Supplemental Material

Inhibition of CaMKII Phosphorylation of RyR2 Prevents Induction of Atrial Fibrillation in FKBP12.6 knock-out Mice

Supplemental Methods

Animals. All animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine conforming the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health. FKBP12.6-deficient (-/-) and R176Q/+ mice were generously provided by Dr. Susan Hamilton (Baylor College of Medicine, TX).[1, 2] RyR2-S2814A knock-in mice (S2814A) were generated as previously described.[3] Serine2814 on RyR2 was substituted with alanine, which inhibits CaMKII phosphorylation of S2814 on RyR2. S2808A knock-in mice were generously provided by Dr. Andrew Marks (Columbia University, NY). [4, 5] Serine2808 on RyR2 was substituted with alanine such that PKA phosphorylation of S2808 on RyR2 was inhibited. FKBP12.6 deficient (-/-) and R176Q/+ mice were intercrossed with S2814A and S2808A mice, to obtain FKBP12.6-/-:S2814A or FKBP12.6-/-:S2808A mice, respectively. These mice were used for electrophysiology, biochemistry, Ca^{2+} imaging and patch clamp studies. S2814A and S2808A mice were also intercrossed with R176Q/+ mice to compound heterozygous R176Q/+:S2814A/+ or R176Q/+:S2808A/+ mice, respectively. All animals were studied at age of 3-4 month.

Optical mapping of intact atria. Whole atria electrophysiological studies were performed in WT (n=6) and FKBP12.6-/- (n=7) mice. Briefly, isolated hearts were Langendorff perfused with oxygenated Tyrode's solution and the voltage sensitive dye di-4-ANEPPS (4 mmol/ l). After 5-7 min of Langendorff perfusion the L and R atria were carefully dissected away from the ventricles and placed in a microscope imaging chamber for optical mapping. All preparations were

continuously superfused with normal Tyrode's solution at 37°C. Voltage dye fluorescence intensity was measured using a high-speed CCD camera that is attached to the microscope imaging system. Spontaneous atrial arrhythmias were defined as any atrial tachyarrhythmia lasting >30 s that developed during a 5 min period of electrophysiological monitoring without external pacing. Statistical differences were assessed with the Chi Square test.

***In vivo* electrophysiology in mice.** Briefly, atrial and ventricular intracardiac electrograms were recorded using an 1.1F octapolar catheter (EPR-800, Millar Instruments, Houston, Texas) inserted via the right jugular vein. Surface and intracardiac electrophysiology parameters were assessed at baseline. Right atrial pacing was performed using 2-ms current pulses delivered by an external stimulator (STG-3008, Multi Channel Systems, Reutlingen, Germany). Sinus node recovery time (SNRT) was measured after applying a 15-s atrial pacing train at a basic cycle length of 100 ms. SNRT is defined as the interval between the last stimulus of the pacing train and the onset of first spontaneous sinus beat. Atrial effective refractory period (AERP) and the effective refractory period of the atrioventricular effective refractory period (AVERP) were determined by applying a series of atrial pacing trains at a fixed basic cycle length of 100 ms (S1) with a S2 premature stimulus. The S1-S2 interval was progressively reduced by 2-ms in each pacing train from 70ms to 20ms. The AERP is defined as the longest S1-S2 coupling interval for atria that failed to generate a propagated beat with S2. The AVERP is defined as the longest S1-S2 coupling interval at which the premature stimulation delivered to the atrium is followed by a His potential but not by a QRS complex. Atrial fibrillation (AF) inducibility was determined by using the protocol described by Sood *et al.*,[6] and defined as the occurrence of rapid and fragmented atrial electrograms with irregular AV-nodal conduction and ventricular rhythm for at least 1 second. Inducibility of AF was considered positive if at least 2 of 3 pacing trials induced AF [3].

Western blot analysis Protein extraction and Western blotting was performed as previously described [3]. Briefly, mouse atrial lysates were subjected to electrophoresis on 5% (for RyR2, SERCA and NCX) and 12% (for PLN and CaMKII) acrylamide gels, and transferred onto polyvinyl difluoride membranes. Membranes were probed with monoclonal anti-RyR2 (1:5,000), monoclonal anti-PLN (1:5,000), polyclonal anti-Thr17-phosphorylated PLN (1:5,000, Badrilla), polyclonal anti-Ser16-phosphorylated PLN (1:5,000, Badrilla), monoclonal anti-Thr287-phosphorylated CaMKII (1:1,000) and polyclonal anti-CaMKIIδ (1:1000) antibodies at room temperature. The polyclonal anti-Ser2808-RyR2 (1:1000) and anti-Ser2814-RyR2 (1:1000) phosphoepitope-specific antibody were custom generated using the peptide C-RTRRI-(pS)-QTSQV corresponding to the PKA phosphorylation site region at serine 2808 on RyR2 and peptide CSQTSQV-(pS)-VD corresponding to RyR2 CaMKII phosphorylated at serine 2814, respectively. Membranes were then incubated with secondary anti-mouse and anti-rabbit antibodies conjugated to Alexa-Fluor 680 (Invitrogen Molecular Probes) and IR800Dye (Rockland Immunochemicals), respectively, and bands were quantified using Image J.

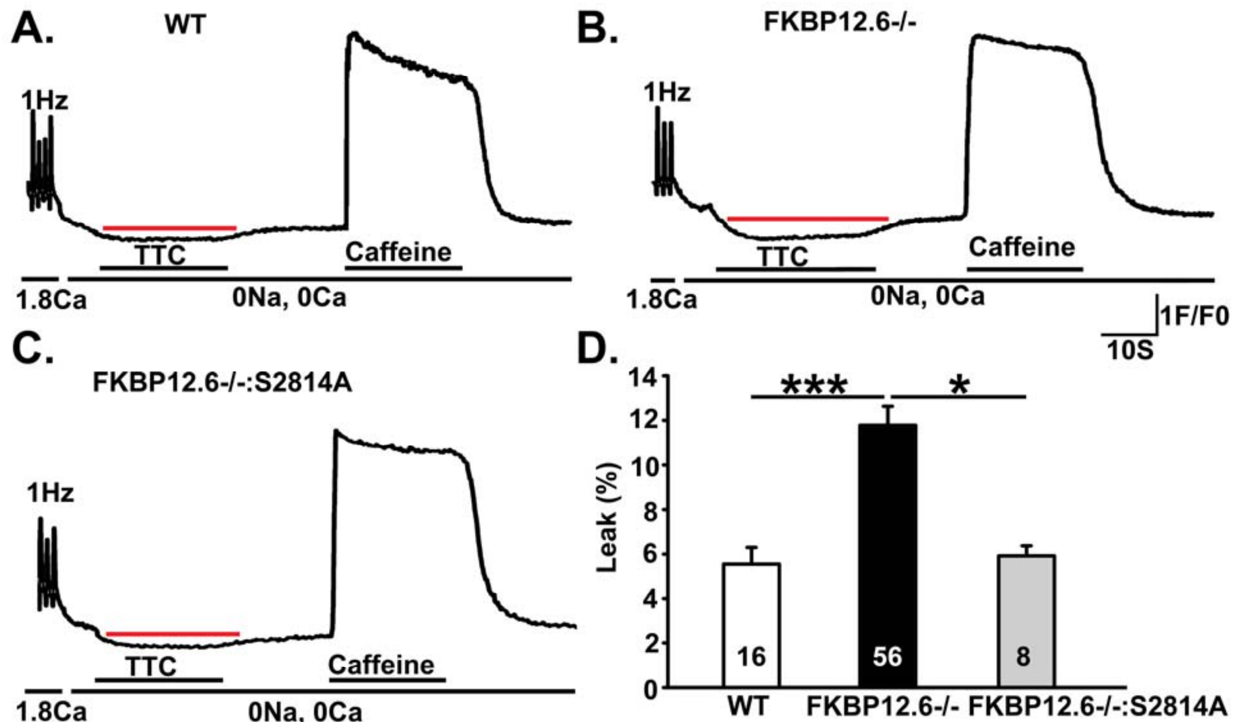
Atrial Myocyte Isolation. Mouse atrial myocytes were isolated as described previously.[3] Briefly, the heart was removed and the blood was washed out with 0 Ca^{2+} Tyrode solution (137 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, 10 mM glucose, 3 mM NaOH, pH 7.4). The heart was cannulated through the aorta and perfused on a Langendorff apparatus with 0 Ca^{2+} Tyrode for 3 to 5 minutes at 37 °C, followed by 0 Ca^{2+} Tyrode containing 20 μg/mL Liberase (Roche, Indianapolis, IN) for 10 to 15 minutes at 37 °C. After digestion, heart was perfused with 5 mL 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). Both left and right atrium were minced in KB solution and gently agitated, then filtered through a 210 mm polyethylene mesh. Atrial myocytes were stored in KB solution at room temperature before use.

Calcium imaging. Only rod-shaped myocytes showing clear striation were selected for further experiments.[6] Atrial myocytes were loaded with 2 μM Fluo-4-AM (Invitrogen, Carlsbad, CA) in normal Tyrode solution containing 1.8 mM Ca^{2+} for 30 minutes at room temperature. Cells were washed with Tyrode solution for 15 minutes for de-esterification and transferred to a chamber equipped with parallel platinum electrodes. For Ca^{2+} sparks recordings, the chamber was placed on a LSM510 confocal microscope (Carl Zeiss, Thornwood, NY). Fluorescence images were recorded in line-scan mode with 1024 pixels per line at 500 Hz. Once steady state Ca^{2+} transient induced by 1Hz-pacing (5 ms, 10 V) was observed, pacing was stopped for 45 seconds and Ca^{2+} sparks were counted. Steady state SR Ca^{2+} content was estimated by rapid application of caffeine (10mmol/L) after pacing. SR Ca^{2+} leak in atrial myocytes were measured as described in detail previously.[7] Myocytes were preconditioned in 1.8 mM Ca^{2+} normal Tyrode solution for 15 seconds by pacing delivered by platinum electrodes. Steady state $[Ca^{2+}]_i$ was then monitored after perfusate was switched to a $0Na^+$, $0Ca^{2+}$ Tyrode solution which blocks the Ca^{2+} exchange via the Na^+/Ca^{2+} exchanger and the L-type Ca^{2+} channel. Acute application of tetracaine (5 μmol/L) was used to rapidly and reversibly block RyR2, followed by application of caffeine (10mmol/L) to estimate steady-state SR Ca^{2+} content. The tetracaine-dependent shift of Ca^{2+} from cytosol to SR is proportional to SR Ca^{2+} leak.

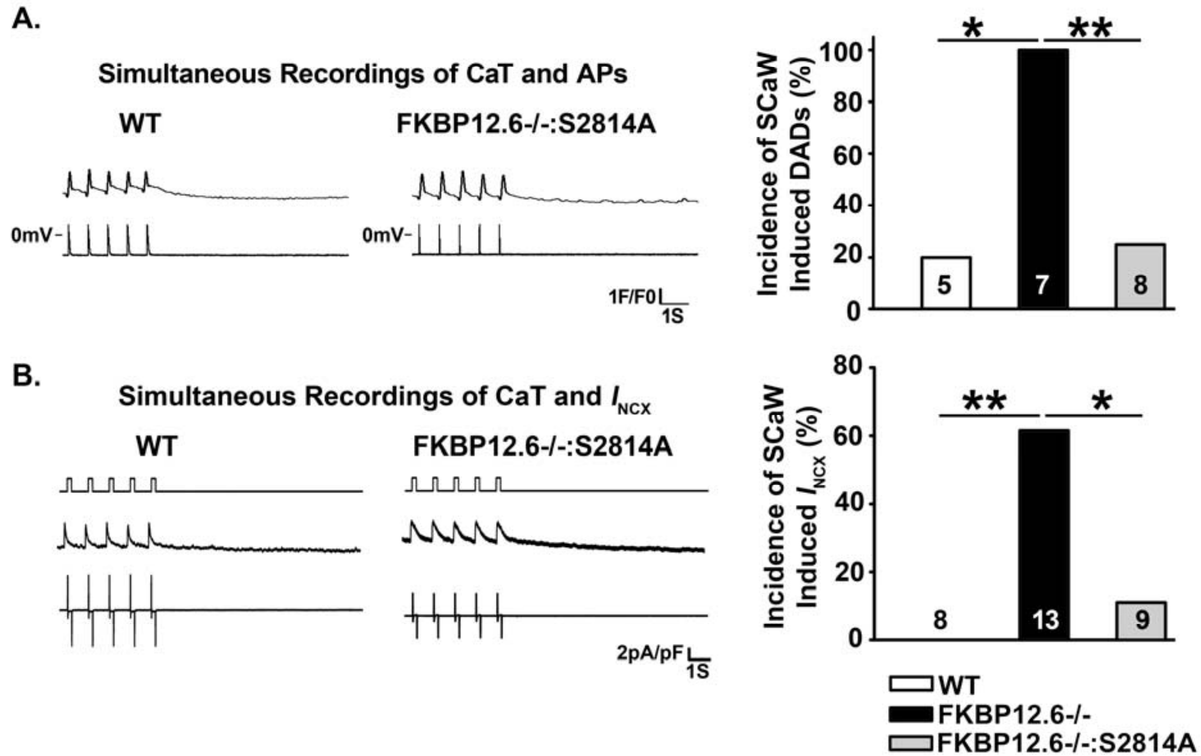
Patch Clamp. Membrane currents and potentials were measured in whole-cell configuration using voltage-clamp and current-clamp techniques, with simultaneous Ca^{2+} transients (CaTs) measurement. The resistances of glass microelectrodes were 2-5 MΩ when filled with pipette solution (mmol/L: K-aspartate 120, KCl 20, $MgCl_2$ 1, Na_2-ATP 5, EGTA 0.02, HEPES 10; pH=7.2). Series resistance and cell capacitance were compensated. For simultaneous recording of CaT and I_{NCX}, myocytes were superfused with a bath solution containing (mmol/L): NaCl 140, KCl 5.4, $MgCl_2$ 1, $CaCl_2$ 3, glucose 10, HEPES 10, $BaCl_2$ 0.5; pH=7.4). CaT was

induced by a 200 ms conditioning pulse from -40 mV to 0mV at 1Hz.

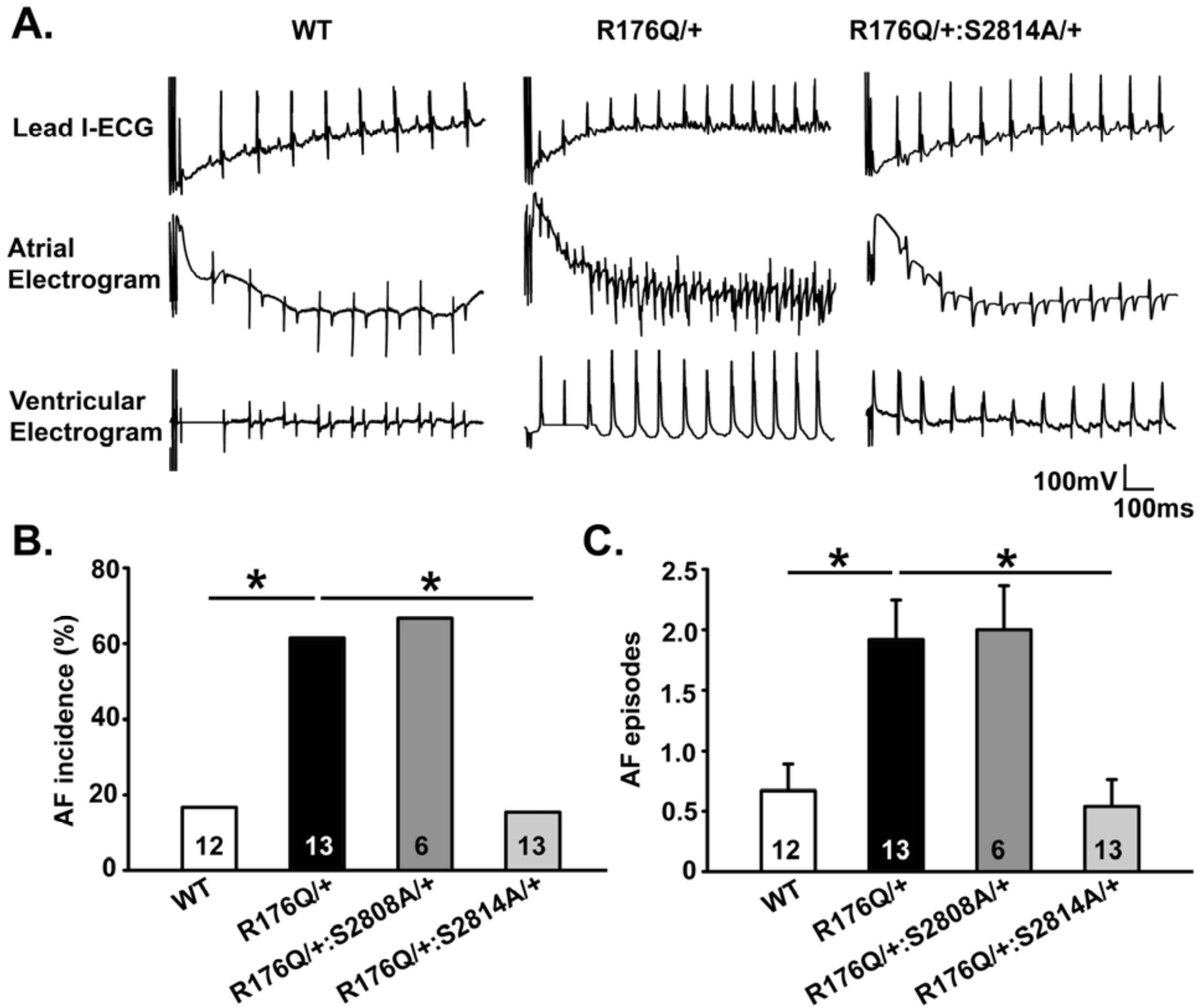
Supplemental Figures and Figure Legends



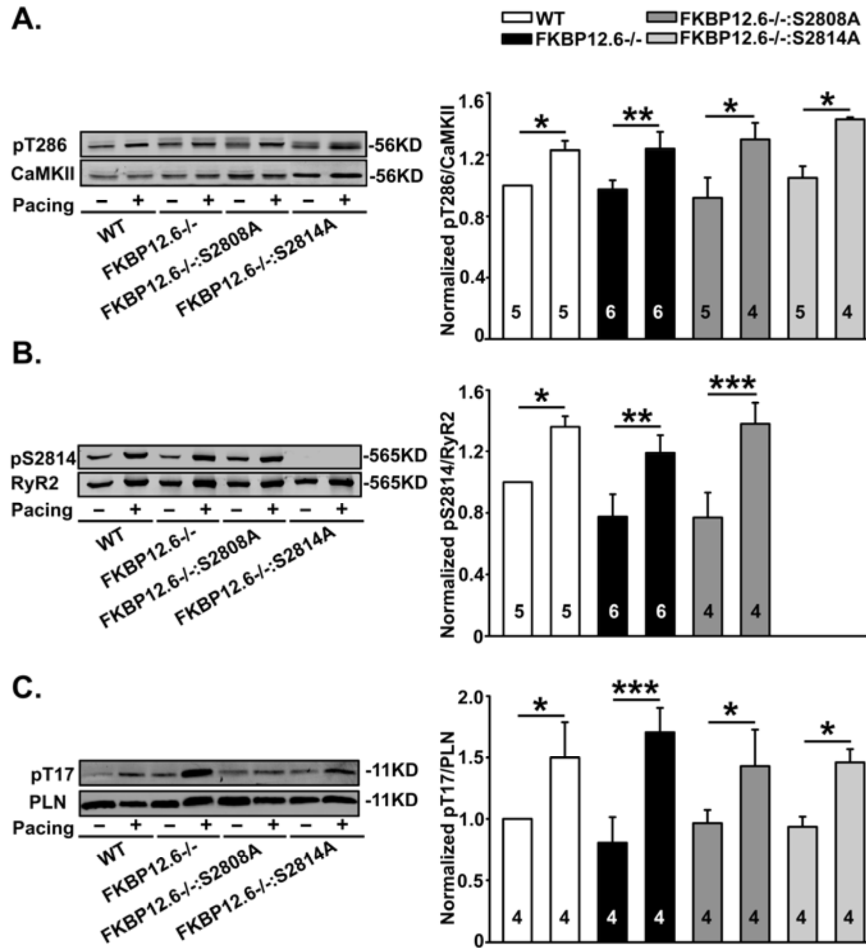
Online Figure I. Increased SR Ca^{2+} leak in FKBP12.6-/- mice depends on RyR2-S2814 phosphorylation level. A-C. Representative $[Ca^{2+}]_i$ tracings from fluo-4-AM loaded atrial myocytes paced at 1 Hz followed by rapid switch to Tyrode containing 0 Na^+, 0 Ca^{2+}, and 1 mmol/L tetracaine (TTC) to block RyR2. SR Ca^{2+} content was measured by adding 10 mmol/L caffeine. SR Ca^{2+} leak (curve below red baseline) was quantified and normalized to SR Ca^{2+} content (**D**). Numbers in bars indicate numbers of cells studied from 3-4 mice in each group. **P* < 0.05, ****P*<0.001.



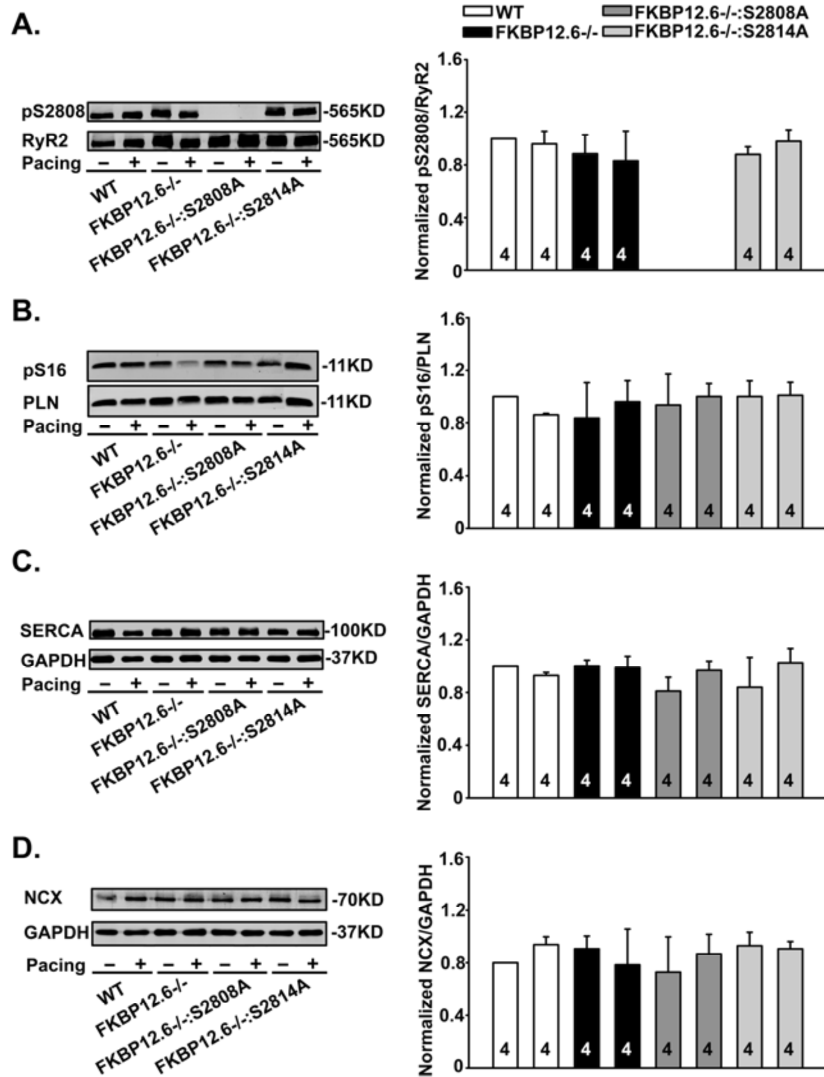
Online Figure II. A. Left panel**:** Representative simultaneous recording of Ca^{2+} transients and membrane potential in WT and FKBP12.6-/-:S2814A atrial myocytes. Right panel: Bar graph summarizing the incidence of SCaW induced DADs. **B.** Left panel**:** Representative simultaneous recording of Ca^{2+} transients and membrane current in WT and FKBP12.6-/-:S2814A atrial myocytes. Right panel: Bar graph summarizing the incidence of SCaW induced I_{NCX}. Numbers in bars indicate numbers of cells tested from 2-3 animals. *P<0.05, **P<0.01



Online Figure III. S2814A but not S2808A mutation prevents pacing-induced AF in R176Q/+ knockin mice. A. Because the S2814A mutation is located in the same gene (i.e., *RYR2*) as the R176Q mutation, compound heterozygous mice were generated with mutation R176Q/+ in one *RYR2* allele, and S2814A (or S2808A) in the other allele. Simultaneous surface ECG (lead 1), intracardiac atrial and ventricular electrograms revealed atrial fibrillation in R176Q/+ mice after burst pacing. WT and R176Q/+:S2814A/+ mice typically had sinus rhythm following rapid pacing. **B-C.** Bar graphs summarizing the incidence (**B**) and number of episodes (**C**) of reproducible AF in the respective genotypes of mice. Numbers in bars indicate numbers of mice tested in each group. *$P < 0.05$.



Online Figure IV. Pacing-induced activation of CaMKII leads to phosphorylation of RyR2 and PLN. A. Western blots were used to measure total CaMKII and T286 phosphorylation levels in atria excised at resting heart rates or immediately after rapid burst pacing. Bar graphs showing T286 phosphorylation normalized to total CaMKII levels. **B.** Western blots were used to measure total RyR2 and S2814 phosphorylation levels in atria excised at resting heart rates or immediately after rapid burst pacing. Bar graphs showing S2814 phosphorylation normalized to total RyR2 levels. **C.** Western blots were used to measure total PLN and T17 phosphorylation levels in atria excised at resting heart rates or immediately after rapid burst pacing. Bar graphs showing phosphorylation level normalized to total levels. Numbers in bars indicate numbers of mice tested in each group. *P < 0.05, **P<0.01, ***P<0.001.



Online Figure V. PKA phosphorylation levels of RyR2 and PLN and protein expressions of SERCA and NCX were unaltered. A-B. Western blots were used to measure PKA phosphorylation levels of RyR2 at S2808 (**A**) and PLN at S16 (**B**) in atria excised at resting heart rates or immediately after rapid burst pacing. Bar graphs showing phosphorylation level normalized to total levels. **C-D.** Western blots were used to measure SERCA (**C**) and NCX (**D**) levels in atria excised at resting heart rates or after rapid burst pacing. Bar graphs showing protein level normalized to housekeeping protein GAPDH levels. Numbers in bars indicate numbers of mice tested in each group.

Supplemental Table

Online Table I. Cardiac electrophysiological parameters

	WT (n = 15)	FK (n = 19)	FK-S2808A (n = 23)	FK-S2814A (n = 22)
HR (bpm)	519 ± 15	547 ± 13	544 ± 16	521 ± 10
PR (ms)	35.9 ± 0.9	35.7 ± 1.0	37.9 ± 0.8	38.5 ± 1.0
QRS (ms)	10.1 ± 0.3	10.4 ± 0.4	10.8 ± 0.4	10.1 ± 0.3
QTc (ms)	24.3 ± 0.9	25.9 ± 0.9	27.2 ± 0.8	26.1 ± 0.7
SNRT (ms)	141.8 ±4.3	142.2 ± 9.9	138.9 ± 3.7	151.9 ± 7.3
AERP (ms)	35.7 ± 1.1	37.9 ± 1.5	37.5 ± 0.9	41.1 ± 1.4
AVERP (ms)	46.1 ± 1.2	45.8 ± 1.9	43.9 ± 1.2	47.3 ± 1.2

Supplemental References

1. Kannankeril PJ, Mitchell BM, Goonasekera SA, Chelu MG, Zhang W, Sood S, Kearney DL, Danila CI, De Biasi M, Wehrens XH, Pautler RG, Roden DM, Taffet GE, Dirksen RT, Anderson ME, Hamilton SL. Mice with the R176Q cardiac ryanodine receptor mutation exhibit catecholamine-induced ventricular tachycardia and cardiomyopathy. *Proc Natl Acad Sci U S A*. 2006;103:12179-12184.
2. Wehrens XH, Lehnart SE, Huang F, Vest JA, Reiken SR, Mohler PJ, Sun J, Guatimosim S, Song LS, Rosemblit N, D'Armiento JM, Napolitano C, Memmi M, Priori SG, Lederer WJ, Marks AR. FKBP12.6 deficiency and defective calcium release channel (ryanodine receptor) function linked to exercise-induced sudden cardiac death. *Cell*. 2003;113:829-840.
3. Chelu MG, Sarma S, Sood S, Wang S, van Oort RJ, Skapura DG, Li N, Santonastasi M, Muller FU, Schmitz W, Schotten U, Anderson ME, Valderrabano M, Dobrev D, Wehrens XH. Calmodulin kinase II-mediated sarcoplasmic reticulum Ca2+ leak promotes atrial fibrillation in mice. *J Clin Invest*. 2009;119:1940-1951.
4. Lehnart SE, Wehrens XH, Reiken S, Warrier S, Belevych AE, Harvey RD, Richter W, Jin SL, Conti M, Marks AR. Phosphodiesterase 4D deficiency in the ryanodine-receptor complex promotes heart failure and arrhythmias. *Cell*. 2005;123:25-35.
5. Wehrens XH, Lehnart SE, Reiken S, Vest JA, Wronska A, Marks AR. Ryanodine receptor/calcium release channel PKA phosphorylation: a critical mediator of heart failure progression. *Proc Natl Acad Sci U S A*. 2006;103:511-518.
6. Sood S, Chelu MG, van Oort RJ, Skapura D, Santonastasi M, Dobrev D, Wehrens XH. Intracellular calcium leak due to FKBP12.6 deficiency in mice facilitates the inducibility of atrial fibrillation. *Heart Rhythm*. 2008;5:1047-1054.

7. Sarma S, Li N, van Oort RJ, Reynolds C, Skapura DG, Wehrens XH. Genetic inhibition of PKA phosphorylation of RyR2 prevents dystrophic cardiomyopathy. *Proc Natl Acad Sci U S A*. 2010;107:13165-13170.