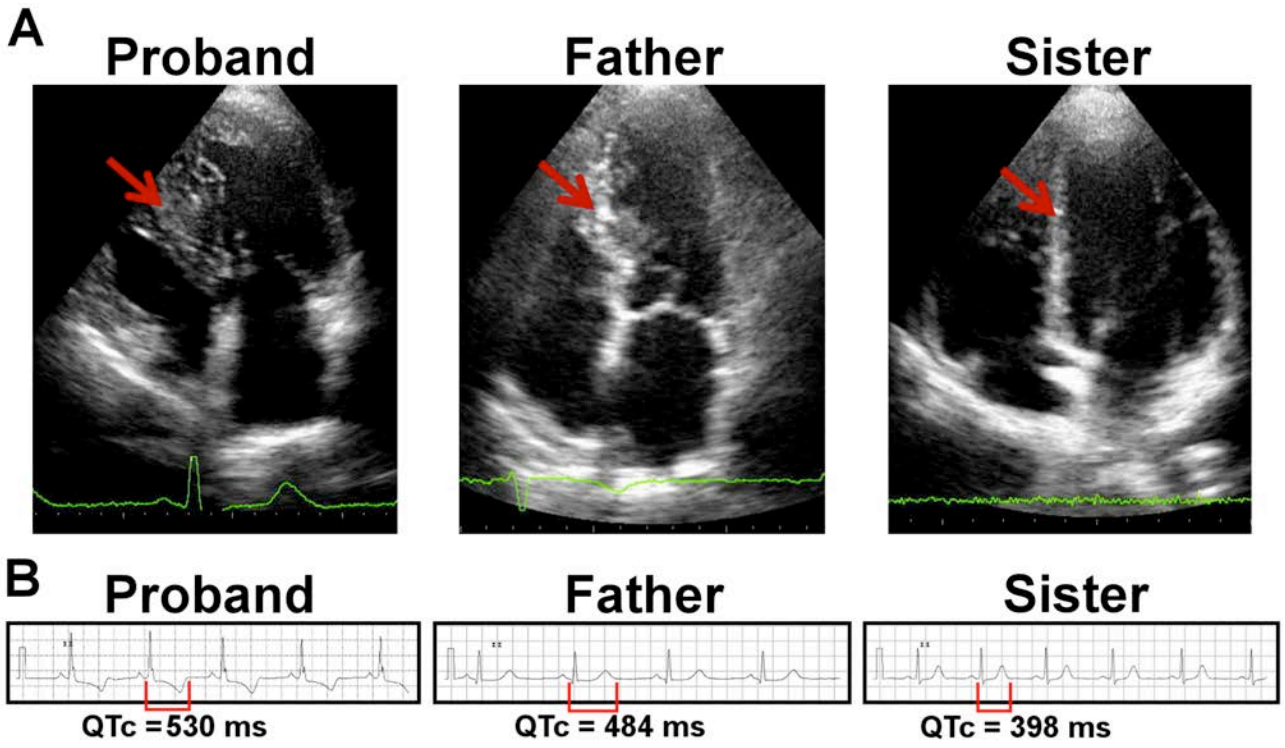
Online Figure S3. Preserved cardiac function and absence of hypertrophy in E169K-PKI mice. A, Ejection fraction in wild-type pseudoknockin (WT-PKI) and E169K pseudoknockin (E169K-PKI) mice compared to non-transgenic (NTG) controls at 2 months of age. **B**, Diastolic left ventricular posterior wall thickness in PKI and NTg mice at 2 months. **C**, Heart weight-to-body weight ratio in PKI mice and NTg controls at 2 months. N=4 mice for all experiments.

Online Figure S4. E169K mutation alters intracellular Ca^{2+} transient properties in atrial myocytes. A, Representative confocal line scan images and tracings showing steady state Ca^{2+} transients and SR Ca^{2+} content in atrial myocytes from WT-PKI, E169K-PKI, and A399S-PKI mice. **B-D**, Bar graphs showing unaltered Ca^{2+} transient amplitude E169K-PKI mice **(B),** reduced SR Ca^{2+} load (0.46 ± 0.04 in E169K-PKI, 0.36 ± 0.02 in WT-PKI) **(C),** and increased fractional SR Ca^{2+} release **(D)** in E169K-PKI mice. * P<0.05.

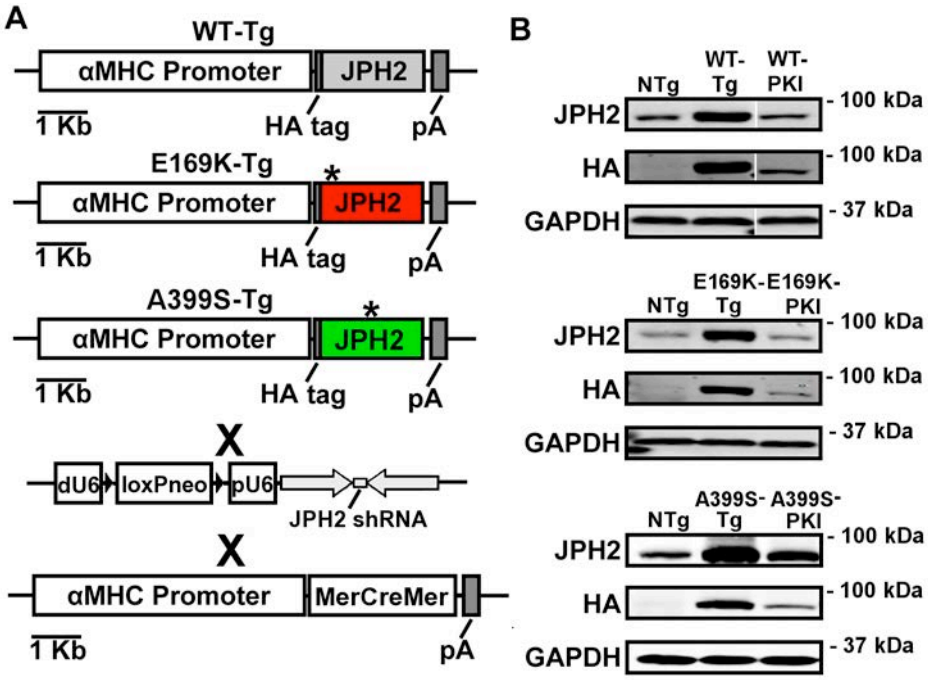
Online Figure S5. Scrambled peptide does not effect RyR2 single channel open probability. Bar graph showing open probabilities of RyR2 isolated from control mice (iαMHC-MerCreMer; MCM) mice and JPH2 knockdown mice (JPH2-KD). Addition of scrambled peptide did not alter the open probability of RyR2 from either groups of mice. Numbers indicate number of channels. * P <0.05

Online Figure S6. JPH2 levels are not correlated with age. Scatter plot of JPH2 protein levels versus age in patients in sinus rhythm (SR), paroxysmal atrial fibrillation (pAF), or healthy donors (Do).

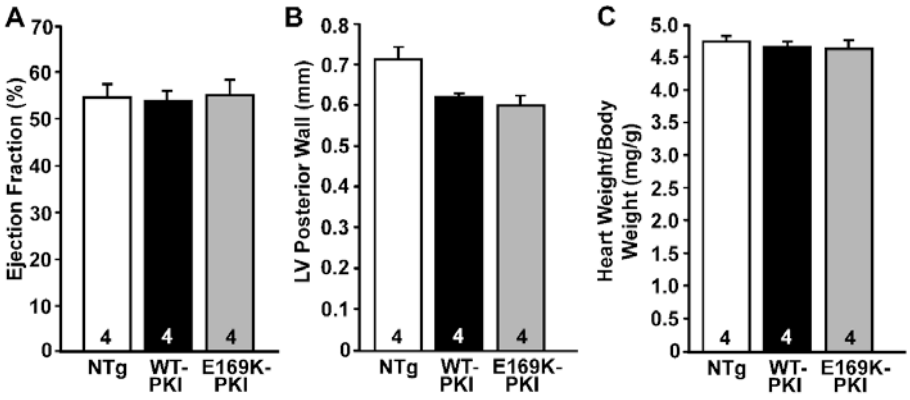
Online Figure S1



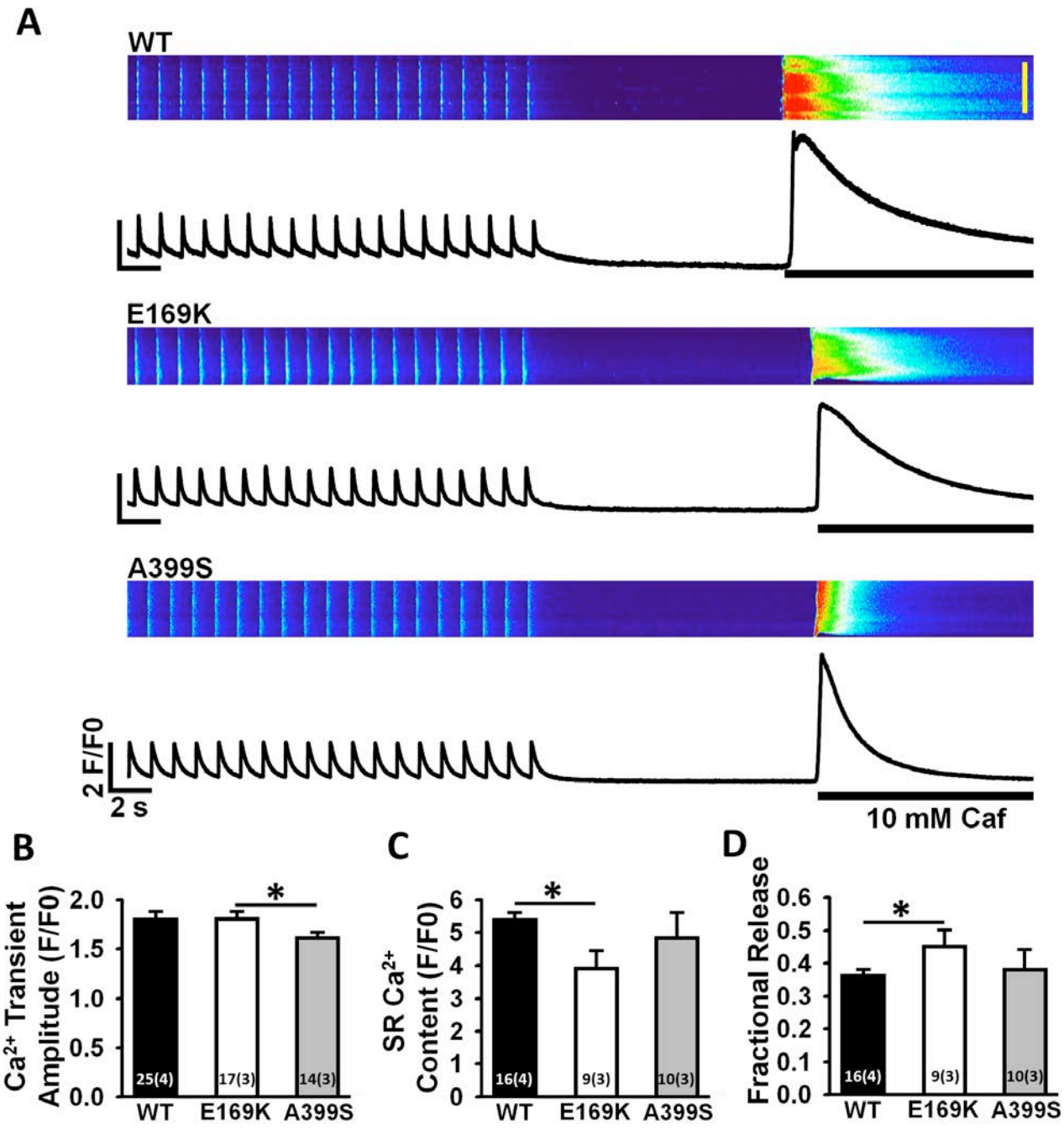
Online Figure S2



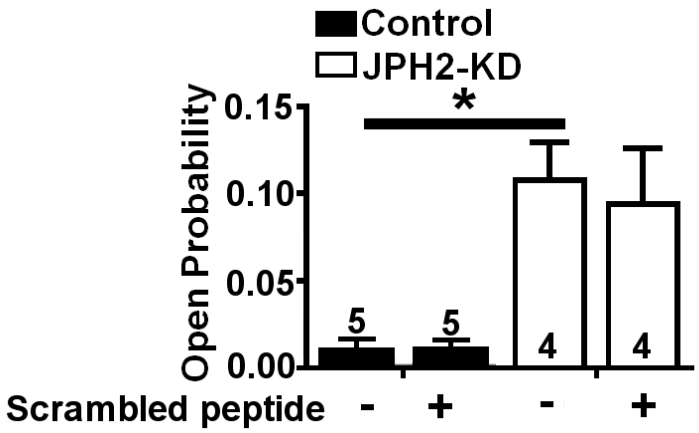
Online Figure S3



Online Figure S4



Online Figure S5



Online Figure S6