SUPPLEMENTAL MATERIAL
SUPPLEMENTAL METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Materials

Unless otherwise stated, all reagents have been purchased from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Munich, Germany).

Experimental animals

Mice with cardiomyocyte-directed overexpression of hemagglutinin (HA)-tagged CREM-IbΔC-X (CREM TG) were described previously\textsuperscript{11,12}. Wild-type (WT) littermates have been used as control. Mice were kept at a room temperature of 22°C, under a 12-h light/dark cycle and received normal diet (Altromin Spezialfutter GmbH, Lage, Germany) and water \textit{ad libitum}. The mice were euthanized by CO\textsubscript{2} inhalation. Animals were handled and maintained according to the rules of local welfare authority and the Directive 2010/63/EU of the European Parliament. The animals were grouped in 4 categories according to age (5-7 and 11-13 weeks) and genotype (WT and TG) as follows: WT\textsubscript{6w}, TG\textsubscript{6w}, WT\textsubscript{12w} and TG\textsubscript{12w}.

\textit{In vivo ECG measurements}

Electrocardiogram (ECG) measurements were performed in isoflurane anesthetized mice to confirm the absence (in WT\textsubscript{6w}, TG\textsubscript{6w} and WT\textsubscript{12w} mice) and the presence of persistent atrial fibrillation (in TG\textsubscript{12w}). Mice were anesthetized with isoflurane (1.2% v/v) and nitrous oxide (66% v/v) while being placed on a warmed pad in supine position. Needle electrodes were attached to obtain limb leads (Einthoven). Short ECGs were recorded for 1 minute after achieving deep anesthesia (in 5 minutes) using a PowerLab hardware and LabChart Pro software (ADInstruments, Bella Vista, Australia). A section of 20 seconds at steady state of
the ECG recordings were analyzed offline using Heart Rate Variability module (HRV) to measure the following parameters: mean heart rate (HR), standard deviation of NN intervals (SDNN), root mean square of the successive differences between neighboring RR intervals (RMSSD). After ECG measurements the hearts were used for cell isolation or biochemical assays.

**Tissue isolation**

The hearts were removed in ice cold calcium free Tyrode’s solution. Both atria were quickly cut from the heart, frozen in liquid nitrogen and stored for further analysis using qRT-PCR, Western blot and Chromatin immunoprecipitation (ChIP) assays.

**Isolation of atrial cardiomyocytes**

Atrial cardiomyocytes were isolated from all 4 groups of mice, using the retrograde perfusion of the hearts to digest the connective tissue with collagenase Type II (230U/mg; Worthington, Lakewood, NJ, USA) as described before. The TG mice exhibiting AF at the age of 5-7 weeks were randomly detected with low incidence. Due to the small number and the aim of our study they were not included or sufficient for further investigation. Prior to cell isolation, it was essential to perform ECG measurements on all mice to test for AF presence or absence for grouping the data after analysis, therefore, not all experimenters were blinded. Moreover, due to the strong atrial phenotype, the quality of cell isolation was poor (few or no cells) in TG mice, meaning that more TG than WT mice had to be sacrificed for this study.

**Electrophysiology**

Ca\(^{2+}\)-tolerant atrial myocytes were used for the measurements of ionic currents using the patch-clamp technique in perforated-patch configuration achieved with amphotericin B (300 μg/mL; Sigma-Aldrich Chemie GmbH, Steinheim, Germany). All recordings have been performed at room temperature (22-24 °C). Borosilicate glass capillaries (Science Products GmbH, Hofheim, Germany) were pulled to a resistance of 3-5MΩ with a horizontal puller (P-
Data were sampled with an 18-bit A/D converter InstruTech ITC-18 and filtered at 10 kHz using an EPC-800 amplifier under the control of the PatchMaster software (HEKA Elektronik, Lambrecht, Germany). The average values for the seal resistances: 1.35 ± 0.15 GΩ (WT6w, n = 56), 0.93 ± 0.18 GΩ (TG6w, n = 37), 1.09 ± 0.13 GΩ (WT12w, n = 56) and 1.07 ± 0.24 GΩ (TG12w, n = 32) were not significantly different between groups. The series resistances reached on average 22.9 ± 1.6 MΩ (WT6w, n = 56), 22.4 ± 1.9 MΩ (TG6w, n = 37), 23.2 ± 1 MΩ (WT12w, n = 56) and 22.6 ± 1.3 MΩ (TG12w, n = 32) and were compensated between 52 % and 66 % in order to insure the stability of the recordings. An average level of compensation of 55 % was achieved in each group.

To record action potentials and K⁺-currents, the cells were perfused with a bath solution containing (in mM): 136 NaCl, 5.4 KCl, 1 CaCl₂, 1 MgCl₂, 5 HEPES, 0.33 NaH₂PO₄ and 10 glucose, adjusted to pH 7.4 with NaOH and the pipettes were filled with a solution containing (in mM): 5 NaCl, 120 KCl, 2.5 MgATP, 1 EGTA and 5 HEPES adjusted to pH 7.2 with KOH. The 10 μM solutions of BaCl₂ or acetyl choline (ACh) were freshly prepared from 100 mM BaCl₂ or 100 mM ACh, respectively, stock solutions in water.

To measure the total outward K⁺ currents, the cells were clamped at -80 mV followed by a 10 ms prepulse to -40 mV to inactivate the sodium channel and a series of 500 ms voltage steps from -80 to +70 mV in 10 mV increment. The depolarizing step was followed by a 300 ms pulse to -120 mV for assessing the inward tail currents flowing through the delayed rectifier currents, including HERG current (I_{Kr}). I_{Ktot} and I_{Kend} current amplitudes were measured as the peak current or the current at the end of the depolarizing pulse minus the mean current at -40 mV. The I_{Ktail} current amplitude was measured as the current at peak minus the current at the end of the 300 ms pulse to -120 mV. The transient outward channel (I_{to}) was inactivated using a 180 ms prepulse to -40 mV followed by a 5 s voltage steps from -
40 to +70 mV in 10 mV increment. The $I_{to}$ currents were obtained after offline subtraction of the depolarizing steps of the two voltage protocols and the amplitudes were measured as peak current minus the current at the end for the subtracted trace. The inward rectifier $I_{K1}$ current was measured as the current at the end of the 500 ms pulses between -120 and -10 mV from -40 mV holding voltage. To assess the BaCl$_2$ and ACh sensitive currents, a voltage ramp between -120 to +60 mV for 1 second, from the holding voltage of -40 mV was applied at 30 seconds interval. Action potentials (AP) were triggered at 1 Hz frequency with a suprathreshold current stimulus of 600 – 1000 pA amplitude and 3 - 6 ms duration. Three to five consecutive AP traces at steady state were averaged, and action potential amplitude, slope, and durations from the peak to 20, 50, 70 and 90 % repolarization using a macro for APD analysis designed by Dr. Jan S. Schulte (University of Münster, Germany)$^{21}$ for Origin 8.1 (OriginLab Corporation, Northampton, MA, USA).

**Western blot**

Atria from six to eight mice have been used from each group. Both left and right atria were pooled from each mouse and homogenized in lysis buffer containing 100 mM NaCl, 20 mM Tris Base, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, pH adjusted to 8.0 and supplemented with phosphatase and protease inhibitor tablets (A32959, ThermoFischer Scientific, Darmstadt, Germany). Protein concentration of the homogenate was measured using the classical Bradford method (Thermo Fisher Scientific). The samples were equally distributed and 40 µg of protein loaded per well of the 10% SDS-PAGE gel. Equal protein load was assessed after transfer using Ponceau stain. The following rabbit polyclonal primary antibodies were used: Kv4.2 (1:200, APC 023 Alomone Labs, Jerusalem, Israel), Kv4.3 (1:200, APC 017 Alomone Labs), KChIP2 (1:200, sc-25685, Santa Cruz Biotechnology Inc, CA, USA) Kir2.1 (1:200, APC 159, Alomone Labs), Kir2.3 (1:1000, APC 032, Alomone Labs), Kir3.1 (1:200, APC 005, Alomone Labs), Kir3.4 (1:200, APC 027, Alomone Labs) and
calsequestrin (CSQ, 1:2500, PA1-913, Thermo Fisher Scientific). As secondary antibodies we used HRP conjugated anti-rabbit IgG antibody (1:10000, A27036 Thermo Fisher Scientific) further developed with SERVALight Helios (42587.03, SERVA Electrophoresis GmbH, Heidelberg, Germany) or SERVALight Eos (42585.02, SERVA Electrophoresis GmbH). Band intensities were quantified using ImageJ 1.49v (National Institute of Health, Bethesda, MD, USA). Each band was normalized for load (to the Ponceau stain intensity of the full lane), for transfer (normalized to the reference considered the averaged signal of the WT<sub>6w</sub> bands of the same membrane) and to CSQ for myocyte content. The normalized values of the samples belonging to the same group were averaged for statistical comparison.

**Quantitative real time PCR (qRT-PCR)**

Eight animals have been used from each group. Total RNA was purified using TRIzol (15596018, ThermoFisher Scientific) according to the manufacturer instructions, and 1 μg was reversely transcribed to cDNA that served as input for real-time PCR. The primers used to probe the ion channel subunits were as follows:

- **Kcnd2:** for 5'-CTGTCACGGAGACACAAAAA-3';
  
  rev 5'-CGGCTGTTGGATAGTGGAGT-3'

- **Kcnd3:** for 5'-TGTACGAACCTCCACCACATCA-3';
  
  rev 5'-AGTGGCTGGACAGAGAAGGA-3'

- **Kcna4:** for 5'-GAAGAAGGGGTCAAGGAATC-3';
  
  rev 5'-TGGCACAGTGGAGAGAACAAT-3'

- **Kcnip2:** for 5'-GACATGATGGGCAAGTACACC-3';
  
  rev 5'-ACGCCGTCCTTGTTTCTGT-3'

- **Kcna5:** for 5'-TCCGACGGCTGGACTCAATAA-3';
  
  rev 5'-CAGATGGCCTTCTTAGGCTGTG-3'

- **Kcnb1:** for 5'-CCACCAGATTCTCCACAGT-3';
Kcnq1: for 5’-GGCTACGGGGATAAGGTACC-3’;
rev 5’-CACCTCCATGCAGTCTGGAT-3’

Kcnh2: for 5’-GTGGAGATCGCCTTCTACCG-3’;
rev 5’-CCCTGTGGGTTGGTGTCAAGA-3’

Kcne1: for 5’-GCAGAGCCTCGACCATTTAG-3’;
rev 5’-GTAGAGCGCCTCAGCCTTGCG-3’

Kcne2: for 5’-CACATTAGCCAACTTTTGACCAG-3’;
rev 5’-GAACATGCGCCTCAGCCTTGAGA-3’

Kcnj2: for 5’-GGGAATTCTCACTTGCTTCG-3’;
rev 5’-AGAGATGGATGCTTTCCGAGA-3’

Kcnj3: for 5’-AGTAGAAAGGGGGTCAAGGAC-3’;
rev 5’-GGCCACCTCTCTACCTTGC-3’

Kcnj4: for 5’-GACCCTCCTCGGCCCTTA-3’;
rev 5’-GACGTTACACTGGCCGTTCT-3’

Kcnj5: for 5’-ATGTCTCGTGCTCAACTGG-3’;
rev 5’-AAGCTCGGTGTCTGGTCTCA-3’

Hprt1: for 5’-ATGAGCGCAAGTGGATCTTG-3’;
rev 5’-GGACGAGCAACTGACATT-3’

Hypoxanthine phosphoribosyltransferase 1 (Hprt1) was used as housekeeping gene.
The primers were designed using Primer3 and synthesized by Eurofins (Eurofins Genomics, Ebersberg bei München, Germany). The outliers in a group were calculated based on the dCt (Ct_target - Ct_hprt) for each gene using Grubbs’ test for outliers (GraphPad Prism Version 8.0.2 for Windows, GraphPad Prism Software, La Jolla, California, USA).
Calculation of relative expression was derived from the $2^{-\Delta\Delta Ct}$ method using the relative expression software tool (REST® Version 2.07).\textsuperscript{25}

**Chromatin immunoprecipitation (ChIP)**

Standard ChIP protocol was performed on atria of TG or WT mouse\textsuperscript{19}. Eight to ten animals have been used per group. The tissue was fixed with 1% formaldehyde, chromatin was fragmented to 700-1000 bp by sonication and immunoprecipitated with a rabbit polyclonal anti-HA antibody (tag, 2.5 µg, ab9110 Abcam, Cambridge, UK). After purification and amplification of the whole genomic DNA, real-time PCR was performed using primers designed around possible functional cyclic AMP response element (CRE) identified using JASPAR2016\textsuperscript{26} in the promoters of genes of interest. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as reference. The primers used to probe the specific promoter region were as follows:

- **Kcnd2**: for 5'-TTTGGGAAGGTGACAAGGAG-3’;
  rev 5'-GGGGCTGAGTTGCATAGAGA-3’
- **Kcnd3**: for 5'-TATTGGAGCGAATCCTGACC-3’;
  rev 5'-ATCCCCACGGAAGACAAACTG-3’
- **Kcna5**: for 5'-CGGTTTCTCTCTTCAGCGAG-3’;
  rev 5'-TGGTAACCAGCTGCCAGAAC-3’
- **Kcnh2**: for 5'-ATGGTCGGTTTGGAGGTGAC-3’;
  rev 5'-ATTTTCTGGCTGTCCCCTGG-3’
- **Kcnj2**: for 5'-ATGGTCGGTTTGGAGGTGAC-3’;
  rev 5'-TTGCCATCTGGCTCCCTGGG-3’
- **Kcnj3**: for 5'-GGGCGGACATGGGAATAGGAG-3’;
  rev 5'-AATGCAGGTGTGTCAGGCTC-3’
- **Kcnj4**: for 5'-ACGTTACACTGGCCGTTCTT-3’
rev 5’-GGTCTCCAAACCGTCCTCTG-3’

*Kcnj5:* for 5’-AAGCCAAAGAAGCCAGCCAGG-3’;

rev 5’-ACATCCCCATTGCCACAGAC-3’

c-*fos:* for 5’-TGCCAAGAGCGGGTTGAAAAG-3’;

rev 5’-TCGAGTCGCCGTGGAGTAGTGG-3’

*smICER:* for 5’-TCTGGCTCTTCAGCTAATTGAGTTCAAA-3’;

rev 5’-GAAAGGAACCTGAATCAATCAACATGACT-3’

*Gapdh:* for 5’-TGCACCACCAACTGCTTA-3’;

rev 5’-GGATGCAAGGATGATGTTC-3’

The primers were designed using Primer3 and synthesized by Eurofins (Eurofins Genomics, Germany). The outliers of each group were calculated based on the dCt (Ct*target*-Ct*hprt*) for each gene using Grubbs’ test for outliers (GraphPad Prism Version 8.0.2 for Windows, GraphPad Prism Software, La Jolla, California, USA). Calculation of relative expression was derived from the 2^ΔΔCt method using the relative expression software tool (REST© Version 2.07).

**Measurement of atrial contraction**

Left atria of all 4 experimental groups of mice were dissected in oxygenated ice cold physiological solution and attached to a force transducer for recording isometric contractions electrically driven by field stimulation at 1 Hz using suprathreshold rectangular pulses of 5 ms duration, as described previously. Before the experimental protocol, atria were pre-stretched with a force of 5 mN and were allowed to equilibrate at 37°C for at least 30 minutes in a bath solution containing (in mM): 120 NaCl, 5.4 KCl, 1.8 CaCl₂, 1 MgCl₂, 22.6 NaHCO₃, 0.42 NaH₂PO₄, 0.28 ascorbic acid, 0.05 Na₂EDTA and 5.5 glucose, adjusted to pH 7.4 with NaOH. To build the dose-response curves, the concentrations of carbachol (CCh) or isoproterenol (ISO) were increased every 5 minutes to the following concentrations (in M): 10⁻⁹, 10⁻⁸, 3x10⁻⁷
8, 10^{-7}, 3 \times 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} \text{ M}, while the force of contraction was monitored continuously. Dose response curves were built using the force of contraction measured for each drug concentration at steady state. Six out of 12 WT\textsubscript{6w} and 10 out of 24 WT\textsubscript{12w} atria did not show a positive staircase in response to ISO application after CCh, therefore these results were excluded from the calculation of half-maximal effective concentration (EC\textsubscript{50}).

**Statistics**

Data are reported as mean values ± standard error (SE) with n indicating the number of cells and N the number of animals. Normal distribution was assessed using Shapiro-Wilk test in GraphPad 8.0 (GraphPad Software, La Jolla, California, USA). According to the Gaussian distribution, student’s t test or Mann-Whitney test or Wilcoxon matched-pairs signed rank test were used for two group comparison, and one-way ANOVA test with Newman-Keuls post-hoc test or Kruskal-Wallis test with Dunn’s post-hoc test were used for multiple groups comparison. Current-voltage relations and dose-response curves were compared using 2-way repeated measures ANOVA with Bonferroni post-hoc test. Relative gene expression ratios were expressed as mean values ± standard error (SE) for qRT-PCR and ChIP experiments as reported by the relative expression software tool (REST\textsuperscript{+}, version 2.07)\textsuperscript{24}. Data were considered significantly different at P<0.05.
Figure S1. *In vivo* electrophysiology of CREM TG mice.

(A-F) Representative electrocardiograms (ECG) recorded from wildtype mice young (6 weeks, WT$_{6w}$, A) and older (12 weeks, WT$_{12w}$, B) and from CREM transgenic (TG) mice young (TG$_{6w}$, C, E) and older (TG$_{12w}$, D and F) showing no AF (C, D) and AF (E, F). Scale bars: vertical 0.2 mV, horizontal 0.1 s. (G-I) Analysis of ECG parameters of heart rate variability (HRV): mean heart rate (G), standard deviation of NN intervals (SDNN, H), root mean square of the successive differences between neighboring RR intervals (RMSSD, I). Data show mean ± SE resulted from 12 (WT$_{6w}$), 36 (TG$_{6w}$), 34 (WT$_{12w}$) and 54 (TG$_{12w}$) ECG traces. *$P<0.05$ for TG$_{6w}$ vs. WT$_{6w}$ (grey), TG$_{12w}$ vs. WT$_{12w}$ (black), #$P<0.05$ TG$_{12w}$ vs. TG$_{6w}$ (black) from parametric or non-parametric 1-way ANOVA with the corresponding post-hoc test.
Figure S2. Basal properties of the action potentials (AP) of atrial myocytes isolated from CREM TG mice.

AP parameters such as AP amplitude (A), slope of depolarization (B), and AP duration (APD) from peak to 20, 50, 70 and 90% repolarization (C-F) were measured in the 4 groups of animals. Data show mean ± SE of the values of parameters calculated for individual cells of the group (average of cells) or the mean value of the averaged parameter calculated per heart (average of mice). In the legend, the numbers in brackets show number of cells / number of mice. *P<0.05 for TG6w vs. WT6w (grey), TG12w vs. WT12w (black), #P<0.05 WT12w vs. WT6w (grey), TG12w vs. TG6w (black) from parametric or non-parametric 1-way ANOVA with the corresponding post-hoc test.
Figure S3. Representative uncut immunoblots presented in Fig.2, showing the transient outward channel components: the forming α-subunits Kv4.2, Kv4.3 and auxiliary subunit KChIP2, vs. the myocyte marker calsequestrin (CSQ) used for normalization.

Two samples of each group have been loaded on the gel, as mentioned above the immunoblots. Number on the right represent the relative size of the proteins.
Figure S4. Total inward K⁺-current of TG atrial myocytes, before and after AF onset.

Data show mean ± SE of averaged I-V plots of the current density at the end of the hyperpolarizing pulse ($I_{K_{ir}}$), recorded from the four experimental groups. n/N = 33–55/11–18. *P<0.05 for TG_{6w} vs. WT_{6w} (grey), TG_{12w} vs. WT_{12w} (black), #P<0.05 TG_{12w} vs. TG_{6w} (black) from 2-way repeated measures ANOVA with Bonferroni post-hoc test.
Figure S5. Action potential (AP) parameters following blocking or activating $I_{K1}$ and $I_{KACH}$, respectively, of atrial myocytes isolated from CREM TG mice.

Data show mean ± SE of AP amplitude (A), slope of depolarization (B), and AP duration (APD) from peak to 20, 50, 70 and 90% repolarization (C-F) measured in the 4 groups of animals. n/N for WT$_6w$ (7/5) TG$_6w$ (5/5), WT$_{12w}$ (11/6) and TG$_{12w}$ (5/4). §$P<0.05$ BaCl$_2$ or ACh vs. normal Tyrode (NT) using Wilcoxon matched pairs signed rank test.