Supplemental Material

Supplemental Methods

Human atrial samples. The collection of human tissue samples was approved by the Institutional Review Board of Medical Faculty Mannheim, Heidelberg University (No. 2011–216 N-MA).[1] Right atrial appendages were obtained from 12 sinus rhythm (Ctl) patients, 6 paroxysmal and 4 long-standing persistent (chronic) AF patients undergoing open-heart surgery. Patient characteristics are provided in Supplemental Table S4. All patients gave informed consents.

Study animals. All animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine conforming to the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health. CREM-IbΔC-X (CREM) transgenic mice were previously established on a FVB/N background[2] and RyR2^{S2814A} (S2814A) knock-in mice were previously established on the C57BL/6 background.[3] In S2814A mice, Serine2814 on RyR2 was substituted with alanine, which inhibits CaMKII phosphorylation of S2814 on RyR2. We intercrossed CREM mice with S2814A mice, to obtain the CREM:S2814A mice. Wild-type (WT), CREM and CREM:S2814A mice on a mixed genetic background were used in the current study.

***Telemetry ECG recordings*.** Mice were implanted with telemeters (Data Sciences International, MN, USA) as previously described.[4] 24-hour ambulatory ECG was monitored in conscious mice at ages of 3, 5 and 7 months. Telemetry ECG recordings were analyzed using ECG-Auto software (emka Technologies). When a mouse exhibited at least one atrial ectopic complex during 24-hour recording, it was considered as atrial ectopy positive. Quantification of spontaneous atrial ectopic events was obtained between 12 p.m. and 1 p.m. to exclude confounding effects of circadian variation. AF was defined by absent P-waves and irregular R-R intervals on telemetry ECG recording for at least 1 second. When a mouse exhibited one

episode of AF longer than 1 second during 24-hour recording, it was considered to show sAF.

Intracardiac electrophysiology in mice. *In vivo* electrophysiology studies were performed in mice at the age of 4-5 months, as previously described.[5] 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). AF inducibility was determined by using an overdriving pacing protocol and defined as the occurrence of rapid and fragmented atrial electrograms with irregular AV-nodal conduction and ventricular rhythm for at least 1 second.

***Transthoracic echocardiography*.** Cardiac function was assessed using a VisualSonics VeVo 770 Imaging System (VisualSonics, Toronto, Canada) equipped with high-frequency 30 MHz probe, as described.[6]

***Mouse atrial myocyte isolation*.** Atrial myocytes were isolated by a modified collagenase method as described.[5] 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.

***Ca^{2+} imaging*.** Atrial myocytes were loaded with 2 μmol/L Fluo-4-AM (Invitrogen, Carlsbad, CA) in normal Tyrode solution containing 1.8 mmol/L 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 20 seconds and Ca^{2+} sparks were counted using SparkMaster.[7] Steady state SR Ca^{2+} content was estimated by rapid application of caffeine (10mmol/L) after pacing.

Sarcoplasmic reticulum preparation. Atrial tissues were homogenized on ice in a solution containing (in mmol/L) HEPES 10, sucrose 500 and EDTA 5, supplemented with NaF 20, Na_3VO_4 1, and protease inhibitor and phosphatase inhibitor cocktails (cOmplete, Mini and PhosSTOP from Roche Applied Science, Indianapolis, IN). The samples were subsequently subjected to three steps of centrifugation: 1) 3,800 g for 15 min at 4 °C; 2) the supernatants from step 1 were collected and centrifuged at 27,900 g for 15 min at 4 °C; 3) the supernatants from step 2 were ultracentrifuged at 110,000 g for 1 hour at 4 °C. The supernatants from step 3 were discarded while the pellets were resuspended in 50 μl of the same solution as for homogenization but without the EDTA. The resultant suspensions were used for single channel recording.

Single-channel recordings of RyR2 channels. Single-channel recordings were obtained under voltage-clamp conditions at 0 mV, as previously described.[8] Atrial SR membrane-

preparations were incorporated into lipid-bilayer membranes comprised of a mixture of phosphatidylethanolamine and phosphatidylserine at a ratio of 3:1 (Avanti Polar Lipids, Alabaster, AL) dissolved in n-decane (25 mg/mL). Bilayers were formed across a 150 μm aperture of a polystyrene cuvette. The *cis* and *trans* chambers correspond to the cytosolic and the luminal sides of the SR, respectively. The *trans* chamber contained (in mmol/L) HEPES 250, KCl 50 and $CaOH_2$ 53. The *cis* chamber contained (in mmol/L) HEPES 250, Tris-base 125, KCl 50, EGTA 1, $CaCl_2$ 0.35, pH=7.35. Data were collected using Digidata 1322A (Molecular Devices, Sunnyvale, CA) and Warner Bilayer Clamp Amplifier BC-535 (Warner Instruments, Hamden, CT) under voltage-clamp conditions. Data were analyzed from digitized current recordings using pCLAMP-9.2 software (Molecular Devices).

***Optical mapping*.** Optical mapping of action potentials in the mouse atria was performed in mice at the age of 3-5months as previously described.[3] Hearts were rapidly removed and retrogradely perfused with oxygenated Tyrode's solution (containing (mM): 137 NaCl, 5.4 KCl, 1 MgCl2, 2 NaH2PO4, 10 HEPES, 10 glucose, pH adjusted to 7.4 with NaOH) containing 1.2 mM CaCl2 at 37°C in a Langendorff apparatus. The perfusion pressure was maintained constant at ~60-65 mmHg and monitored using an in-line physiological pressure transducer connected to a Bridge Amplifier (AD Instruments Inc). To prevent motion artifacts, blebbistatin at a final concentration of 5 μmol/L was applied as an excitationcontraction uncoupler. The heart was then stained with the voltage-sensitive dye RH237 (0.33 μM) for 10 minutes to monitor changes in membrane potential. After staining, atria were cut and imaged in a chamber perfused with Tyrode containing 1.2 mM CaCl2. Green light (520±20nm) from a 532 nm laser light source (B&W TEK Inc) was shone directly onto the atrial tissue. Emitted fluorescence was collected using electron-multiplying CCD camera (Cascade 128+, Photometrics) covering the same mapped field. A grid was used to calibrate the field of view of the CCD camera. For membrane

voltage, passed fluorescence was collected through a 710 nm long pass filter.

Whole cell patch clamp. L-type Ca^{2+} currents were recorded using the patch clamp technique in the whole-cell configuration. Patch pipettes were pulled on a horizontal Flaming Brown micropipette puller (P-87, Sutter Instruments, Inc.) and the tips were coated with wax (KERR Sticky Wax). When filled with pipette solution, the resistance of the patch pipettes was in a range of 3-5 MΩ. Pipette solution contained (in mM): 140 NMDG, 20 HEPES, 5 EGTA, 10 HCl. The bath solution contained (in mM): 130 TEAOH, 1 $MgCl_2$, 10 HEPES, 10 $CaCl_2$. The pH of the solutions was adjusted to 7.2 with MES. Experiments were performed at room temperature (20-22^{o}C). Data were acquired with an Axopatch 200B amplifier (Molecular Devices). Currents were subsequently filtered by an 8-pole Bessel filter (Frequency Device, Inc.) at 5 kHz and sampled at 200 kHz with an 18-bit A/D converter (Instrutech ITC-18). A P/8 protocol was used for leak subtraction with a holding potential of -100 mV. Data were digitally filtered at 1 kHz during analysis with Igor Pro (WaveMetrics, Inc.).

Western blotting. Western blot analysis was performed in atrial samples of study animals at the ages of 1 month (no atrial arrhythmias), 3 months (atrial ectopy prior to the onset of spontaneous AF), and 7 months (long-lasting spontaneous AF). Protein extraction and western blotting was performed as previously described.[5] Briefly, mouse atrial lysates were subjected to electrophoresis on 5% (for RyR2) and 10 or 12% (for other proteins) acrylamide gels, and transferred onto polyvinyl difluoride membranes. Membranes were probed with mouse anti-RyR2 (1:5,000, #MA3-916, ABR), mouse anti-Thr287-phosphorylated CaMKII (1:1,000, #10011438, Cayman Chemical), rabbit anti-CaMKIIδ (1:1,000, #S2169, Epitomics), rabbit anti-Thr17-phosphorylated PLN (1:5000, #A010-13, Badrilla), mouse anti-PLN (1:5,000, #MA3-922, Pierce), rabbit anti-Cx40 (1:1,000, #AB1726, Millipore), and mouse anti-GAPDH (1:10,000, #MAB-374, Millipore) antibodies at room temperature. The rabbit anti-Ser2808-RyR2 (1:1,000)

and anti-Ser2814-RyR2 (1:1,000) 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.

***Histology*.** Whole hearts were excised, washed briefly in normal saline, blotted dry and then fixed in 4% buffered formaldehyde for 48h. Longitudinal 5μm sections were stained with Masson-Trichrome for fibrosis. Fibrosis were quantified as previously described.[9]

***Statistical analysis*.** Continuous variables are presented as mean±SEM and categorical data are presented as percentages. Statistical analysis was performed using SPSS Statistics (IBM, USA). One-way ANOVA followed by the post-hoc Bonferroni t-test was applied for multiple group non-repeated measures data and paired t-test for single repeated measures. Fisher's exact test was used to compare categorical data. To compare continuous variables with a skewed distribution, the Mann-Whitney test was applied. $P<0.05$ was considered statistically significant.

Supplemental Tables

Table S1. Characteristics of Ca^{2+} sparks

	WT (n=157)	CREM (n=360)	CREM:S2814A (n=228)
Amplitude (F/F0)	0.23 ± 0.01	0.27 ± 0.004***	0.25 ± 0.01#
FWHM	1.52 ± 0.05	1.46 ± 0.03	1.56 ± 0.04#
FDHM	17.2 ± 0.5	27.8 ± 0.7***	21.1 ± 0.6###
Full Width	2.48 ± 0.09	2.30 ± 0.06	2.7 ± 0.09###
Full Duration	35.2 ± 1.3	48.0 ± 1.3***	42.3 ± 1.5##
TdP	12.4 ± 0.5	15.8 ± 0.6***	14.0 ± 0.6#
Dvdt	26.3 ± 0.8	27.0 ± 0.4	28.3 ± 0.9
tau	15.9 ± 0.8	32.2 ± 1.3***	20.2 ± 0.9###

FWHM: Full width at half maximum of full width; FDHM: Full duration at half maximum.

*P<0.05, **P<0.01, ***P<0.001 vs WT; #P<0.05, ##P<0.01, ###P<0.001 vs CREM.

Table S2. Cardiac electrophysiological parameters at the age of 5 months.

	WT (n=7)	CREM (n=12)	CREM:S2814A (n=12)
HR (bpm)	622 ± 14	598 ± 18	602 ± 17
PR (ms)	33.9 ± 1.5	43.9 ± 1.0*	39.8 ± 1.3#
QRS (ms)	9.8 ± 0.2	10.1 ± 0.3	10.2 ± 0.4
QTc (ms)	26.6 ± 2.4	27.0 ± 0.7	28.2 ± 0.7
cSNRT (ms)	9.2 ± 1.4	13.4 ± 2.6	12.5 ± 4.5
AVERP (ms)	48.7 ± 1.4	48.0 ± 1.6	48.2 ± 1.8
AERP (ms)	38.2 ± 1.1	33.5 ± 1.9	37.6 ± 2.1

*P<0.05 vs WT; #P<0.05 vs CREM

Table S3. Echocardiographic parameters of WT, CREM, and CREM:S2814A mice at 3 and 5 months of age.

	3 months			5 months		
	WT (n=9)	CREM (n=9)	CREM:S2814A (n=7)	WT (n=7)	CREM (n=6)	CREM:S2814A (n=10)
HR (bpm)	483.9±15.7	501.3±14.6	486.9±21.2	477.1±12.3	455.7±16.6	463.0±14.9
EF (%)	59.0±1.5	54.6±1.9	59.3±1.8	57.9±2.7	61.2±1.9	56.0±1.7$^{\#\#}$
FS (%)	31.0±1.0	28.1±1.2	31.1±1.2	30.3±1.8	32.6±1.4	28.9±1.1$^{\#\#}$
$LVAW_d$ (mm)	0.72±0.04	0.77±0.04	0.69±0.03	0.76±0.02	0.76±0.03	0.79±0.03
$LVID_d$ (mm)	4.08±0.07	4.21±0.11	3.96±0.04	4.11±0.14	4.14±0.11	4.03±0.08
$LVPW_d$ (mm)	0.61±0.03	0.61±0.01	0.57±0.02*	0.60±0.03	0.60±0.04	0.62±0.03
$LVAW_s$ (mm)	0.98±0.06	1.01±0.04	1.05±0.05	1.07±0.04	0.97±0.08	0.92±0.05*
$LVID_s$ (mm)	2.82±0.07	3.04±0.12	2.74±0.04	2.87±0.12	2.69±0.07	2.85±0.11$^{\#}$
$LVPW_s$ (mm)	0.93±0.05	0.90±0.03	0.92±0.05	0.88±0.04	0.94±0.04	0.95±0.05

Data are expressed as mean ± SEM. *P<0.05 vs WT, and $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs CREM. BW= body weight; HR = heart rate; EF = ejection fraction; FS = left ventricular fractional shortening; LVAW = left ventricular anterior wall thickness; LVPW = left ventricular posterior wall thickness. Subscript letters represent during diastole or systole.

Table S4. Characteristics of patients.

	Ctl	pAF	cAF
Patients, n	12	6	4
Gender, M/F	8/4	3/3	4/0
Age, y	67.6 ± 2.2	76.7 ± 2.4*	66.8 ± 0.2
Body mass index, kg/m^2	29.2 ± 1.1	26.7 ± 1.6	29.3 ± 1.1
CAD, n	9	3	4
MVD/AVD, n	0	1	0
CAD+MVD/AVD, n	3	2	0
Hypertension, n	10	5	2
Diabetes, n	2	0	0
Hyperlipidemia, n	8	3	2
LVEF, %	53.3 ± 4.4	53.3 ± 4.1	50.8 ± 6.6
Digitalis, n	1	1	2
ACE inhibitors, n	8	3	3
AT1 blockers, n	1	0	0
β-Blockers, n	8	5	3
Dihydropyridines, n	3	3	1
Diuretics, n	3	3	2
Nitrates, n	1	2	1
Lipid-lowering drugs, n	9	3	2

Ctl, control patients in sinus rhythm; pAF, paroxysmal atrial fibrillation; cAF, chronic atrial fibrillation; 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 Ctl from unpaired Student's *t*-test for continuous variables and from Fisher's exact test for categorical variables.

Supplemental References

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Supplemental Figures

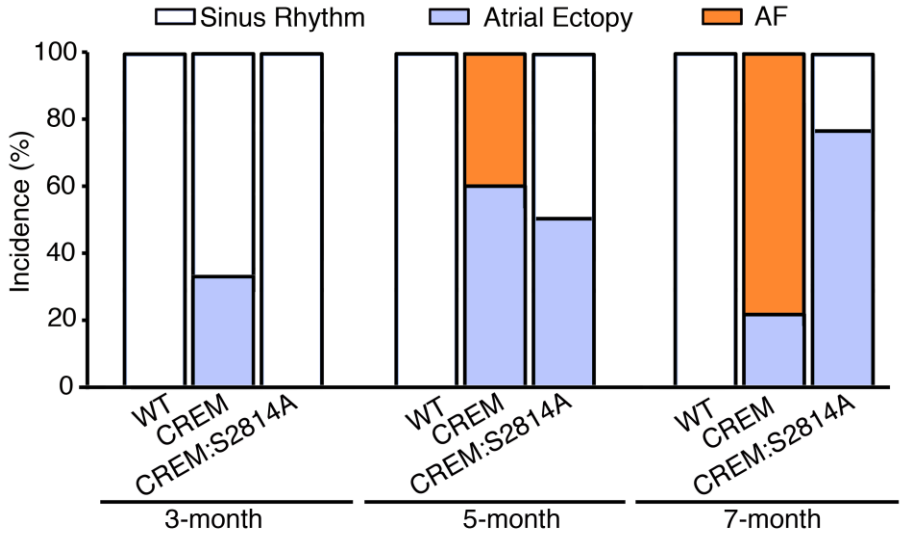

Figure S1. Stacked bar graphs summarizing the incidence of spontaneous atrial ectopy and AF in WT, CREM and CREM:S2814A mice at 3 different ages.

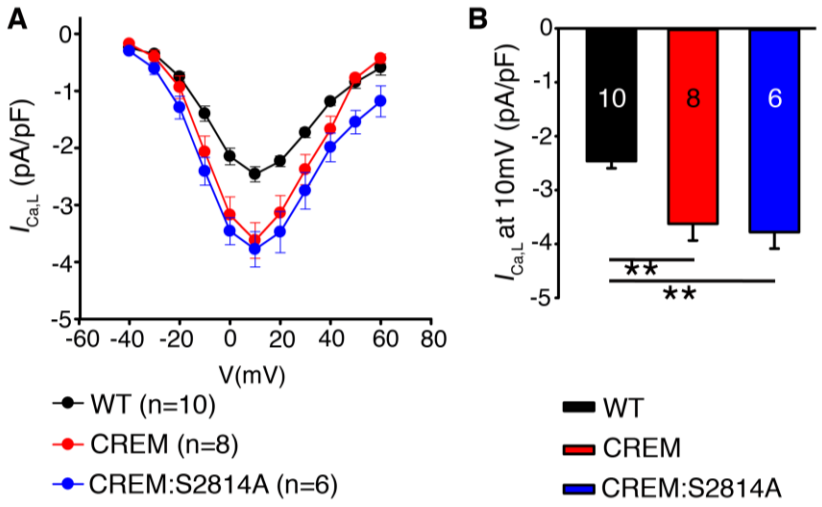

Figure S2. Enhanced L-type Ca^{2+} currents in atrial myocytes**. A.** Current-voltage relationships of $I_{Ca,L}$ obtained in atrial myocytes from WT, CREM and CREM:S2814A. **B.** The peak amplitude of $I_{Ca,L}$ obtained at 10mV. Numbers in the graph indicated number of cells studied from 3-4 mice in each group. **P<0.01.

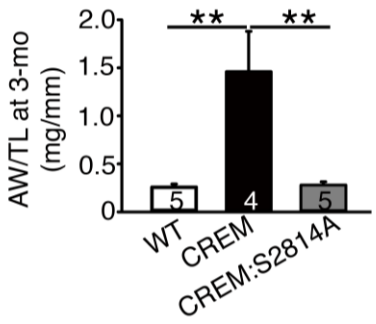

Figure S3. Increased AW/TL in CREM mice at the age of 3 months. **P<0.01.

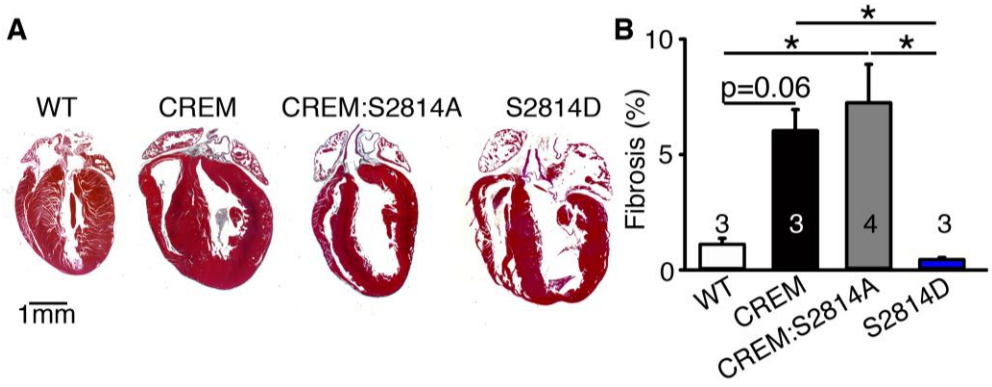

Figure S4. Increased fibrosis in CREM and CREM:S2814A mice. **A.** Masson-Trichrome staining of fibrosis in longitudinal sections. **B.** Quantification of ventricular fibrosis**.** Numbers in the bars indicate the number of animals studied. *P<0.05.