

Supplemental Information

Froese *et al.* : Popeye domain containing proteins are essential for stress-mediated modulation of cardiac pacemaking in mice

1. Supplemental Figures

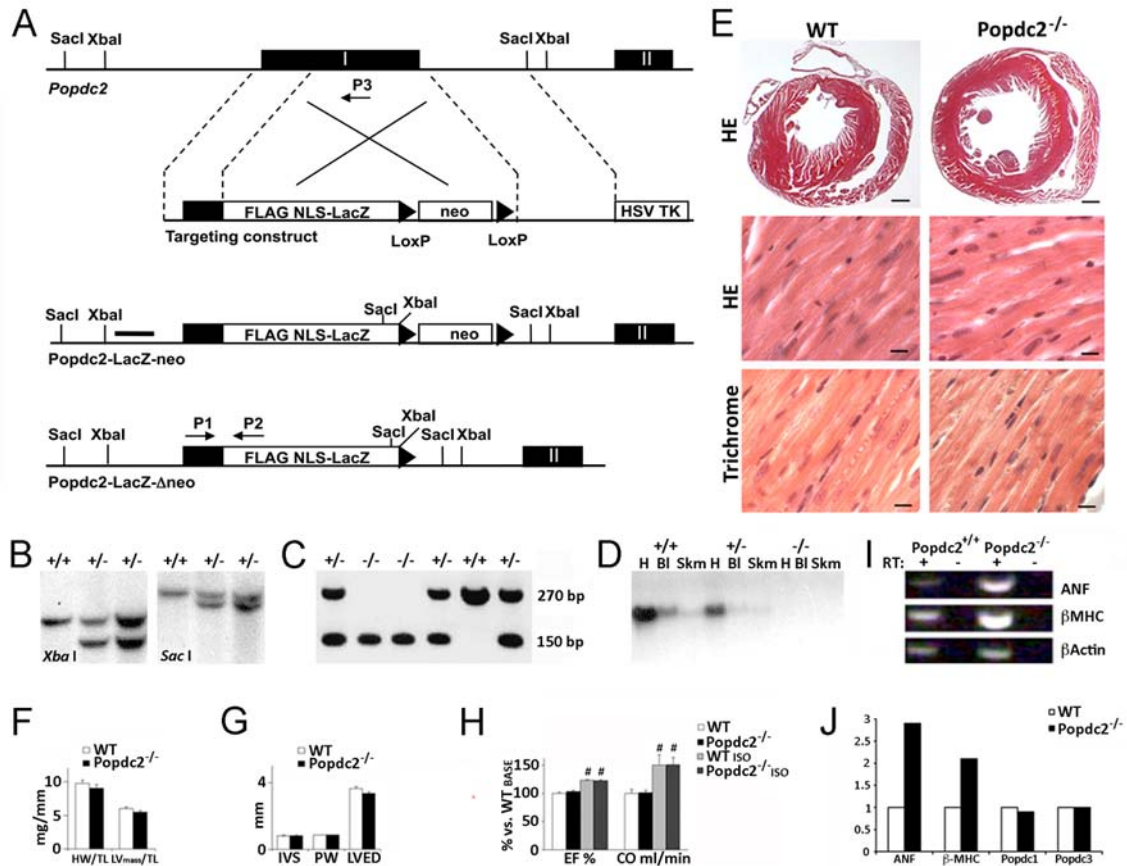


Figure S1: Generation and characterization of the *Popdc2*^{-/-} mice. (A) Targeting strategy for the *Popdc2* locus, only exon 1 and 2 are depicted. The targeting construct consists of 2.2 kb and 2.8 kb left and right homology arms, respectively. Arrows depict the approximate locations of primer sequences (P1, P2, and P3), which were used for genotyping. Black bar depicts the approximate location of the probe used in Southern blot analysis. Neomycin was excised generating the *Popdc2-LacZ-Δneo* allele by breeding heterozygous animals with *Zp3-cre* mice. (B) Southern blot analysis of ES cell clones. The restriction pattern of WT and heterozygous (+/-) cell clones is shown. (C) PCR analysis using DNA isolated from tail biopsies and primers P1 and P2 and P3. The WT and mutant alleles produced 270 bp and 150 bp fragments, respectively. (D) Northern blot analysis using total RNA isolated from skeletal muscle (Skm), heart (H), and bladder (Bl) of homozygous (*Popdc2*^{-/-}), heterozygous (*Popdc2*^{+/-}), and WT (*Popdc2*^{+/+}) mice. (E) Sections of WT (left) and *Popdc2*^{-/-} (left) mutant hearts stained with hematoxylin and eosin, or Masson's Trichrome demonstrating normal histology and absence of fibrosis in

mutant hearts. **(F)** Ratios of heart weight (HW) to tibia length (TL) and left ventricular mass to tibia length in 3 month old WT (n=8) and *Popdc2*^{-/-} mice (n=10). Error bars are +/- SEM. **(G)** End-diastolic diameter of the interventricular septum (IVS), posterior wall (PW), and left ventricle (LVED) in 3 month old WT (n=10) and *Popdc2*^{-/-} mice (n=12). Error bars are +/- SEM. **(H)** Ejection fraction (EF) of the left ventricle and cardiac output (CO), hash p<0.05 BASE vs. ISO. Error bars are +/- SEM. **(I)** End-point RT-PCR analysis of β -MHC and ANF expression in adult (3 month old) WT and *Popdc2*^{-/-} littermates. **(J)** Real time PCR analysis of *Popdc1*, *Popdc3*, β -MHC and ANF expression in adult (3 month old) WT and *Popdc2*^{-/-} littermates.

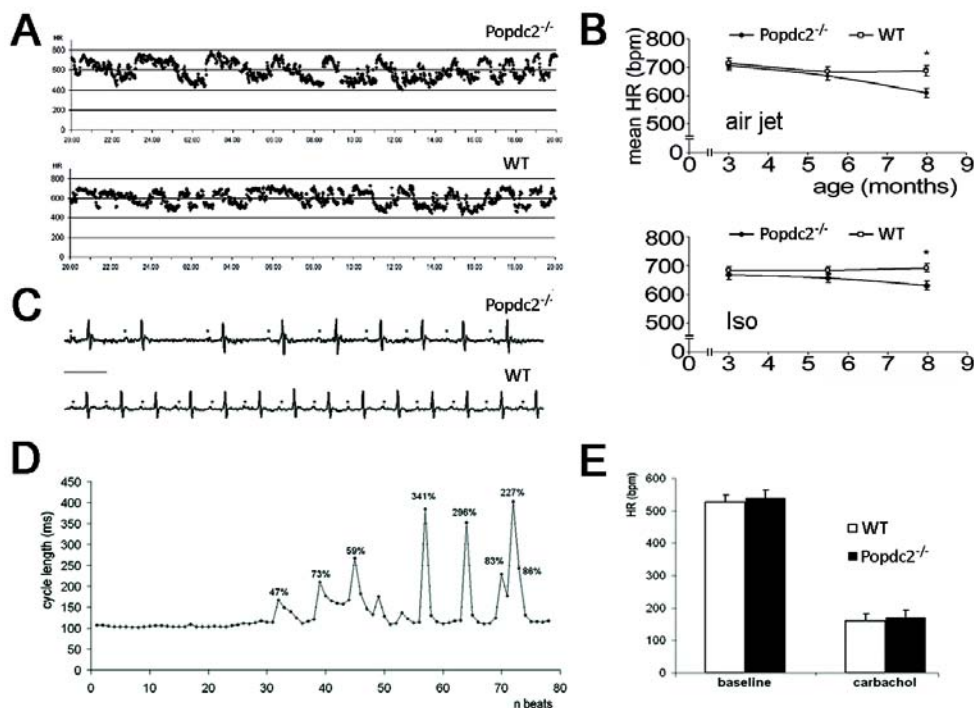


Figure S2: Age-dependent bradycardia in the *Popdc2* null mutant mice. **(A)** Representative 24h telemetry profile of sedentary WT and *Popdc2*^{-/-} mice at 8 month of age (n=5). **(B)** Age-dependent mean heart rates of WT (blank) and *Popdc2*^{-/-} (filled) mice during air jet stress and β -adrenergic stimulation (Iso), respectively. Asterisks p<0.05 (n=5-12 per genotype and age). Error bars are +/- SEM. **(C)** Representative ECG recording of *Popdc2*^{-/-} (upper trace) and WT (lower trace) mice during airjet stress test at 8 month of age. Asterisks indicate P waves. Horizontal bar indicates 100 ms. **(D)** Example of pause cycle length variation. **(E)** Heart rate reduction after carbachol treatment. (n = 5, age 8 month), p<0.05 for carbachol vs. baseline, p= n.s. for WT vs. *Popdc2*^{-/-}. Error bars are +/- SEM.

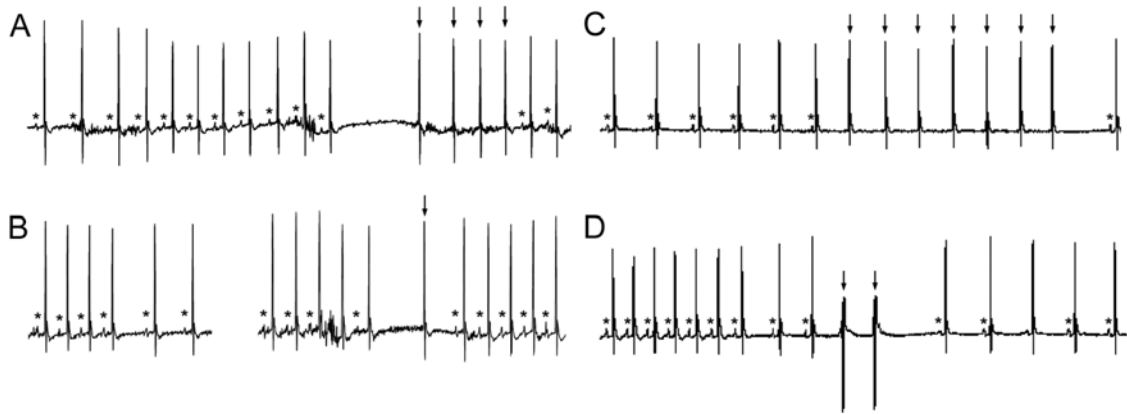


Figure S3 Escape rhythms in *Popdc* null mutants. ECG recordings of 8 month old (**A,B**) *Popdc1*^{-/-} and (**C,D**) *Popdc2*^{-/-} mice. Asterisks indicate P waves, arrows in (**A-C**) indicate AV nodal escape beats and arrow in (**E**) points to ventricular escape beats. (**B**) Example of an animal showing a pause with a sinus nodal escape beat (left part) followed by a pause with an AV nodal or high ventricular narrow QRS complex escape beat (right part).

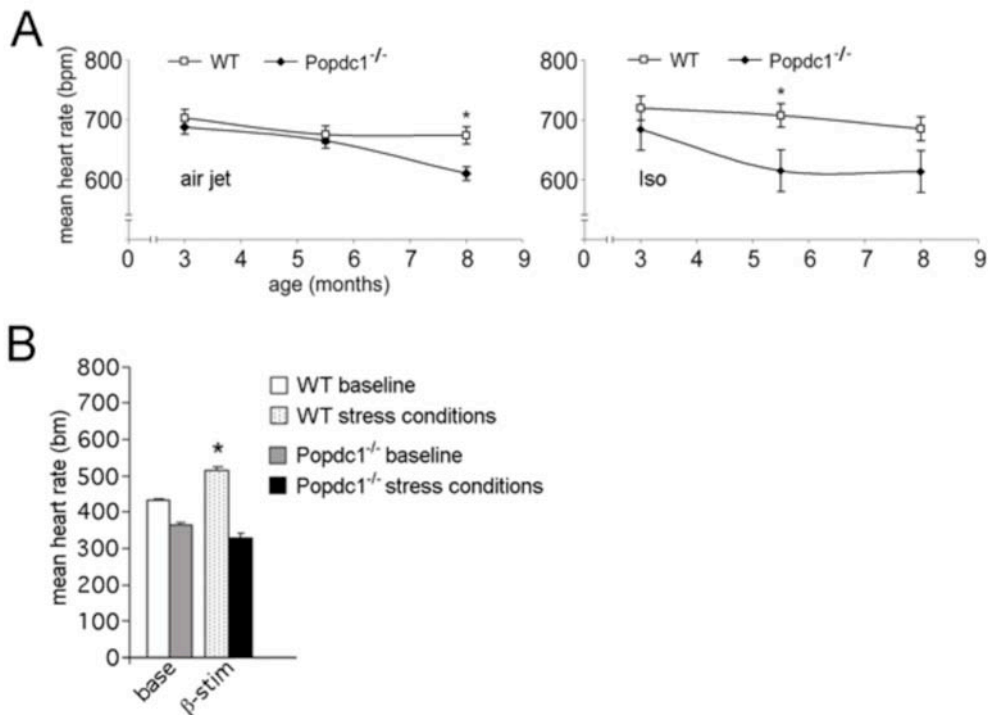


Figure S4: Blunted heart rate response to physiological stress and catecholamines in *Popdc1* null mutants. (**A**) Age-dependent mean heart rates of WT (blank) and *Popdc1*^{-/-} (filled) mice during air jet stress (left panel) and β -adrenergic receptor stimulation (right panel). Mean heart rates (y-axes), age in month (x-axes). (n=5-12 per genotype and age). (**B**) Mean heart rates of isolated, beating, Langendorff-perfused heart preparations of WT and *Popdc1*^{-/-} mice (n=5 per genotype). Mean heart rates are given at baseline and during perfusion with the β -adrenergic agonist orciprenaline (1.6 μ M; Kirchhof et al. 2006). Asterisks indicate a statistically significant difference (p<0.05) between genotypes. Error bars are +/- SEM.

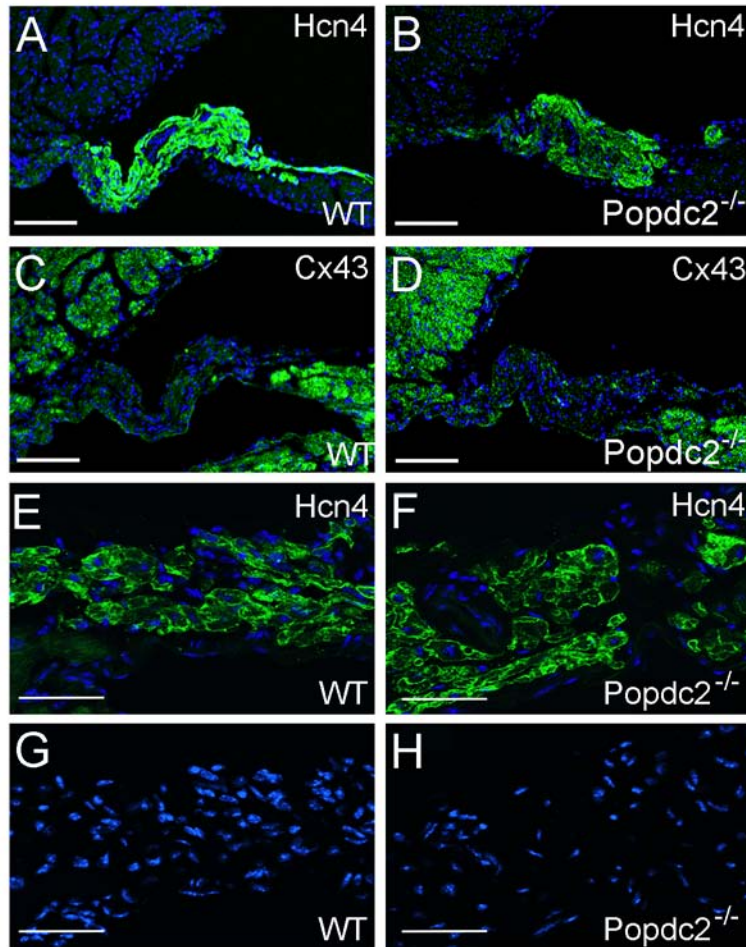


Figure S5: Expression of Cx43 and HCN4 in the sinus node of *Popdc2*^{-/-} mice. Sections through the sinus node of (A,C,E,G) WT and (B,D,F,H) *Popdc2*^{-/-} mice are stained for (A,B,E,F) HCN4, or (C,D) Cx43 and HCN4, or counterstained with DAPI. Sections depicted in (A-D) are from the superior and (E-H) from the inferior part of the sinus node. In (G,H) only DAPI stained nuclei of panel (E,F) are shown in order to demonstrate the reduction in cell number in the inferior part of the mutant sinus node. Scale bars at the lower left in each panel = 100 μ m in A-D; 50 μ m in E-H.

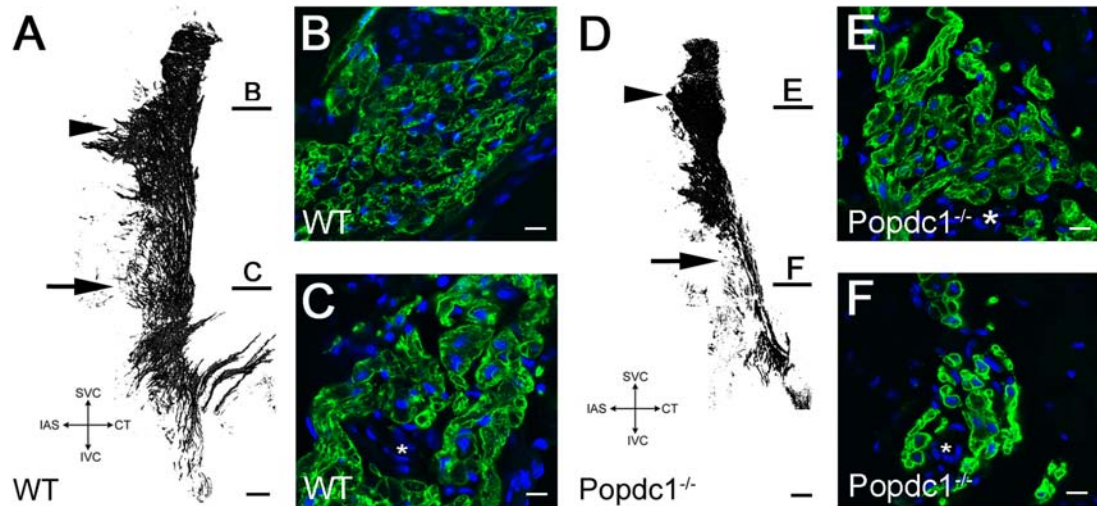


Figure S6: Aberrant morphology of the sinus node in *Popdc1*^{-/-} mice. (A,D) Whole mount immunofluorescence detection of HCN4 in the sinus node of (A) WT and (D) *Popdc1*^{-/-} mutant. In *Popdc1*^{-/-} mutants the superior part of the sinus node is more compact (arrowhead) and a loss of pacemaker tissue can be seen in the inferior part (arrow). (B,C,E,F) Immunohistochemical detection of HCN4 on sections through the (B,E) superior and (C,F) inferior part of the sinus node. The genotype of the tissue is indicated at the lower left of each panel. Nuclei are stained with DAPI. Scale bars = 200 μm in A,D; 100 μm in B,C,E,F.

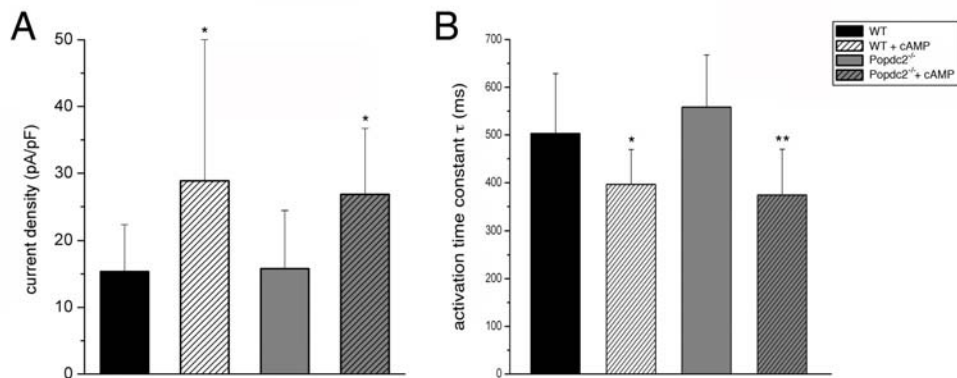


Figure S7: The pacemaker current I_f cells is unaltered in sinus node cells of *Popdc2*^{-/-} mice. (A) I_f -current density (WT: n=29; *Popdc2*^{-/-}: n=27), and (B) I_f -activation kinetic (WT n=9; *Popdc2*^{-/-}: n=12) in sinus node myocytes isolated from WT and *Popdc2*^{-/-} mice under basal conditions and after 8-Br-cAMP treatment. Statistically significant differences ($p < 0.05$) between 8-Br-cAMP treatment vs. baseline condition are marked with an asterisk ($p < 0.05$), or two asterisks ($p < 0.001$), $p = n.s.$ for WT vs. *Popdc2*^{-/-}. Error bars are \pm SD.

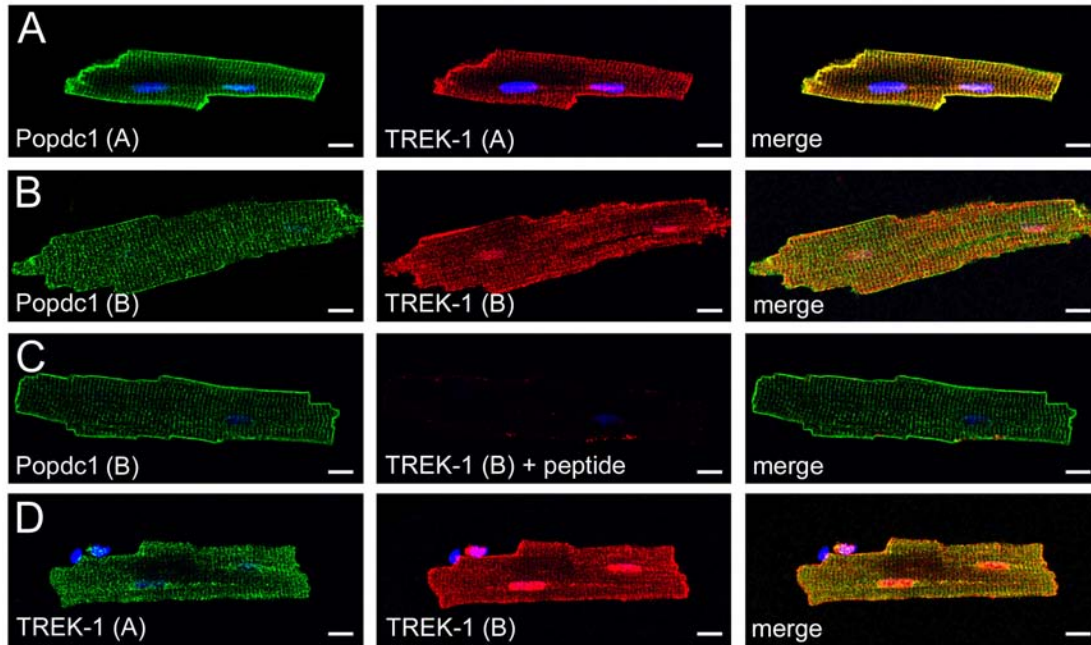


Figure S8: Co-expression of Popdc1 and TREK-1 in isolated adult cardiac myocytes. Isolated cardiac myocytes are stained with antibodies directed against **(A-C)** Popdc1 (green, left) and TREK-1 (red, middle) and a merged image (right). **(A)** Popdc1 (C20 antibody), TREK-1 (H75 antibody), **(B)** Popdc1 (HPA014788 antibody), TREK-1 (N20 antibody), **(C)** Popdc1 (HPA014788 antibody), TREK-1 (N20 antibody preadsorbed with immunogenic peptide). **(D)** TREK- (H75 antibody) and TREK-1 (N20 antibody). Nuclei were stained with DAPI (blue label). Bar 10 μm .

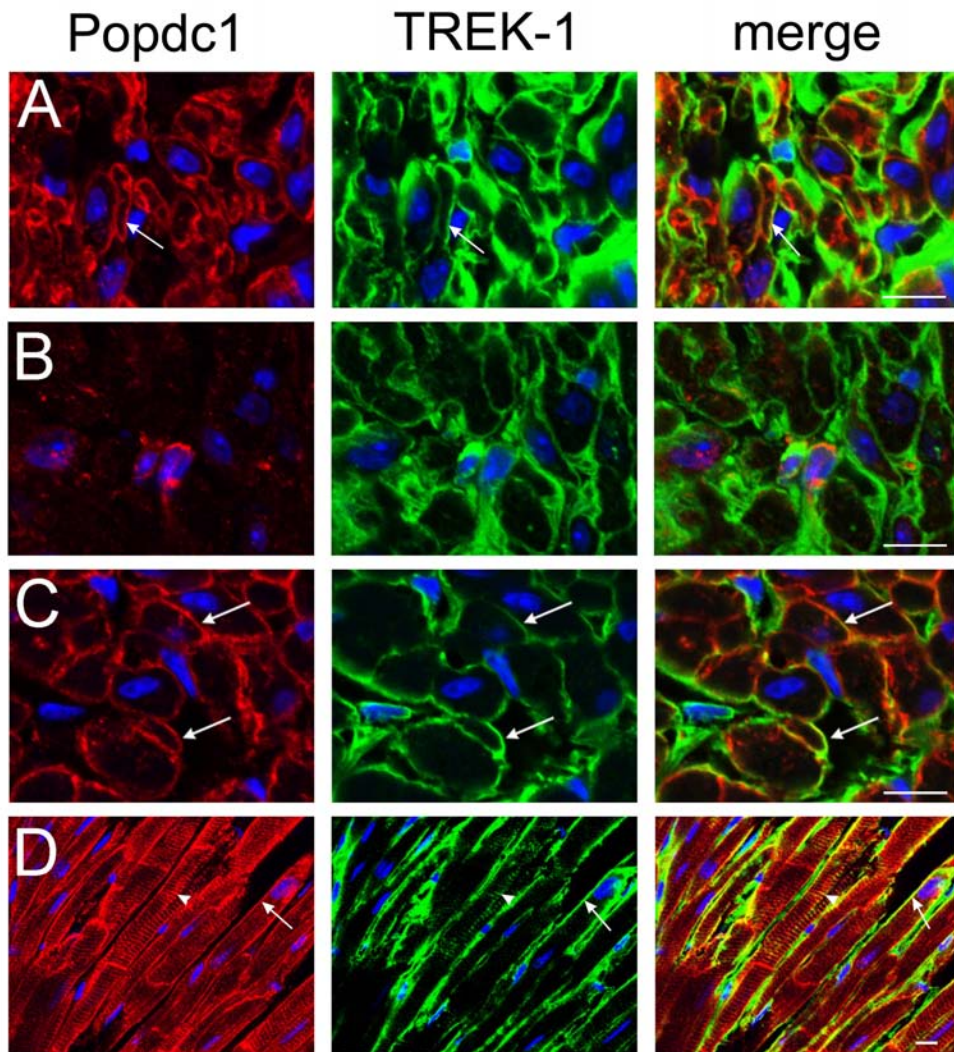


Figure S9: Overlapping expression of Popdc1 and TREK-1 in cardiac myocytes. (A-E) Staining of Popdc1 (C20 antibody, red, left), TREK-1 (H75 antibody, green, middle) and a merged image (right) of sinus node tissue of (A) WT, (B) Popdc1^{-/-} mice, (C) atrial, and (D) ventricular tissue of WT mice. Nuclei were stained with DAPI (blue label). Bar 10 μm. Arrow in (A,C,D) examples of co-localized staining in the plasma membrane. Arrowheads in (D) colocalization of Popdc1 and TREK-1 in T-tubules.

Table S1: Heart rates in freely roaming Popdc2^{-/-} and WT animals under different experimental conditions, Related to Figure 1

Treatment	Age	Mean HR		0.1 Quantile		0.9 Quantile	
		WT	Popdc2 ^{-/-}	WT	Popdc2 ^{-/-}	WT	Popdc2 ^{-/-}
Rest	8	527 ± 42	539 ± 19	444 ± 36	441 ± 23	613 ± 38	641 ± 15
Carbachol	8	160 ± 10	169 ± 12	148 ± 7	158 ± 13	175 ± 18	184 ± 13
Airjet	3	715 ± 20	707 ± 11	666 ± 29	669 ± 14	756 ± 8	732 ± 13
	5.5	685 ± 10	669 ± 6	617 ± 23	616 ± 11	725 ± 6	708 ± 8
	8	687 ± 14	610 ± 18*	609 ± 29	514 ± 26*	741 ± 9	695 ± 12*
	3	697 ± 08	687 ± 15	632 ± 16	625 ± 24	737 ± 7	724 ± 16
Swim stress	5.5	710 ± 08	664 ± 6*	659 ± 14	618 ± 11*	756 ± 11	700 ± 8*
	8	712 ± 04	593 ± 19*	670 ± 8	441 ± 45*	742 ± 5	684 ± 6*
Isoproterenol	3	684 ± 05	668 ± 9	663 ± 8	654 ± 7	712 ± 5	690 ± 12
	5.5	681 ± 07	658 ± 12	656 ± 16	643 ± 12	712 ± 5	683 ± 14
	8	691 ± 05	651 ± 11*	674 ± 5	630 ± 11*	723 ± 6	683 ± 5*

*p<0.05 WT vs. Popdc2^{-/-}, n=5-12 per genotype and age. Data are mean +/- SEM.

2. Supplemental Methods

A. Generation of *Popdc2* Mutant Mice

The targeting construct was assembled in the pPNT-2loxP vector. The *LacZ* gene was isolated from pPD46.21 vector and subcloned into the *Bam*HI and *Not*I sites of pPNT-2loxP (Fig. S1a). Two fragments of homologous sequence flanking the protein coding sequence of exon 1 of the *Popdc2* gene were isolated from cosmid clone MPMGc1211101192Q2, which was derived from a 129/ola mouse cosmid library (RZPD, Berlin). The left arm (2.2 kb) was PCR amplified and subcloned into *Sa*II and *Bam*HI upstream of the *LacZ* gene. The 3' end of this fragment contained an ATG start codon followed by a Flag epitope sequence, which after subcloning, was in frame with the *LacZ* reporter gene. The right arm (2.8 kb) was isolated as an *Eco*RI restriction fragment and inserted downstream of the neomycin gene. The targeting vector was linearized with *Not*I and electroporated into 129 SVJ (TBV2)-derived embryonic stem (ES) cells. Selection was with G418 and gancyclovir. A total of 400 clones were picked and 9 clones were identified by PCR analysis to have a recombined *Popdc2* locus. Upon targeting 490 bp of coding sequence of exon 1 and 27 bp of intron 1 were replaced with NLS-*LacZ* (nuclear localization signal of SV40 large T-antigen followed by the *E. coli* β -galactosidase gene) having a FLAG epitope in frame (Fig. S1a). These clones were further analyzed by Southern blot analysis of genomic DNA digested with *Xba*I and *Sac*I, respectively, using an external probe (Fig. S1b). Blastocyst injection of one the clones generated chimeras, which transmitted the recombinant locus. Genotyping of *Popdc2* mice was performed with two different PCR reactions and genomic tail DNA using oligonucleotides p1 (5'-GTTTTGGAGCCTGCAAAGGC-3'), p2 (5'-CGATTAGTTGGGT AACGCC-3'), and p3 (5'-TTCTACATCCGGTTTCCAGC-3') to produce 120 bp and 270 bp bands from the WT and targeted alleles, respectively (Fig. S1a and c). F1 mice were bred with *Zp3-cre* mice to delete the floxed *neo* cassette (Lewandoski et al., 1997). The resulting *Popdc2* neo mice (in the following referred as *Popdc2*) were backcrossed with C57BL/6J mice for at least six generations (>98% C57BL/6J background) and were maintained on the C57BL/6J background. Animal colonies were kept in

pathogen-free conditions at the animal facilities of the Universities of Braunschweig, Münster and Würzburg.

Physiological Experiments

Experiments were performed following institutional guidelines, approved by the institutional animal care and use committee (G61/99, G83/2004 at University of Münster). The study adhered to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH publication No. 85-23, revised 1996). Functional analyses were performed and analyzed blinded to genotype in sex-matched wild-type and *Popdc2^{-/-}* mice aged 3 to 8 month.

B. Echocardiography

To assess cardiac size, morphology, and function, two-dimensional echocardiography and Doppler measurements were performed during isoflurane sedation using 13.5 MHz 2 D and M Mode and 12 MHz Doppler transducers (HP Sonos) and a small animal ultrasound platform (VEVO770) equipped with a 50 MHz transducer following published protocols (Kirchhof et al., 2006; Kuhlmann et al., 2006). Measurements were obtained at baseline and after injection of isoproterenol (2mg/kg body weight) intraperitoneally in mice aged 2-3 month (10 weeks) and 6-7 month (26 weeks).

C. ECG and Telemetry

Six-lead surface electrocardiograms (ECG) were recorded in littermate pairs of 3 month-old mice during isoflurane inhalation (1-1.5 % plus oxygen). Recordings of 10 s were used for signal averaging. To record an ECG in freely roaming animals, a telemetric ECG transmitter (DSI, St. Paul, MN) was implanted and between 5-12 per genotype and age, data are represented as mean +/- SEM values). The telemetric ECG was analyzed during normal activity in the standard laboratory cage over a 24-hour period, and during defined stress tests, i.e. swimming, isoproterenol injection, or warm air jets, according to published protocols (Harzheim et al., 2008; Knollmann et al., 2003). To unmask potential chronotropic incompetence, we did not use

intermittent, but used continuous stress by 6 minutes of warm air jets in this study. Recordings were performed at 3 month, 5.5 month, and 8 month of age. All recordings were digitized (EMKA, Falls Church, VA) at 2 kHz and analyzed off-line. The telemetric ECG was analyzed during normal activity in the standard laboratory. Paired and unpaired t-tests, univariate analysis of variance (ANOVA) and ANOVA analyses for multiple comparisons and repetitive measurements were used for comparison of continuous parameters at different cycle lengths in WT and mutant mice at baseline and with drugs (SPSS, version 12.0G for Windows). Fisher's Exact test was used to compare hearts with occurrence of sinus pauses. Values are reported as mean \pm SEM throughout the text if not indicated otherwise.

For invasive electrophysiological testing, mice were anaesthetized with intraperitoneal 2,5% Avertin (100 μ l/10g BW) for a terminal procedure. An octapolar 2-French EP catheter was advanced via a cutdown approach of the right internal jugular vein into the right atrium and right ventricle. With the proximal electrodes we were able to pace and sense from the atrium, with the distal electrodes we were able to pace and record in the ventricle. AV nodal conduction properties were obtained by atrial incremental pacing until the first stimulated beat was blocked at the AV node (Wenckebach point).

D. Electrophysiological Study in the Isolated Heart

To assess intrinsic heart rate and pauses, hearts of 8 month old mice were rapidly excised and perfused using a warm, oxygenated modified Krebs-Henseleit solution on a vertical Langendorff apparatus (Harzheim et al., 2008; Knollmann et al., 2003). Atrial and ventricular monophasic action potentials, electrograms, and tissue bath ECGs were simultaneously recorded during spontaneous rhythm at baseline and during perfusion with orciprenaline (1.7 μ M) following published techniques (Kuhlmann et al., 2006). All original recordings were manually scrutinized for pauses. We analyzed heart rate by manual analysis of 15 beats and by semiautomatic analysis of several minutes. 2:1 Sino-atrial block was defined following accepted prespecified clinical criteria, i.e. a sudden twofold increase of PP interval. All analyses were blinded to

genotype (Harzheim et al., 2008). Sinus pauses were defined as a sudden increase in PP interval to more than 60% of the 0.9 quantile of the average heart rate in a given protocol part. Whole heart weight of isolated hearts was assessed immediately after the Langendorff procedure prior to preparation of the sinus node. Tibiae length was measured in each mouse after excision of the heart.

E. Electrophysiology of isolated sinus node Myocytes.

Single sinus node myocytes were isolated and prepared for electrophysiological recordings as described (Mangoni and Nargeot, 2001; Stieber et al., 2006). I_f was recorded with the whole cell patch clamp recording technique at a temperature of $22 \pm 2^\circ\text{C}$. Patch pipettes were pulled from borosilicate glass and had a resistance of 2-5 $\text{M}\Omega$ when filled with intracellular solution, containing: 10 mM NaCl, 30 mM KCl, 90 mM potassium aspartate, 1 mM MgSO_4 , 5 mM EGTA, 10 mM HEPES, pH adjusted to 7.4 with KOH. During the recordings, cells were continuously superfused with extracellular (bath) solution, containing: 140 mM NaCl, 5.4 mM KCl, 1 mM MgCl_2 , 1.8 mM CaCl_2 , 10 mM 4-(2-hydroxyethyl)-1-piperazine-N'-2-ethanesulphonic acid (HEPES), 10 mM Glucose, 2 mM BaCl_2 , 0.3 mM CdCl_2 , pH adjusted to 7.4 with NaOH. Integrity of the cell and identity of I_f was tested by applying 2 mM cesium followed by a washout with bath solution which restored the original current amplitude. 8-Br-cAMP (Sigma-Aldrich) was dissolved in bath solution to a concentration of 100 μM on the day of the experiment. Cells were superfused with 8-Br-cAMP containing bath solution at least 10 minutes prior to the currents recordings. Data were acquired using a MultiClamp 700A/B amplifier and pClamp10 software (all Axon Instruments/ Molecular Devices, Union City, USA). Analysis was done offline with Origin 6.1. (MicroCal Software, Northampton, USA) or Clampfit10 (Molecular Devices, Union City, USA). Standard I_f parameters were calculated as described (Stieber et al., 2006). The current amplitude was defined as the amplitude at the end of the activation pulse minus the amplitude of the initial lag, current density was obtained by dividing the amplitude through the capacity of the cell. Time constants of activation (τ) were obtained by fitting the current traces after the initial lag with

the sum of two exponential functions: $y_0 + A_1e^{(-x/t_1)} + A_2e^{(-x/t_2)}$, where t_1 and t_2 are the fast and slow time constants of activation, respectively; t_1 is consequently referred to as t since the slow component (A_2) generally accounts for <20% of the current amplitude.

F. Enzyme Histochemistry

For LacZ staining the hearts were fixed for 1 hr at room temperature in fixation solution (4% formaldehyde, 0.2% glutaraldehyde, 5 mM EGTA, 2 mM MgCl₂ in phosphate-buffered saline, pH 7.2-7.4 (PBS)). Organs were rinsed two times in washing buffer (0,01% (v/v) sodium desoxycholate, 0,02% (v/v) Nonidet P-40 (NP-40), 5 mM EGTA, 2 mM MgCl₂ in PBS) and stained for varying length of time at 37°C in the dark with LacZ staining solution (5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 1 mg/ml 5-bromo-4-chloro-3-indolyl-2-D-galactopyranoside (X-gal) in washing buffer). Following staining, specimens were washed at least three times with washing buffer, post-fixed in 4% (w/v) paraformaldehyde (PFA) and washed again in PBS. For LacZ staining on sections, hearts were embedded in OCT (Polysciences) and frozen on dry ice. Sections were cut in a cryostat and fixed for 5 min in fixative solution, rinsed in washing solution, and incubated for varying length of time in LacZ staining solution. For acetylcholinesterase (AChE) staining, sections were incubated with substrate solution containing 65 mM sodium acetate (pH 6.0), 0.5 mg/ml acetylthiocholine iodide, 0.5 mM potassium ferricyanide, 0.3 mM copper sulphate and 5 mM tri-sodium citrate for 2 hours at 37°C. All sections were passed through an ascending series of ethanol, immersed in xylene, and coverslipped with Entellan (E. Merck).

G. Immunohistochemistry

10µm frozen sections were mounted on Superfrost Plus glass slides (Menzel, Braunschweig). Cardiac myocytes were isolated from mouse hearts and fixed on chamber slides with 4% paraformaldehyde. For immunohistochemistry the following primary antibodies were used: HCN4 (5 µg/ml, Alomone Labs), Popdc1 (A) C20 antibody (4 µg/ml, Santa Cruz), Popdc1 (B) HPA014788 antibody (4 µg/ml, SIGMA), TREK-1 (A) H75 (4µg/ml, Santa Cruz), TREK-1 (B) N20 (4 µg/ml, Santa Cruz). As a control, the H75 antibody was preincubated

with 20 µg/ml blocking peptide for 1 hr at room temperature. For the detection of primary antibodies the following secondary antibodies were employed: Alexa Fluor donkey anti-goat 555 (Invitrogen), Alexa Fluor donkey anti-mouse 555 (Invitrogen), Alexa Fluor donkey anti-rabbit 488 (Invitrogen) all 10µg/ml, Anti-goat HRP (5µg/ml, Vector Laboratories), Anti-mouse Fab FITC (15µg/ml Dianova), Anti-mouse HRP (15µg/ml, Vector Laboratories). The fluorescent signal was analyzed by confocal microscopy. For paraffin embedding, hearts were dehydrated through an ethanol series followed by xylene and processed into paraffin blocks for sectioning. Serial 10 µm sections were stained with hematoxylin and eosin (HE) or Masson's trichrome staining.

H. Whole-mount *in situ* Hybridization

The inflow tract of adult mice was fixed overnight with 4% (w/v) PFA in PBS solution, dehydrated through a methanol series for prolonged storage. After rehydration the tissue was bleached in 6% (v/v) H₂O₂ for 1 hour and subjected to 5 min Proteinase K (10 µg/ml) treatment. The tissue was refixed in 4% PFA solution and the hybridization was done overnight at 70°C as described (Andrée et al., 2000). After extensive washing, the tissue was blocked in 10% sheep serum (Sigma-Aldrich), incubated in an anti DIG-antibody 1:2000 (Roche) overnight and stained with NBT/BCIP (Roche).

I. RNA Isolation, RT-PCR and qPCR Analysis

Preparation of total RNA (TRIzol reagent; Invitrogen) and first-strand cDNA synthesis (SuperScript™ First-Strand Synthesis System; Invitrogen) were done according to manufacturer's instructions. Quantitative real-time PCR was performed using the Platinum SYBR Green Supermix-UDG Kit (Invitrogen). The expression ratios were calculated by normalization to *Gapdh* expression and every individual sample was measured three times. The melting curve analysis was used to rule out unspecific PCR products and only cycle threshold (C_t) values of products showing a single melting point were taken into account. Reverse-transcription PCR (RT-PCR) was performed with the following primers:

ANF-fw: 5'-GAGAGACGGCAGTGCTTCTAGGC-3'

ANF-rev: 5'-CGTGACACACCACAAGGGCTTAGG-3'

βMHC-fw: 5'-GCCAACACCAACCTGTCCAAGTTC-3'
βMHC-rev: 5'-TGCAAAGGCTCCAGGTCTGAGGGC-3'
β-actin-fw: 5'-TGGAATCCTGTGGCATCCATGAAAC-3'
β-actin-rev: 5'-TAAAACGCAGCTCAGTAACAGTCCG-3'
Popdc2-fw: 5'-GCCTGCACCACTTTCTGC-3'
Popdc2-rev: 5'-CTCGATTGGCTTCATCTTGG-3'
Popdc3-fw: 5'-CCTGAGTGGGATTCGCTAAG-3'
Popdc3-rev: 5'-CGGTGTCTGCTGTGAGAGTT-3'

J. Mutagenesis

The mutants *Popdc1*^{D200A}, *Popdc1*^{P202A}, *Popdc1*^{E203A}, *Popdc1*^{V217F}, and *Popdc2*^{D184A}, were generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). Primer sequences (mutant nucleotides are underlined) that were used for mutagenesis are:

Popdc1-D200A_{fw}: 5'-TACCCGTGTGCCTTTATAGCTTCTCCCGAATTCAG-3'
Popdc1-D200A_{rv}: 5'-CTGAATTCGGGAGAAGCTATAAAGGCACACGGGTA-3'
Popdc1-P202A_{fw}: 5'-CCCGTGTGCCTTTATAGATTCTGCCGAATTCAGAT-3'
Popdc1-P202A_{rv}: 5'-ATCTGAATTCGGCGAGAATCTATAAAGGCACACGGG-3'
Popdc1-E203A_{fw}: 5'-GCCTTTATAGATTCTCCCGCATTTCAGATCAACGCAGATG-3'
Popdc1-E203A_{rv}: 5'-CATCTGCGTTGATCTGAATGCGGGAGAATCTATAAAGGC-3'
Popdc1-V217F_{fw}: 5'-CAAAGGCGAAAAATTCCAGTTACCATTGTTGCAGATGA-3'
Popdc1-V217F_{rv}: 5'-TCATCTGCAACAATGGTGAACTGGAATTTTTTCGCCTTTG-3'
Popdc2-D184A_{fw}: 5'-TCCGTATCAGTTCATGGCCTCTCCTGAATGGGAAT-3'
Popdc2-D184A_{rv}: 5'-ATTCCCATTTCAGGAGAGGCCATGAACTGATACGGA-3'

The presence of mutant sequences was confirmed by sequence analysis. For injection into *Xenopus* oocytes, wild-type and mutant *Popdc1*, as well as *Popdc2* and *Popdc3* cDNAs were subcloned into psGEM (Villmann et al., 1997).

K. Preparation of Tissue and Cell Lysates

Hearts of 12 to 14 days old chicken embryos were dissected and homogenized in 1 ml/heart of lysis buffer (20 mM HEPES pH 7.4, 20 mM NaCl, 0.5 mM EDTA, 5 mM EGTA, 0.5% Triton X-100, 1 mM DTT and protease inhibitor cocktail (Roche). Cos-7 cells were transiently transfected either with *Popdc1*^{wt},

Popdc1^{D200A}, Popdc1^{P202A}, Popdc1^{E203A}, Popdc1^{V217F}, or TREK-1c-Flag using Lipofectamine 2000 reagent (Invitrogen) and harvested 24 hours after transfection and lysed in a buffer containing 20 mM Tris pH 8.0, 150mM NaCl, 2mM EDTA pH 8.0, 1% (v/v) Triton X-100, 0,05% (w/v) SDS, 1.0 mg/ml BSA, 1 mM DTT, and protease inhibitors. Cells or tissue lysates were cleared by centrifugation at >18,000 x g at 4°C for 10 min. For the generation of TREK-1-Flag, human TREK-1 was cloned in frame with a Flag epitope in pcDNA3.1(+) (Invitrogen).

L. cAMP-Affinity Precipitation

IBMX (3-Isobutyl-1-Methylxanthine (Sigma-Aldrich) was added to a final concentration of 1 mM to the cell or tissue lysates. To prevent unspecific binding the lysates were preincubated with 50 µl ethanolamine-agarose (BioLog) for 1 h with rotation at 4°C. After centrifugation at 500 x g at 4°C, the supernatant was added to 4.8 mg cAMP-agarose (C₈-linked cAMP; A0144, Sigma-Aldrich), and incubated for 2 hrs with rotation at 4°C. As a negative control, lysates were incubated with ethanolamine agarose. After centrifugation at 500 x g at 4°C the pellets were washed four times with wash buffer (10 mM HEPES, pH 7.4, 1.5 mM MgCl₂, 10 mM KCl, 0,1% (v/v) Igepal CA-630, 1 mM DTT, and protease inhibitor cocktail) and taken up in SDS loading buffer or subjected to the cAMP elution protocol. As a positive control 5 µl of total cell or tissue lysate was loaded directly on an SDS gel. To elute protein from cAMP-agarose, 5, 10, 20 and 20 mM 6-MB-cAMP (BioLog) in a total volume of 50 µl in wash buffer, containing 1 mM IBMX was added to the pellet and incubated for 30 minutes at 4°C with rotation. After centrifugation, SDS loading buffer was added to the supernatant and the cAMP agarose pellets, respectively, and subjected to Western blot analysis.

M. GST-Pulldown

GST-Bves harbouring the cytoplasmic portion (aa 115-347) of Popdc1 (Osler et al., 2005) and GST-E12 were kind gifts of David Bader, Vanderbilt University, Nashville, TN and Manfred Gessler, University of Würzburg, Germany, respectively. Glutathione beads (GE Healthcare) were prepared according to

(Smith et al., 2008). Cell lysate of Cos-7 cells transiently transfected with TREK-1-Flag was added to GST-Bves, GST-E12, or GST bound to glutathione-sepharose 4B beads and incubated overnight at 4°C. The beads were washed 5 times with 200 µl PBS and bound proteins were eluted and subjected to Western blot analysis.

N. Gel Electrophoresis and Western Blot

Proteins were size separated and transferred by electroblotting onto nitrocellulose membrane (BioTrace NT, Pall). The membrane was washed with TBST (50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween-20) and blocked in 5% (w/v) low-fat milk in TBST for 1 hr at room temperature or overnight at 4°C. For immunodetection of chick *Popdc1*, a monoclonal antibody directed against chick *Popdc1* (Pop1 3F11) (DiAngelo et al., 2001) was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. For immunodetection of mouse *Popdc1*, 1 µg/ml of a polyclonal goat anti-Bves antibody (BVES (C-20); Santa Cruz) was used. A mouse anti-myc antibody (Abcam) and a mouse anti-Flag antibody (Sigma-Aldrich) were employed to detect myc and Flag epitope fusion proteins, respectively. After several washes, the blots were incubated for 1 hr at room temperature with horseradish peroxidase-coupled anti-mouse or anti-rabbit monoclonal antibodies (Vector). After washing, signals were detected using an enhanced chemiluminescence protein detection method (Thermo Scientific).

O. Measurement of TREK-1 Current in *Xenopus* Oocytes

Human TREK-1 and TASK-1 as well as mouse *Popdc1*, *Popdc2*, and *Popdc3* and human POPDC2 and POPDC2^{W188X} cDNAs were subcloned into the psGEM vector (Villmann et al., 1997). Capped run-off poly(A⁺) cRNA transcripts from linearised cDNAs were synthesised. The ovarian lobes were dissected from mature *Xenopus laevis* anesthetized with tricaine and treated with collagenase (1 mg/ml, Worthington, type II) in OR2 solution (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM HEPES, pH 7.4) for 120 min. Stage IV and V oocytes were injected with cRNAs encoding TREK-1 (0.25 ng) or TASK-1 (1.5

ng), alone or together with Popdc1, Popdc2 and Popdc3 cRNAs (0.05 or 0.25 ng). The injected oocytes were stored at 18°C in ND96 recording solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES), supplemented with 275 mg/l Na-pyruvate, 90 mg/l theophylline, and 50 mg/l gentamicine, pH 7.4 and assayed 24-72 hrs post-injection. Standard two-microelectrode voltage-clamp recordings were performed at room temperature (21-22°C). Microelectrodes were fabricated from glass pipettes filled with 3 M KCl and had a resistance of 0.2-1.0 MΩ. Voltage-clamp recordings were performed at room temperature (21–22°C) with an Axoclamp 900A amplifier, a Digidata 1440A and pClamp10 software (Axon Instruments). Some measurements were made with an EPC9 (Heka) patch clamp amplifier, PULSE/PULSEFIT software (Heka), and data analysis was performed with IGOR software (WaveMetrics, Lake Oswego, USA). The holding potential was always –80 mV.

P. Surface expression analysis

Surface expression of HA-tagged TREK-1 channel constructs was analyzed in *Xenopus* oocytes as described previously (36). Three days after cRNA injection, oocytes were incubated for 30 min in ND96 solution containing 1% BSA at 4°C to block non-specific binding of antibodies. Subsequently, oocytes were incubated for 60 min at 4°C with 1 mg/mL rat monoclonal anti-HA antibody (clone 3F10, Roche Pharmaceuticals, Basel, Switzerland) in 1% BSA/ND96, washed six times at 4°C with 1% BSA/ND96 and incubated with 2 µg/µL peroxidase-conjugated affinity-purified F(ab)₂ fragment goat anti-rat IgG antibody (Dianova) in 1% BSA/ND96 for 60 min. Oocytes were washed thoroughly, initially in 1% BSA/ND96 (at 4°C for 60 min) and then in ND96 without BSA (at 4°C for 15 min). Individual oocytes were placed in 20 µl SuperSignal Elisa Femto solution (Pierce) and chemiluminescence was quantitated in a luminometer (Promega). The luminescence produced by noninjected oocytes (ni) was used as a reference signal (negative control).

Q. Modeling of the Popeye domain

The secondary structure of the Popeye domain A was predicted by the PSIPRED program (<http://bioinf4.cs.ucl.ac.uk:3000/psipred/>). Modeling of the Popeye domain of human Popdc1 (residues Ser125 to Asp266) was performed starting from a crude model built by the web-based alignment/building tool Phyre (<http://www.sbg.bio.ic.ac.uk/phyre/html/index.html>). Based on the experimental observations that the Popeye domain is likely a cAMP-binding module, the Phyre results were screened for domains with similarity to cAMP-binding proteins/domains. Of the ten hits with known structures, six protein domains sharing significant sequence similarities with Popdc1 belong to the class of cAMP-binding proteins. The representative (PDB entry 2PQQ: N-terminal domain of a transcriptional regulator from *Streptomyces coelicolor*) with the highest sequence identity (20% identity over 149 amino acid residues) was chosen for further model building. At first, the model, which was lacking several loop segments, was refined by manually building, and missing residues were added using the ProteinDesign tool of the software package Quanta2006 (Accelrys Inc, San Diego). The completed model was subsequently subject to energy minimization to remove bad van der Waals contacts between side chain as well as main chain atoms. Steric clashes between side chains were removed by performing rotamer searches using the XBuild tool of the Quanta2006 software for the side chains involved in the close contact. Subsequently, energy minimization was performed, keeping the main chain atoms fixed and side chain atoms with a weak harmonic potential (force constant: $5 \text{ kcal mol}^{-1} \text{ \AA}^{-2}$) using only geometric energy terms (no electrostatic potential). After 500 steps of steepest gradient minimization, main chains atoms were also relaxed stepwise by first employing a strong harmonic potential with a force constant of $50 \text{ kcal mol}^{-1} \text{ \AA}^{-2}$ and in a second round of $5 \text{ kcal mol}^{-1} \text{ \AA}^{-2}$. This model was structurally superimposed onto the cAMP-binding domain of the cAMP-dependent protein kinase (PKA; PDB entry 1CX4). The cAMP-binding loop of our Popdc1 model was rebuilt on the basis of sequence and structural similarity with 1CX4. A cAMP-moiety was placed in the putative binding pocket of the Popeye domain using the contacts identified in the structure of the cAMP-binding domain of protein kinase A. The improved model

for the Popeye domain of Popdc1 was again subjected to energy minimization as described above. The final model shows good overall geometry for backbone and side chain geometries and had no bad van der Waals contacts.

R. Fluorescence Resonance Energy Transfer (FRET) Assay

Popdc1 cDNA was cloned into the pECFP-N1 vector (Clontech) to obtain Popdc1-CFP. hTREK-1c and EYFP cDNAs were amplified by PCR and cloned into pcDNA3.1(+) to produce YFP-TREK-1. 293A cells were transiently transfected with Popdc1-CFP and YFP-TREK-1 constructs using Effectene Reagent (Qiagen), and FRET measurements were performed 24 hr later. For fluorescent microscopy, glass coverslips with adherent cells were mounted in an experimental chamber, placed on a Zeiss Axiovert 200 inverted microscope equipped with an oil-immersion 63x objective, a polychrome IV light source (Till Photonics, Planegg, Germany), a DualView imaging system and a CoolSNAP-HQ CCD-camera (Visitron Systems, Puchheim, Germany). FRET was monitored using MetaFluor 5.0 software (Molecular Devices, Sunnyvale, USA) as the ratio between emission at 535 ± 20 nm (YFP) and emission at 480 ± 15 nm (CFP), upon excitation at 436 ± 10 nm. The imaging data were analyzed by MetaMorph 5.0 (Molecular Devices) and Origin (OriginLab Corporation, Northampton, USA) software, corrected for spillover of CFP into the 535 nm channel and direct YFP-excitation to give corrected YFP/CFP ratios. Images were acquired every 5 s, with 10-20 ms illumination time, which resulted in negligible photobleaching.

S. Radioligand-binding Assay

Purified GST-C-terminal Popdc1 protein (1-2 μ g) was incubated for 1 hr at 37°C in 200 μ l of the assay buffer (20 mM Tris, 100 mM NaCl, 1mM EDTA, pH 7.6) containing 0.1-1.0 nM [3 H]-cAMP (Amersham, Freiburg, Germany) and increasing concentrations of unlabeled cAMP or cGMP (Sigma-Aldrich). Reactions were terminated by vacuum filtration through GF/B glass fibre filters (Millipore, Schwalbach, Germany) and the bound radioactivity was measured.

Supplemental References

- Andrée, B., Hillemann, T., Kessler-Icekson, G., Schmitt-John, T., Jockusch, H., Arnold, H.H., and Brand, T. (2000). Isolation and characterization of the novel popeye gene family expressed in skeletal muscle and heart. *Dev Biol* 223, 371-382.
- DiAngelo, J.R., Vasavada, T.K., Cain, W., and Duncan, M.K. (2001). Production of monoclonal antibodies against chicken Pop1 (BVES). *Hybrid Hybridomics* 20, 377-381.
- Harzheim, D., Pfeiffer, K.H., Fabritz, L., Kremmer, E., Buch, T., Waisman, A., Kirchhof, P., Kaupp, U.B., and Seifert, R. (2008). Cardiac pacemaker function of HCN4 channels in mice is confined to embryonic development and requires cyclic AMP. *EMBO J* 27, 692-703.
- Kirchhof, P., Fabritz, L., Zwiener, M., Witt, H., Schafers, M., Zellerhoff, S., Paul, M., Athai, T., Hiller, K.H., Baba, H.A., *et al.* (2006). Age- and training-dependent development of arrhythmogenic right ventricular cardiomyopathy in heterozygous plakoglobin-deficient mice. *Circulation* 114, 1799-1806.
- Knollmann, B.C., Kirchhof, P., Sirenko, S.G., Degen, H., Greene, A.E., Schober, T., Mackow, J.C., Fabritz, L., Potter, J.D., and Morad, M. (2003). Familial hypertrophic cardiomyopathy-linked mutant troponin T causes stress-induced ventricular tachycardia and Ca²⁺-dependent action potential remodeling. *Circ Res* 92, 428-436.
- Kuhlmann, M.T., Kirchhof, P., Klocke, R., Hasib, L., Stypmann, J., Fabritz, L., Stelljes, M., Tian, W., Zwiener, M., Mueller, M., *et al.* (2006). G-CSF/SCF reduces inducible arrhythmias in the infarcted heart potentially via increased connexin43 expression and arteriogenesis. *J Exp Med* 203, 87-97.
- Lewandoski, M., Wassarman, K.M., and Martin, G.R. (1997). Zp3-cre, a transgenic mouse line for the activation or inactivation of loxP-flanked target genes specifically in the female germ line. *Curr Biol* 7, 148-151.
- Mangoni, M.E., and Nargeot, J. (2001). Properties of the hyperpolarization-activated current (I_f) in isolated mouse sino-atrial cells. *Cardiovasc Res* 52, 51-64.
- Osler, M.E., Chang, M.S., and Bader, D.M. (2005). Bves modulates epithelial integrity through an interaction at the tight junction. *J Cell Sci* 118, 4667-4678.

Smith, T.K., Hager, H.A., Francis, R., Kilkenny, D.M., Lo, C.W., and Bader, D.M. (2008). Bves directly interacts with GEFT, and controls cell shape and movement through regulation of Rac1/Cdc42 activity. *Proc Natl Acad Sci U S A*.

Stieber, J., Wieland, K., Stockl, G., Ludwig, A., and Hofmann, F. (2006). Bradycardic and proarrhythmic properties of sinus node inhibitors. *Mol Pharmacol* 69, 1328-1337.

Villmann, C., Bull, L., and Hollmann, M. (1997). Kainate binding proteins possess functional ion channel domains. *J Neurosci* 17, 7634-7643.