Ion channel-kinase TRPM7 is required for maintaining cardiac automaticity

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Sick sinus syndrome and atrioventricular block are common clinical problems, often necessitating permanent pacemaker placement, yet the pathophysiology of these conditions remains poorly understood. Here we show that Transient Receptor Potential Melastatin 7 (TRPM7), a divalent-permeant channel-kinase of unknown function, is highly expressed in embryonic myocardium and sinoatrial node (SAN) and is required for cardiac automaticity in these specialized tissues. TRPM7 disruption in vitro, in cultured embryonic cardiomyocytes, significantly reduces spontaneous Ca2+ transient firing rates and is associated with robust down-regulation of Hcn4, Ca3.1, and SERCA2a mRNA. TRMP7 knockout in zebrafish, global murine cardiac Trpm7 deletion (KOmHHC-Cre), and tamoxifen-inducible SAN restricted Trmp7 deletion (KOmCNA-CreERT2) disrupts cardiac automaticity in vivo. Telemetered and sedated KOmHHC-Cre and KOmCNA-CreERT2 mice show episodes of sinus pauses and atrioventricular block. Isolated SAN from KOmHHC-Cre mice exhibit diminished Ca2+ transient firing rates with a blunted diastolic increase in Ca2+. Action potential firing rates are diminished owing to slower diastolic depolarization. Accordingly, Hcn4 mRNA and the pacemaker current, If, are diminished in SAN from both KOmHHC-Cre and KOmCNA-CreERT2 mice. Moreover, heart rates of KOmHHC-Cre mice are less sensitive to the selective If blocker ivabradine, and acute application of the recently identified TRPM7 blocker FTY720 has no effect on action potential firing rates of wild-type SAN cells. We conclude that TRPM7 influences diastolic membrane depolarization and automaticity in SAN indirectly via regulation of Hcn4 expression.

arrhythmia | electrocardiogram | electrophysiology | confocal

Significance

Transient Receptor Potential Melastatin 7 (TRPM7) is a divalent-permeant channel-kinase of unknown function expressed in human atrial myocytes and fibroblasts and recently implicated in atrial arrhythmias. We show that TRPM7 is highly expressed in embryonic myocardium and sinoatrial node (SAN). Trmp7 disruption in vitro, in cultured embryonic cardiomyocytes, and in vivo in zebrafish and in mice impairs cardiac automaticity. We show that this occurs via reductions in Hcn4 mRNA and the pacemaker current, If, in SAN. We conclude that TRPM7 influences diastolic membrane depolarization and automaticity in SAN via regulation of Hcn4 expression.

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day (E)9.5 embryos (7) and becomes ubiquitously expressed in later embryonic development (7) and into adulthood (5, 6). Because TRPM7 is first expressed in the developing embryonic heart (7), we examined TRPM7 current in cultured EVM isolated from E13.5–14 Trpm7fl/fl (7) embryos and compared these with TRPM7 current in freshly dissociated AVM. A large TRPM7-like current is activated in EVM (I_{Trpm7,AVM} = 64.2 ± 16.7 pA/pF, n = 5), ~eightfold larger than AVM (I_{Trpm7,AVM} = 8.3 ± 0.9 pA/pF, n = 5, P < 0.05; Fig. 1 A and B) under whole-cell conditions. These TRPM7-like currents are initially absent on “break-in” but then run up over the course of 10 min of cell dialysis to a steady-state level (Fig. S1). To confirm that these TRPM7-like currents are indeed TRPM7, we used Cre-loxP technology to genetically ablate Trpm7 in murine EVM. The conditional Trpm7 allele contained loxP sites flanking exon 17, and Cre-mediated recombination induces a frame shift that prevents expression of the ion channel and kinase domains of TRPM7 (7). We introduced Cre to cultured Trpm7fl/fl EVM using adenoviruses (Ad) that drive Cre expression under either cytomegalovirus (CMV: ubiquitous) or troponin-T (TnT: cardiac-specific) promoters (Ad-CMV-Cre/Ad-TnT-Cre). An adenovirus expressing β-galactosidase (Ad-CMV-Lacz) served as a control. Using EVM isolated from E14 ROSA26TrpM7EG embryos, we confirmed high-efficiency Cre transduction in EVM (~95%) by both Ad-CMV-Cre and Ad-TnT-Cre (Fig. 1C, only Ad-TnT-Cre shown). Genetic ablation of Trpm7 exon 17 in Trpm7fl/fl EVM is evident by PCR within 2 d after adenoviral transduction with both Ad-TnT-Cre (Fig. 1D, lane 1) and Ad-CMV-Cre (lane 2) but not with Ad-CMV-Lacz (lane 3), according to the presence of the expected size of the deletion product in EVM genomic DNA. Finally, TRPM7 current is largely abolished in Trpm7fl/fl EVM treated with Ad-CMV-Cre when patch-clamped 4–5 d after adenoviral transduction (Fig. 1 E and F).

After 4 d in culture, Ad-CMV-Lacz–treated Trpm7fl/fl EVMs contract rhythmically at a significantly higher rate than either Ad-CMV-Cre- or Ad-TnT-Cre–treated EVM. To quantify this difference, we loaded EVM with Fluo-4AM and measured intracellular Ca^{2+} transients in clusters of Ad-CMV-Lacz-, Ad-CMV-Cre-, and Ad-TnT-Cre–treated Trpm7fl/fl EVM using high-speed laser scanning confocal microscopy (Fig. 2A). Ca^{2+} transient firing frequency is reduced equivalently ~twofold in EVM upon deletion of Trpm7 by Ad-CMV-Cre (102 ± 18/min, n = 18, P < 0.01) and Ad-TnT-Cre (103 ± 17/min, n = 13, P < 0.01) compared with Ad-CMV-Lacz controls (206 ± 23/min, n = 18; Fig. 2B). Accordingly, cycle length increased ~twofold after Trpm7 deletion (Fig. 2C). To control for possible off-target effects of Cre expression, we also compared cycle lengths of Trpm7fl/+ EVM treated with Ad-CMV-Cre and Ad-TnT-Cre (Fig. S2). We found these no different from the Ad-CMV-Lacz controls. There is a small but statistically significant increase in peak Ca^{2+}
transient amplitude upon Trpm7 deletion with Ad-CMV-Cre (F/F<sub>CVM-Cre</sub> = 2.2 ± 0.1, n = 19, P < 0.05), but this is not observed in Ad-TnT-Cre (F/F<sub>TnT-Cre</sub> = 1.7 ± 0.1, n = 12) treated Trpm7<sup>fl/fl</sup> EVM compared with Ad-CMV-Lacz controls (F/F<sub>CVM-Lacz</sub> = 1.8 ± 0.1, n = 19; Fig. 2D). Additionally, Ca<sup>2+</sup> transient duration is significantly lengthened in Trpm7-deleted EVM by both Ad-CMV-Cre (664 ± 96 ms, n = 20, P < 0.001) and Ad-TnT-Cre (471 ± 59 ms, n = 12, P < 0.05) compared with Ad-CMV-Lacz (291 ± 43, n = 19; Fig. 2E). Thus, TRPM7 deletion seems to slow spontaneous contractions and Ca<sup>2+</sup> transient firing, in a cell autonomous fashion in cultured embryonic cardiomyocytes.

**Trpm7 Loss of Function in Vivo Disrupts Automaticity in Zebrafish and Induces Sinus Pauses and AVB in Mouse.** We next examined whether this effect of TRPM7 knockout on embryonic cardiac automaticity is also present in vivo in embryonic zebrafish. We find that the Trpm7 morphtolino (MO) zebrafish recapitulate the previously described phenotype of melanocyte deficiency and loss of touch responsiveness (12, 13) compared with water-injected controls. In addition, heart rates are ~25% lower in Trpm7 MO zebrafish (HR, Trpm7 MO = 80 ± 2 bpm, n = 30 embryos, P < 0.001) vs. controls (HR, H<sub>20</sub> = 107 ± 2 bpm, n = 27 embryos; Fig. 3A), suggesting that the negative chronotropic effect of TRPM7 disruption is not restricted to cultured murine EVM but also occurs in intact zebrafish embryos.

To determine whether TRPM7 is also important for automaticity in the adult mouse heart we turned to global cardiac TRPM7 knockout mice generated by crossing αMHC-Cre mice with either αMHC-Cre mice (KO<sub>αMHC-Cre</sub>). Conscious electrocardiograms recorded over a 24-h period in freely moving telemetered KO<sub>αMHC-Cre</sub> mice reveal frequent SPs (Fig. 3 C and E and Fig. S3 B and F) and AVB (Fig. 3 D and F and Fig. S3D) compared with no SP or AVB in WT mice (Fig. 3 B and Fig. S3 A and E). KO<sup>αMHC-Cre</sup> mice also exhibit SP with atrial bigeminy (Fig. S3 C and F) and ectopic atrial foci (Fig. S3 B and G) in both conscious, telemetered mice as well as in sedated mice. Despite the presence of these bradyarrhythmias in KO<sup>αMHC-Cre</sup> mice, the mean heart rates over a 24-h period are not statistically different from WT (Fig. 3G).

**TRPM7 Is Highly Expressed in Murine Sinoatrial Nodal Cells and Is Required for Normal SAN Automaticity.** The SPs and AVB observed in KO<sup>αMHC-Cre</sup> mice suggest that Trpm7 deletion affects SAN and AVN function, because these are the specialized myocardin cell types that exhibit automaticity in the adult heart. To determine whether this was due to a direct effect of TRPM7 activity in SAN, we next examined TRPM7 current in freshly isolated SAN and assessed whether TRPM7 is efficiently deleted in SAN cells of KO<sup>αMHC-Cre</sup> mice. We first confirmed robust Cre expression in all observed SAN cells by crossing αMHC-Cre mice with the mTmG reporter mouse line (ROSA26<sup>tm1GtmG</sup>), in which membrane-targeted green fluorescent protein expression (mg) is induced only after Cre-mediated recombination (14) (Fig. 4A, single SAN shown). Indeed, αMHC-Cre induces recombination in all myocardial cells (15). Next we confirmed deletion of Trpm7 exon 17 from genomic DNA isolated from SAN by PCR in KO<sup>αMHC-Cre</sup> mice (Fig. 4B) according to the presence of the expected size of the deletion product. Finally we measured TRPM7 current from isolated SAN (I<sub>Trpm7</sub>, WT SAN = 32.0 ± 6.2 pA/pF at +100 mV, n = 6) and found them to be ~fourfold larger than TRPM7 in AVM (I<sub>Trpm7</sub>, AVM = 8.3 ± 0.9 pA/pF, n = 5) and absent in KO<sup>αMHC-Cre</sup> SAN (I<sub>Trpm7</sub>, KO SAN = 0.4 ± 0.2 pA/pF, n = 4, P < 0.01; Fig. 4 C and D). Similar to EVM, the initial TRPM7 current on break-in before SAN dialysis is negligible and

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**Fig. 3.** Trpm7 deletion in vivo disrupts automaticity in zebrafish and induces SPs and AVB in mice. (A) (Left) Images of zebrafish embryos: water-injected (Upper) and Trpm7 MO-injected (Lower). (Right) Trpm7 MO zebrafish (n = 30) and water-injected zebrafish (n = 27) heart rates. (B) Normal sinus rhythm (p denotes P waves; atrial depolarization) with intact atrioventricular conduction in the ECG of a telemetered, conscious WT mouse. (C) Representative ECG showing an episode of SP observed in a KO<sup>αMHC-Cre</sup> mouse. Solid arrows denote location of expected p waves. (D) Representative ECGs demonstrating AVB observed in KO<sup>αMHC-Cre</sup> mice (broken black arrow, conducted QRS complexes; broken gray arrow, expected location of QRS complex). (E and F) Box plots with overlying data points showing the distribution of the frequency of (E) SPs and (F) AVB observed over 24 h of telemetric monitoring in WT (n = 8) and KO<sup>αMHC-Cre</sup> (n = 8) mice. (G) Mean heart rates of WT and KO<sup>αMHC-Cre</sup> over a 24-h period were not statistically different. In box plots, error bars represent the SD of the mean. Box height represents the SE. **P < 0.01, ***P < 0.001.
only activates fully after ~10 min of cell dialysis (Fig. S4). Thus, measuring TRPM7 current in SAN, under these conditions, serves primarily as an assay to quantify the amount of functional TRPM7 channels in the membrane of a given cell, as opposed to showing the active current present under physiological conditions.

As with EVM, we next assessed automaticity in WT (Fig. 5A) and KO\textsuperscript{MHC-Cre} SAN (Fig. 5B) using confocal microscopy to measure spontaneous Ca\textsuperscript{2+} transients. Ca\textsuperscript{2+} transient frequency is significantly diminished in KO\textsuperscript{MHC-Cre} SAN (Ca\textsuperscript{2+} transient rate \(k_0 = 32 \pm 5 \text{ min}^{-1} \), n = 34) compared with WT (Ca\textsuperscript{2+} transient rate \(k_T = 72 \pm 11 \text{ min}^{-1} \), n = 26, \(P < 0.01 \)); Fig. 5C), and Ca\textsuperscript{2+} transients are lengthened (Fig. 5D), as observed in TRPM7-depleted EVM (Fig. 2). Isoproterenol (ISO) increases the Ca\textsuperscript{2+} transient frequency in both WT and KO\textsuperscript{MHC-Cre} cells, but the maximal rate reached by KO\textsuperscript{MHC-Cre} cells in ISO remains slower than in WT counterparts. Similar results, although less marked, are obtained by measuring the frequency of Ca\textsuperscript{2+} transients in individual pacemaker cells within the intact, undissociated SAN upon TRPM7 deletion (Fig. S5A). Despite these clear differences in Ca\textsuperscript{2+} transient firing frequency, peak Ca\textsuperscript{2+} transients are no different in KO\textsuperscript{MHC-Cre} SAN compared with WT (Fig. S5B), consistent with unchanged SR Ca\textsuperscript{2+} load as assessed by 10 mM caffeine application (Fig. S5C).

Cardiac automaticity is determined by diastolic depolarization arising from the combined effects of voltage-gated ion channels (Hcn2/Hcn4/Ca\textsubscript{3.1}/Ca\textsubscript{1.3}) and intracellular Ca\textsuperscript{2+} cycling (diastolic Ca\textsuperscript{2+} and Ncx2) (3). Thus, we next measured the rate of rise of diastolic Ca\textsuperscript{2+} leading up to each Ca\textsuperscript{2+} transient and found this to be significantly blunted in KO\textsuperscript{MHC-Cre} SAN (Fig. 5E, Right) compared with WT (Fig. 5E, Left) and also only minimally responsive to ISOstimulation (Fig. 5F). These data suggest that TRPM7 is required for maintenance of normal automaticity in murine SAN and that it can influence diastolic Ca\textsuperscript{2+} release, either directly or indirectly, and thereby contribute to diastolic depolarization in SAN (3).

**DSSL is Diminished in KO\textsuperscript{MHC-Cre} SAN Cells:** Hcn4 and Ir Are Down-Regulated. To further examine the mechanism by which Trpm7 deletion impairs SAN cell automaticity we measured spontaneous action potentials (APs) in freshly isolated SAN using the perforated patch-clamp technique. Consistent with the Ca\textsuperscript{2+} imaging experiments, AP firing rates are significantly diminished in KO\textsuperscript{MHC-Cre} SAN compared with WT SAN (Fig. 6A and B) and largely reversed by ISO. By measuring membrane potential, we quantified the DSSL (Fig. 6C), an important determinant of SAN firing rate (4), in WT and KO\textsuperscript{MHC-Cre} SAN. DSSL was significantly lower in KO\textsuperscript{MHC-Cre} SAN (DSSL \(k_0 = 0.03 \pm 0.01 \text{ mV/min} \), n = 5, \(P < 0.05 \)) compared with WT (DSSL WT = 0.09 mV/min \(\pm 0.01 \), n = 7), and this difference was reversed by 100 nM ISO (Fig. 6D and Table S1).

Rising diastolic Ca\textsuperscript{2+} is thought to contribute, in part, to diastolic depolarization in SAN via forward mode Na\textsuperscript{+}–Ca\textsuperscript{2+} exchange current and calcium-induced Ca\textsuperscript{2+} release (3, 16), but other membrane currents, including the hyperpolarization-activated pacemaker current (\(I_h\), encoded by Hcn1/Hcn2/Hcn4), T-type Ca\textsuperscript{2+} current (\(I_{Ca,L}\), encoded by Ca\textsubscript{3.1}) and L-type Ca\textsuperscript{2+} current (\(I_{Ca,T}\), encoded by Ca\textsubscript{1.2} (2–4, 17) also participate. In fact, acute blockade of \(I_h\) alone in isolated SAN, using the selective \(I_h\) blocker ivabradine (18), was recently shown to be capable of slowing intracellular Ca\textsuperscript{2+} cycling kinetics and prolonging the period of spontaneous local Ca\textsuperscript{2+} releases occurring during diastolic depolarization (19). Thus, much of what we observe with respect to Ca\textsuperscript{2+} cycling in KO\textsuperscript{MHC-Cre} SAN may be explained via a mechanism of Hcn4 and \(I_h\) reduction. Accordingly, we examined the change in relative expression levels of a panel of genes implicated in cardiac automaticity, both in vitro in cultured Trpm7-depleted EVM as well as in dissected SAN from WT and KO\textsuperscript{MHC-Cre} hearts. In Trpm7-depleted EVM, Hcn4, Ca\textsubscript{3.1}, and Serc2a2a mRNA are robustly down-regulated eightfold, sixfold, and sixfold respectively, relative to WT ECM, whereas Trpm4, Hcn2, and Ca\textsubscript{1.3} are not significantly differentially expressed (Fig. 7A). As expected, Trpm7 mRNA is 19-fold reduced in these Trpm7-deleted cells. Similarly, in SAN dissected from KO\textsuperscript{MHC-Cre} hearts, Hcn4 mRNA is reduced twofold, and Trpm7 mRNA is threefold reduced relative to WT SAN. However, neither Hcn2, Trpm4, Ca\textsubscript{3.1}, Ca\textsubscript{1.3}, nor Serc2a2a mRNA are significantly down-regulated in KO\textsuperscript{MHC-Cre} SAN (Fig. 7B). We next measured the hyperpolarization-activated current, \(I_h\), in freshly isolated SAN to establish whether these reductions in Hcn4 mRNA indeed result in diminished functional \(I_h\) current (Fig. 7C and D). We find \(I_h\) to be on average significantly diminished in KO\textsuperscript{MHC-Cre} SAN (\(I_h\), KO\textsuperscript{MHC-Cre} = -11.9 ± 2.6 pA/PF @ \(-160 \text{ mV} \), n = 27) compared with WT (\(I_h\), WT = -28.7 ± 6.6 pA/PF @ \(-160 \text{ mV} \), n = 14, \(P < 0.01 \)). Furthermore, functional mosaicism is observed in which some KO\textsuperscript{MHC-Cre} SAN have virtually no \(I_h\) (9/27 = 33% < -3 pA/PF at \(-160 \text{ mV} \))), whereas other KO\textsuperscript{MHC-Cre} SAN have nearly normal \(I_h\) current densities (Fig. 7E). On the other hand, no WT SAN (0 of 14) have \(I_h\) current densities below 3 pA/PF.

Because Ca\textsubscript{3.1} mRNA is also significantly down-regulated upon Trpm7 deletion in cultured EVM but not in isolated KO\textsuperscript{MHC-Cre} SAN, we examined whether T-type Ca\textsuperscript{2+} current (\(I_{Ca,T}\)) or R-type Ca\textsuperscript{2+} current (\(I_{Ca,R}\)) are altered in isolated KO\textsuperscript{MHC-Cre} SAN, because these currents are also thought to contribute to both diastolic Ca\textsuperscript{2+} influx and diastolic depolarization in SAN. We measured \(I_{Ca,T}\) by subtracting \(I_{Ca,L}\) (Fig. 7F, Right) from total Ca\textsuperscript{2+} current, \(I_{Ca,T} + I_{Ca,L}\) (Fig. 7F, Left) and generated the current–voltage relationship for \(I_{Ca,T}\) shown in Fig. 7G. \(I_{Ca,T}\), \(I_{Ca,T} + I_{Ca,L}\), and \(I_{Ca,L}\) are not significantly different in KO\textsuperscript{MHC-Cre} compared with WT SAN at all membrane potentials examined. Collectively these data suggest that the negative chronotropic effects of Trpm7 deletion in SAN are mediated largely via down-regulation of Hcn4 and \(I_h\), which decreases the slope of diastolic depolarization and thereby slows
automaticity. Consistent with this notion, KO\(^{\text{MHC-Cre}}\) mice are less sensitive than WT counterparts, in a dose-dependent fashion, to the negative chronotropic effects of the ivabradine (Fig. 7H). Furthermore, the recently identified TRPM7 blocker FTY720 has no effect on SAN firing rates and AP durations when acutely applied to WT SAN cells at a concentration shown to nearly completely block endogenous TRPM7 in human atrial broblasts (500 nM) (20) (Fig. S6).

**Postdevelopmental, SAN-Restricted Trpm7 Deletion Recapitulates the Phenotype Observed in KO\(^{\text{MHC-Cre}}\) Mice.** Because \(\alpha\)MHC-Cre is expressed and recombines in cardiomyocytes as early as E12-14 (21), it is possible that these findings in adult KO\(^{\text{MHC-Cre}}\) SAN result from developmental effects of Trpm7 on SAN maturation or differentiation, consistent with our previous findings in lymphocytes (7) and embryonic tissues (15, 22). Furthermore, \(\alpha\)MHC-Cre deletes globally in heart, so it is also conceivable that KO\(^{\text{MHC-Cre}}\) SAN are affected in a paracrine fashion by Trpm7 deletion in neighboring, non-SAN cells. To address these alternative mechanisms, we crossed Hcn4-CreERT2 mice (23) with Trpm7\(^{fl/fl}\) or Trpm7\(^{fl}\) mice to generate a line of mice (KO\(^{Hcn4-CreERT2}\)) that provides tamoxifen (Tm)-inducible, SAN/AVN-restricted Trpm7 deletion (Fig. 8A). Using Hcn4-CreERT2-ROSA26mTmG mice, we demonstrate efficient recombination that is restricted to the SAN region (green, mGFP) in 6-wk-old Hcn4-CreERT2-ROSA26mTmG mouse right atrium (red, mTomato) (Fig. 8B) after Tm gavage (40 mg/kg) × 4 d (Fig. 8A). In addition, genomic DNA isolated from KO\(^{Hcn4-CreERT2}\) SAN reveals efficient deletion of Trpm7 exon 17 according to the presence of the expected size of the deletion product on PCR (Fig. 8C). Finally, TRPM7 current measured by whole-cell patch clamp of KO\(^{Hcn4-CreERT2}\) SAN is eliminated (\(I_{\text{Trpm7 KO}, \text{Hcn4-CreERT2}}\) SAN = 0.1 ± 0.1 nA/pF, \(n = 4, P < 0.01;\) Fig. 8D and E). Having established effective, postdevelopmental, SAN-restricted Trpm7 deletion in KO\(^{Hcn4-CreERT2}\) mice, we next performed telemetric studies to assess for bradycardias, as observed in KO\(^{\text{MHC-Cre}}\) mice. Strikingly, KO\(^{Hcn4-CreERT2}\) mice also exhibit SPs with ectopic atrial escape rhythms (Fig. 8F and G, Left) as well as AVB (Fig. 8G, Right), whereas the mean heart rate over 24 h is unchanged, similar to KO\(^{\text{MHC-Cre}}\) (Fig. 3C–F and Fig. S3). Likewise, \(I_f\) in patch-clamped KO\(^{Hcn4-CreERT2}\) SAN is significantly diminished (\(I_f, \text{KO}_\text{Hcn4-CreERT2} = -6.9 \pm 2.2\) pA/pF, \(n = 25\)) relative to WT.

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**Fig. 5.** Trpm7 deleted SAN exhibit impaired automaticity with slowed diastolic Ca\(^{2+}\) rise. Ca\(^{2+}\) transients measured by high-speed line-scanning confocal microscopy in (A) WT SAN and (B) KO\(^{\text{MHC-Cre}}\) SAN under basal conditions (Top) and after stimulation with ISO: 2 nM (Middle) and 100 nM (Bottom). (C) Mean Ca\(^{2+}\) transient frequency and (D) Ca\(^{2+}\) transient length in WT SAN compared with KO\(^{\text{MHC-Cre}}\) SAN under basal conditions and after stimulation with 2 nM and 100 nM ISO. (E) Representative Ca\(^{2+}\) transients from WT (Left) and KO\(^{\text{MHC-Cre}}\) (Right) showing the slope of diastolic Ca\(^{2+}\) rise (Ca\(^{2+}\) ramp). (F) Mean slope of the diastolic Ca\(^{2+}\) ramp in WT and KO\(^{\text{MHC-Cre}}\) under increasing ISO concentrations. *\(P < 0.05, **P < 0.01.\)
SAN ($I_f$, WT SAN) = $-21.8 \pm 4.9$ pA/pF, $n = 11$, $P < 0.01$, Fig. 8H and I) and comparable to KO-MHC-Cre ($I_f$, KO-MHC-Cre) = $-11.9 \pm 2.6$ pA/pF @ $-160$ mV, $n = 27$, $P = 0.14$ compared with $I_f$, KOHcn4-CreERT2. Also similar to KO-MHC-Cre SAN was the percentage of KOHcn4-CreERT2 SAN with near-zero $I_f$ amplitudes (14 of 25 or 56% < $-3$ pA/pF at $-160$ mV) vs. (0 of 11) in WT SAN. Taken together, these data show that Trpm7 is required for the maintenance of normal cardiac automaticity via transcriptional regulation of Hcn4 and $I_f$ in adult murine SAN and embryonic myocardium.

**Discussion**

We sought to determine the contribution of TRPM7 to myocardial function by studying the consequences of cardiac-targeted Trpm7 deletion in vitro and in vivo. We show that TRPM7, when activated, forms a significant outwardly rectifying current in several different myocardial subtypes: with highest current densities in EVM > SAN > AVM. TRPM7 deletion in cultured EVM slows cardiac automaticity in vitro, as does TRPM7 knockdown in zebrafish. Moreover, in both global cardiac (KO-MHC-Cre) and inducible SAN-restricted (KOHcn4-CreERT2), TRPM7 deletion disrupts automaticity of isolated SAN and intact SAN (and likely AVN), manifesting as SPs and AVB in the adult mouse heart. Given that TRPM7 is known to conduct Ca$^{2+}$ (8), an obvious potential mechanism is that TRPM7 provides a Ca$^{2+}$ influx pathway important for cardiac automaticity. However, it important to note that TRPM7 currents are typically recorded under nonphysiological conditions, becoming fully activated after ~10 min of cell dialysis (i.e., Mg$^{2+}$ chelation with EDTA/Na$_2$ATP), in nominally free external Mg$^{2+}$. Under these conditions, TRPM7 current density is used to assay the total amount of functional protein at the cell membrane as opposed to physiologically relevant current magnitudes. Under more physiological conditions, TRPM7 current is essentially inactive in SAN and EVM, as demonstrated by the initial currents on "break-in" (Figs. S1 and S4). Thus, it is unlikely that TRPM7 contributes in a meaningful way to diastolic Ca$^{2+}$ influx under basal conditions, given the strong outward rectification characteristic of TRPM7 and its further inhibition by physiological concentrations of extracellular Mg$^{2+}$. Finally, TRPM7 blockade using FTY720 had no effect on WT SAN firing rates under basal physiological conditions (Fig. S6).

There are a number of other ion channels, Hcn4, Hcn2, Ca.3.1, and Ca.1.3, nonetheless, that are considered important for cardiac automaticity (4). Indeed, expression analysis for these “automaticity” genes revealed significant reductions in Hcn4, Ca.3.1, and SERCA2a mRNA in EVM upon TRPM7 deletion, whereas in SAN only Hcn4 mRNA was reduced. Consistent with these findings, the pacemaker current, $I_p$ (encoded by Hcn4), is significantly reduced in isolated SAN and associated with slowed diastolic depolarization. Furthermore, KO-MHC-Cre mice were less sensitive than WT mice to the negative chronotropic effects of the $I_f$ blocker ivabradine. Thus we conclude that TRPM7 is required for maintenance of cardiac automaticity via transcriptional regulation of Hcn4 expression (via an as yet unidentified pathway). It is well established that the hyperpolarization-activated pacemaker current, $I_p$, (encoded by Hcn4) contributes to basal automaticity in mouse SAN (24–26) and embryonic heart (27). However, there is controversy as to the extent of this contribution. Hcn4 knockout studies have demonstrated phenotypes ranging from profound bradycardia with AV block (24) to modest SPs (25), with no significant effect on mean heart rates in telemetered mice, similar to what we observe. Also consistent with our results, both of these studies show clear effects on firing rates and diastolic depolarization of isolated SAN cells. Furthermore, with maximal $\beta$-adrenergic stimulation (100 nM ISO), KO-MHC-Cre SAN largely overcome impairments in automaticity. This finding is consistent with the notion that $I_f$ current is an important determinant of spontaneous activity under basal conditions (25) but does not play an exclusive role in $\beta$-adrenergic
Another member of the TRPM family, Trpm4, has been recently found to be mutated in forms of conduction system disease in humans, including progressive familial heart block I (28, 29). These are thought to be gain-of-function mutations resulting in impaired TRPM4 endocytosis secondary to constitutive SUMOylation. The mechanism for this gain of function was deduced on the basis of heterologous expression studies in HEK cells and the observation that TRPM4 is enriched in human (28) and bovine (29) Purkinje cells. However, the mechanisms underlying TRPM4 gain-of-function contributions to conduction block has yet to be established. Because TRPM4 is a Ca²⁺-activated, sodium-selective current, one possibility is that increased TRPM4 current densities result in further membrane depolarization in Purkinje cells, inactivating excitatory voltage-gated sodium currents (30). This results in an increase in the depolarizing current shifts the membrane potential to a more depolarized level, which further depolarizes the membrane, resulting in a positive feedback mechanism that contributes to the extent of the conduction block.

Fig. 7. Hcn4 mRNA and pacemaker current are down-regulated upon Trpm7-deletion in EVM and SAN cells. mRNA expression profile of genes implicated in automaticity in (A) cultured Trpm7-deleted EVM (n = 10 samples: 6 Ad-TnT-Cre and 4 Ad-CMV-Cre) relative to WT EVM (n = 5 samples: Ad-Lacz-Cre) and in (B) KO-MHC-Cre (n = 4 samples, 4–6 pooled SAN/sample) relative to WT SAN (n = 3 samples, 4–6 pooled SAN/sample). (C) Representative hyperpolarization-activated “funny” current traces, Iₚ from WT SAN (Middle) and KO-MHC-Cre SAN (Bottom). Voltage protocols are shown above current traces. (D) Iₚ current–voltage relationship from WT SAN (n = 14) compared with KO-MHC-Cre SAN (n = 27). (E) Box plots with overlying data points showing the mean and distribution of Iₚ current densities at -160 mV from WT SAN (n = 14) compared with KO-MHC-Cre SAN (n = 27). (F) Representative Ca²⁺ current traces of total Ca²⁺ (I_cal, Left) and L-type Ca²⁺ current (I_cal, Right) from WT SAN (Middle) and KO-MHC-Cre SAN (Bottom). Voltage protocols are shown above current traces. (G) Current-voltage relationship of T-type Ca²⁺ currents (I_CaT) obtained by subtracting I_cal from I_cal) from WT SAN (n = 6) compared with KO-MHC-Cre SAN (n = 10). (H) Box plots with overlying data points showing the mean and distribution of heart rates after ivabradine (Left: 3 mg/kg; Right: 6 mg/kg) in WT mice (n = 5) compared with WT, using an unpaired Student t test. *P < 0.05, **P < 0.01, ***P < 0.001.
gated ionic currents and reducing the contribution of hyperpolarization-activated currents \((\iota_f)\) (25). To date, no gain-of-function mouse model data are available, and the \(Trpm4^{−/−}\) mouse, aside from hypertension (30), has not yet been shown to develop other cardiovascular phenotypes.

This article reports a TRP channel influencing the expression level of another ion channel(s) in SAN and EVM. The connection between TRPM7 and \(Hcn4\) expression in SAN and EVM remains an intriguing question. We showed recently that TRPM7 is required for early events in cardiogenesis, and perturbations in TRPM7 function during ventricular development impair myocardial function, atrophicventricular conduction, and repolarization (15). Thus, we surmised that embryonic deletion of TRPM7 in SAN/AVN tissue in \(KO^{Hcn4-CreERT2}\) mice might alter the maturation of SAN/AVN cells and consequently the expression of \(Hcn4\). However, SAN/AVN-restricted TRPM7 deletion in adult \(KO^{Hcn4-CreERT2}\) mice recapitulated the phenotype observed in \(KO^{αMHC-Cre}\) mice, suggesting that the effect of TRPM7 on \(Hcn4\) expression and automaticity in SAN represents, instead, a post-developmental effect. In this regard, it is noteworthy that SAN cells are more fetal-like, in that they are smaller, mononucleated, and retain automaticity, similar to early embryonic myocardium and in contrast to the larger, quiescent, multinucleated adult ventricular cells. Perhaps it is retention of this “embryonic” nature of SAN cells that allows them to remain sensitive to the effects of TRPM7 after the developmental phase.

A number of studies now show that the T-box transcription factor \(Tbx3\) is critical in mediating the developmental programs leading to formation and function of SAN, AVN, and conduction system in murine heart (31–34). Indeed, the most marked differential expression gene upon manipulation of \(Tbx3\) expression levels in myocardium is \(Hcn4\) (33). In addition, \(Tbx3\)-deficient mice exhibit both SAN and AVN dysfunction, manifested as SPs in both SAN and AVN (36), and the \(Mef2a^{−/−}\) mouse develops sinus arrhythmia and conduction block (37). We speculate that \(TRPM7\) may influence these transcriptional pathways in heart, either directly or indirectly, to affect...
cellular differentiation. We showed recently that TRPM7 C-terminal kinase is cleaved from the channel in LN-18 cells undergoing Fas-induced apoptosis, and this cleavage induced an increase in TRPM7 channel activity that is required for apoptosis (38). Indeed, cleaved TRPM7 C-terminal kinase has been observed in other cell types (38), potentially freeing it to interact with cellular partners beyond the plasma membrane to modify transcriptional programs. We are currently testing these hypotheses.

In summary, we showed that TRPM7 is most highly expressed in EVM and SAN myocytes, and genetic ablation of Trpm7 in these cells severely disrupts automaticity in vitro and in vivo. Impaired automaticity in Trpm7-deleted murine SAN cells arises from a lower and flatter slope of diastolic depolarization, associated with a slowed diastolic Ca$^{2+}$ rise and reduced pacemaker current $I_f$ (encoded by Hcn4). The next goal will be to determine how TRPM7 transcriptionally regulates genes in SAN and in embryonic myocardium to affect cardiac automaticity.

**Methods**

Refer to SI Methods for full methods.

**Cardiac-Targeted Trpm7 Knockout Mice.** All animal procedures have been reviewed and approved by the Institutional Animal Care and Use Committee at Children’s Hospital Boston. Animals were housed under standard conditions and allowed access to food and water ad libitum. Cardiac-targeted knockout mice were generated by crossing Trpm7fl/fl and Ad-CMV-Cre reporter mice. We thank Dr. William T. Pu for generously providing Ad-CMV-Lacz, Ad-CMV-Cre, and Ad-DrTnT-Cre, kind gifts from William Pu, Boston Children’s Hospital, Boston, MA. EVM were incubated differentiation media (DM) with adenovirus (multiplicity of infection 100) for 24 h and then washed twice with DM. Experiments were performed 2–5 d after viral transduction.

**Embrocydonic Cardiomyocyte Isolation, Culture, and Adenoviral Transduction.** Trpm7fl/fl embryonic hearts (6–11) were removed from E13.5–14.0 embryos obtained from pregnant Trpm7fl/fl female mice mated with a Trpm7fl/fl male. Adenoviruses, Ad-CMV-Lacz, Ad-CMV-Cre, and Ad-DrTnT-Cre, were kind gifts from William Pu, Boston Children’s Hospital, Boston, MA. EVM were incubated differentiation media (DM) with adenovirus (multiplicity of infection 100) for 24 h and then washed twice with DM. Experiments were performed 2–5 d after viral transduction.

**Ventricular Cardiomyocyte Isolation.** Ventricular myocytes were isolated by enzymatic digestion using either a solution of 0.07 mg/mL Liberase Blendzyme (Roche Diagnostics) or a mixture of 0.4 mg/mL Collagenase B (Roche), 0.3 mg/mL Collagenase D (Roche), and 0.025–0.05 mg/mL Protease XVI (Sigma), in nominally Ca$^{2+}$-free Tyrode’s solution, as previously described (39).

**Isolation of SAN Myocytes.** SAN myocytes were isolated as described by Marger et al. (40).

**Zebrafish Morpholino.** Disruption of Trpm7 in zebrafish by MO injection was performed as previously described (41).

**Cellular Physiology.** Embryonic cardiomyocytes were imaged on a 10-mm glass-bottom coverslip culture dishes were loaded with 5–10 µM Fluo-4 for 30 min at 37 °C in DM. The cells were imaged using an FV1000 confocal microscope.

**Mouse Telemetry.** Mouse telemetry was performed as described previously (42).

**Statistical Analysis.** All values were calculated using the two-samples, independent Student’s $t$ test with the exception of the data from Fig. 3 E and F and Fig. 8G, which used the nonparametric Mann-Whitney test.

**ACKNOWLEDGMENTS.** We thank Dr. William T. Pu for generously providing Ad-Lacz, Ad-CMV-Cre, and Ad-DrTnT-Cre adenoviruses. This study was supported by Agence Nationale pour la Recherche (ANR) Grants ANR-2010-BLAN-1128-01 and ANR-09 GENO-034 (to M.E.M.). R.S. was supported by grants from the Leadership Council in Cardiovascular Care and the American Heart Association Fellow-to-Faculty transition award. The Intitut de Genomique Fonctionnelle group is a member of the Laboratory of Excellence, Ion Channel Science and Therapeutics, and is supported by a grant from ANR.
Supporting Information

Sah et al. 10.1073/pnas.1311865110

SI Methods
Cardiac-Targeted Trpm7 Knockout Mice. All animal procedures have been reviewed and approved by the Institutional Animal Care and Use Committee at Children’s Hospital Boston. Animals were housed under standard conditions and allowed access to food and water ad libitum. Cardiac-targeted knockout mice were generated by crossing Trpm7fl/fl and Trpm7fl/fl mice described previously (1) with αMHC-Cre (provided by Michael Schneider, Imperial College London, London, England) or Hcn4-Cre-ERT2 (2) (provided by Andreas Ludwig, Institut für Experimentelle Klinische Pharmakologie und Toxikologie, Erlangen, Germany) mice. Mice were maintained on 129/SvEvTac mixed genetic background. In the case of Hcn4-Cre-ERT2 mice, Cre recombination was achieved by administering tamoxifen (Tm) at 40 mg/kg by gavage for 4 d. Cre recombinase expression and function was assessed by crossing the above cardiac Cre lines with ROSA26<sup> reporters mice (3) and imaging isolated cardiomyocytes using an FV1000 confocal microscope (Olympus). Mice were genotyped from tail tip DNA using the following: αMHC-Cre-specific primers (αMHC-Cre-F: 5′-ATG ACA GAC AGA TCC CTC CTA CCT CC-3′; αMHC-Cre-R: 5′-CCTC ATC CTG TGT AGC TAC GAC-3′), Hcn4-CreERT2-Cre specific primers (Hcn4-CreERT2-F: 5′-GGG AAC GAG GCC TCC TCA CTG GC-3′; Hcn4-CreERT2-R: 5′-ACC GAC GAT GAA GCA TTA GCT GGG-3′), and primers flanking exon 17 of Trpm7 (1) (M7 exon17-F: 5′-GCC ATC TCT CCT CTG GTT TT-3′; M7 exon17-R: 5′-GAT GAG CTA TAT ACT AGG TAC ATG G-3′). Transnet Inc performed some of the genotyping. To assess for deletion of Trpm7 at the level of genomic DNA, DNA was isolated using Purelink Genomic DNA Mini kit (Invitrogen) and PCR performed using primers flanking exon 17 (above).

Embryonic Cardiomyocyte Isolation, Culture, and Adenoviral Transduction. Trpm7fl/fl embryonic hearts (6–11) were removed from embryonic day 13.5–14.0 embryos obtained from pregnant Trpm7fl/fl female mice mated with a Trpm7fl/fl male. The ventricles were cut into <1-mm pieces in PBS and washed with fresh PBS to remove blood. The embryonic heart pieces were then digested for 30 min at 37 °C in 1 mL Heps-buffered saline solution (HBSS: PBS, 10 m Hepes, 20% (vol/vol) FBS) with 10 mg of Collagenase A (Roche, catalog no. 11088758103) and 10 mg Collagenase B (Roche, catalog no. 11088723103), vortexing gently every 10 min. The digested heart pieces were then triturated with a 1-mL pipette tip and 10 mL of HBSS added to inactivate the enzyme. The freed embryonic ventricular cardiomyocytes (EVM) were then spun down at 700 rpm for 5 min. The supernatant was discarded and the cell pellet was resuspended in 1–2 mL of differentiation media [DM: Iscove’s modified Dulbecco’s medium with added: 1% (wt/vol) t-glutamin, 2% (vol/vol) Pen/Strep, 15% (vol/vol) FBS, 1% (wt/vol) ascorbic acid, and 0.001% monothioglycerol]. Cells were then preplated in 1–2 × 35-mm culture dishes for ~45 min to remove fibroblasts. Media enriched in EVM was then removed and plated on either 35-mm culture dishes or fibronectin-coated 10-mm glass coverslips or 10-mm glass coverslip culture dishes (In Vitro Scientific) for further experiments. Adenoviruses, Ad-CMV-Lacz, Ad-CMV-Cre, and Ad-TnT-Cre, were kind gifts from William Pu, Boston Children’s Hospital, Boston, MA. EVM were incubated DM with adenovirus (multiplicity of infection, 100) for 24 h and then washed twice with DM. Experiments were performed 2–5 d after viral transduction.

Ventricular Cardiomyocyte Isolation. Hearts were excised from Avertin-anesthetized, heparin-anticoagulated mice and aortas cannulated with an 18-gauge needle connected to a peristaltic pump. Hearts were then retrograde perfused for 8–10 min at a perfusion pressure of 70–100 mm Hg with either a solution of 0.066 mg/ml Liberase Blendzyme (Roche Diagnostics) or a mixture of 0.4 mg/ml Collagenase B (Roche), 0.3 mg/ml Collagenase D (Roche), and 0.025-0.05 mg/ml Protease XVI (Sigma), in nominally Ca<sup>2+</sup>-free Tyrode’s solution (in mM: 140 NaCl, 5.4 KCl, 10 Hepes, 10 glucose, 1 MgCl<sub>2</sub>; pH 7.4 with NaOH) warmed to 36 °C. After digestion, hearts were perfused with high [K<sup>+</sup>] solution (KB, in mM: 120 K<sup>+</sup>, 20 KCl, 20 HEPES-KOH, 10 glucose; pH 7.4 with KOH), minced with scissors, suspended in KB solution by gentle pipetting, and filtered with a 300-μm mesh cell strainer. Freshly dissociated cardiomyocytes were then used for electrophysiological studies within 6–8 h.

Isolation of Sinoatrial Node Myocytes. Sinoatrial node (SAN) myocytes were isolated as described by Marger et al. (4). Briefly, hearts were removed under general anesthesia, consisting of 10 mg/kg of xylazine (Rompun 2%; Bayer AG) and 100 mg/kg of ketamine or using Avertin anesthesia. The SAN regions were excised in prewarmed (35 °C) Tyrode’s solution containing (in mM/L): 140 NaCl, 5.4 KCl, 1.8 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 5 Hepes-NaOH, and 5.5 p-glucose; adjusted to pH 7.4 with NaOH. SAN tissue strips were then be transferred into a “low-Ca<sup>2+</sup>–low-Mg<sup>2+</sup> solution containing (in mM/L): 140 NaCl, 5.4 KCl, 0.5 MgCl<sub>2</sub>, 0.2 CaCl<sub>2</sub>, 1.2 KH2PO4, 50 taunine, 5.5 D-glucose, 1 mg/mL BSA, and 5 HEPS-NaOH; adjusted to pH 6.9 with NaOH. Tissue was digested by Liberase (259 U/mL; Roche), elastase (1.9 U/mL; Boehringer Mannheim), and 200 μM CaCl<sub>2</sub>. Digestion was carried out for 9–13 min at 35 °C, under manual mechanical agitation. Tissue strips were then washed and transferred into a modified “Kraftbrühe” (KB) medium containing (in mM/L): 70 t-glutamin acid, 20 KCl, 80 KOH, 10 (±)-p-OH-butyr acid, 10 KH2PO<sub>4</sub>, 10 taunine, 1 mg/mL BSA, and 10 Hepes-KOH; adjusted to pH 7.4 with KOH. Single SAN and AVN myocytes were then isolated by agitation in KB solution at 35 °C. Cellular automaticity was restored by readapting the cells to a physiological extracellular Ca<sup>2+</sup> concentration by addition of a solution containing (in mM/L): 10 NaCl, 1.8 CaCl<sub>2</sub>, and normal Tyrode’s solution containing BSA (1 mg/mL). The final cell storage solution (in mM/L): 100 NaCl, 35 KCl, 1.3 CaCl<sub>2</sub>, 0.7 MgCl<sub>2</sub>, 14 t-glutamin acid, 2 (±)-p-OH-butyr acid, 2 KH2PO<sub>4</sub>, 2 taunine, 1 mg/mL BSA (pH 7.4), and gentamycin (50 μg/mL).

Zebrafish Morpholino. Morpholino targeting the translation start site of zebrafish trpm7, designed by Gene Tools, LLC, was diluted to a final concentration of 500 μM in water containing 0.1% phenol red. Wild-type Tubingen strain embryos were injected at the one-cell stage with 0.5 μL morpholino or the same volume of water containing 0.1% (vol/vol) phenol red as a negative control, using a PLJ-100 pico-injector (Harvard Instruments) and subsequently raised at 28.5 °C. Morpholino sequence was ATCCAGGACTTGGGACATTCT. Heart rates were determined at 20–21 °C at 48 h by counting contractions over 60 s.

Ca<sup>2+</sup> Imaging of Embryonic Cardiomyocytes. EVMs plated on 10-mm glass-bottom coverslip culture dishes were loaded with 5–10 μM Fluo-4 for 30 min at 37 °C in DM (see above). The cells were
were washed twice with DM and then placed on the stage of an FV1000 confocal microscope (Olympus), enclosed in a temperature- and CO\textsubscript{2}-controlled chamber set at 37 °C, and maintained at 5% CO\textsubscript{2}. Ca\textsuperscript{2+} transients were measured from groups of

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Imaging of SAN Myocytes. In isolated SAN cells, where a 40

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were recorded in both enzymatically isolated primary SAN cells

and in individual cells of intact SAN tissue loaded with Fluo-

AM under control (Tyrode’s) or isoproterenol at 36 °C. In ex-

periments carried with SAN intact tissue, movement artifacts

were avoided by adding 25 μM cytochalasin-D. To detect Ca\textsuperscript{2+} signals, the SAN intact preparation was loaded with the Ca

indicator Fluo-4AM (20 μM) for 60 min. SAN myocytes were distinguished from other excitable cell types (i.e., atrial cells) by their morphology (spindle and elongated shape) and size (~10 μm diameter). To ensure that recordings obtained in intact SAN tissue came from the dominant pacemaker region, we focused on the central nodal area described by Verheijck et al. (5). Images were obtained with confocal microscopy (Zeiss LSM 780) by scanning the cell with an argon laser in line scan configuration (1.54-μm line rate); fluorescence was excited at 488 nm, and emissions were collected at >505 nm. A 63x water immersion objective (N.A. 1.2) and a 63x oil immersion objective were used to record [Ca\textsuperscript{2+}]; in isolated SAN cells, whereas a 40× objective (N.A. 1.2) was used for intact SAN tissue. Image analyses were performed by ImageJ software. Images were corrected for the background fluorescence and the fluorescence values (F) were normalized by the basal fluorescence (F0) to obtain the fluorescence ratio (F/F0). Integrals of light intensity were analyzed by pClamp software (ver. 9, Axon Instruments Inc.), and [Ca\textsuperscript{2+}] transient parameters were analyzed as described previously (6). [Ca\textsuperscript{2+}], transient duration was measured from the peak of the [Ca\textsuperscript{2+}], transient to 90% decay.

Electrocardiography. ECGs were performed in supine mice se-
dated using Avertin anesthesia. Dissection pins were placed s.c. in the right arm and left leg. The ECG signal was amplified using a speaker amplifier/mixer and then digitized using a Digidata 1320 board (Molecular Devices) acquired with Clampex (pClamp9; Molecular Devices) and analyzed with Clampfit (Molecular Devices) and Origin software (OriginLab Corporation).

Mouse Telemetry. For telemetric ECG recording, adult male mice were anesthetized with 2% isoflurane. A midline incision was made on the back along the spine to insert a telemetric trans-
mitter (TA100EA-F20; Data Sciences International) into a s.c. pocket with paired wire electrodes placed over the thorax (chest bipolar ECG lead). Local anesthesia was obtained with lidocaine (1%) injected s.c. at the sites of electrodes and transmitter implantation. To manage possible postsurgery pain, Advil (para-cetamol and ibuprofen, 7 mL/L) was added to the drinking water for 4 d after implantation. Experiments were initiated at least 8 d after recovery from surgical implantation. Mice were housed in individual cages with ad libitum access to food and water and were exposed to standard 12-h light/dark cycles in a thermostatically controlled room. ECG signals were recorded using a telemetry receiver and an analog-to-digital conversion data acquisition system for display and analysis by Dataquest A.R.T. software (Data Sciences International). Heart rates were determined from interbeat (RR) intervals of the ECG. Mean heart rate values were obtained in each mouse for an overall 24-h period from 8:30 AM to 8:30 PM. AVB and SP were counted in 1-min-long stretches of recordings each hour for 24 h and averaged.

Cellular Electrophysiology. TRPM7 current was measured in iso-
lated cardiomyocytes in the whole-cell configuration as previously described (1). Extracellular solution composition was (in mM): 135 NaCl, 5.4 CsCl, 10 Hepes, 10 glucose, 0.1 CdCl\textsubscript{2}, and 1 CaCl\textsubscript{2}; pH 4.0–7.4 with NaOH. To measure Mg\textsuperscript{2+}-inhibited current, 10 mM MgCl\textsubscript{2} was added to the solution and perfused onto cells. Pipette solutions contained (in mM): 120 1-t.-aspartic acid, 20 CsCl, 2.5 EGTA or 1.2-bis(o-aminophenoxy)ethane-

NN,N′-N′-tetracetic acid, 2.5 EDTA, 10 Hepes, 120 CsOH, 5 Na\textsubscript{2}ATP, and 0.5 Na\textsubscript{2}GTP; pH 7.2 in CsOH. The voltage protocol (shown in figure insets) held the myocytes at 0 mV, stepped to +100 mV for 40 ms and then ramped to –80 mV over 500 ms, holding at –80 mV for 40 ms before stepping back to 0 mV. This protocol was repeated every 4–6 s, and recordings continued for 10–15 min per cell until a steady-state TRPM7 current was obtained. After establishing the whole-cell configuration, cell capaci-
tance transients were recorded by applying 40-μs voltage steps from 0 mV to 10 mV, and cell capacitance was calculated offline.

For measuring the I\textsubscript{f} current in SAN cells, the extracellular solution was standard Tyrode’s solution with 2 mM BaCl\textsubscript{2} added to block K\textsubscript{1} (in mM): 140 NaCl, 4 KCl, 10 Hepes, 10 glucose, 1.2 CaCl\textsubscript{2}; pH 4.0–7.4 with NaOH. The pipette solution contained (in mM): 135 KCl, 1 MgCl\textsubscript{2}, 0.1 CaCl\textsubscript{2}, 10 EGTA, 10 Hepes, 5 MgATP, 0.3 Na\textsubscript{2}GTP, and 6.6 phosphocreatine; pH 7.2 with KOH. I\textsubscript{f} was defined as the time-dependent, sustained current component at the end of the hyperpolarizing pulse measured in the absence of tetrodotoxin. For measuring I\textsubscript{a} and I\textsubscript{f} in SAN cells we used an intracellular solution containing (in mM): 120 1-t.-aspartic acid, 20 CsCl, 5 EGTA, 10 Hepes, 1 MgCl\textsubscript{2}, 120 CsOH, 2.5 Mg\textsubscript{2}ATP, and 0.1 Na\textsubscript{2}GTP; pH 7.2 in CsOH. The extracellular solution contained (in mM): 140 CsCl, 10 Hepes, 10 glucose, 2 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}; pH 7.4 with CsOH.

Cellular automaticity was recorded under perforated-patch conditions using β-escin to allow proper voltage- and current-clamp conditions and preservation of the intracellular environment. Recordings were performed in extracellular Tyrode’s solution (at 36 °C) containing (in mM): 140 NaCl, 5.4 KCl, 1.8 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 5 Hepes-NaOH, 5.5 and 5-gluce; adjusted to pH 7.4 with NaOH. The composition of the pipette solution allowed recording of K\textsuperscript{+}-dependent ionic currents and contained (in mM): 130 KCl, 10 NaCl, 2 ATP-Na\textsuperscript{+} salt, 6.6 creatine phosphate, 0.1 GTP-Mg\textsuperscript{2+}, 0.04 CaCl\textsubscript{2} (pCa = 7), and 10 Hepes-

KO\textsubscript{H}; adjusted to pH 7.2 with KOH. All voltage values were corrected for the appropriate liquid junction potential. Action potential (AP) durations were measured from the peak of the AP to 90% repolarization. All currents were recorded using an Axopatch 200B amplifier (Molecular Devices), acquired with Clampex (pClamp9; Molecular Devices), and analyzed with Origin software (OriginLab Corporation). Signals were sampled at 10 kHz and low-pass filtered at 5 kHz. Statistical analysis used the two-sample independent Student t test.

Quantitative RT-PCR Expression Analysis. mRNA was quantified by quantitative RT-PCR (qRT-PCR) from total RNA isolated from pooled SAN (4–6 SAN/sample) dissected from KO or WT hearts, or from 35-mm dishes of beating cultured embryonic ventricular myocytes . Random hexamer primed cDNA synthesis was performed using the SuperScript III First Strand Synthesis System for RT-PCR (Invitrogen) using 2–4 μg total RNA. Primers were synthesized by Integrated DNA Technologies, validated by standard PCR, and quality controlled by melting curve analysis after qRT-PCR reactions. Real-time PCR reactions were run in triplicate using the SYBR Green method (ROX as passive reference dye; Affymetrix) in an Eppendorf Mastercycler epgradient S Re-

alplex4 qRT-PCRycler (Eppendorf) for 40 cycles, and data were analyzed in Microsoft Excel. Quantification was normalized to GAPDH expression.

The primers sequences for the qRT-PCR reactions are as follows:


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**Fig. S1.** Activation of native TRPM7 current in cultured EVMs. Representative examples of whole-cell recordings from cultured EVMs (EVM #1 and EVM #2) demonstrating the current on “break-in” (Initial Current) and after 7–10 min of cell dialysis (Post run-up). Note that TRPM7 current is nearly absent on break-in.

**Fig. S2.** Ca²⁺ transient cycle lengths in *Trpm7*fl/fl and *Trpm7*fl/+ EVMs. Ca²⁺ transient cycle lengths measured by confocal microscopy using Fluo-4AM in EVM transduced with Ad-CMV-Lacz-Trpm7fl/fl (n = 18), Ad-CMV-Lacz-Trpm7fl/+ (n = 6), Ad-TnT-Cre-Trpm7fl/fl (n = 13), with Ad-TnT-Cre-Trpm7fl/+ (n = 12), Ad-CMV-Cre-Trpm7fl/fl (n = 18), and Ad-CMV-Lacz-Trpm7fl/+ (n = 10). *P < 0.05 compared with Ad-CMV-Lacz-Trpm7fl/fl. NS, not statistically different compared with Ad-CMV-Lacz-Trpm7fl/fl.

<table>
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Fig. S3. ECGs by telemetry and in sedated mice. Representative ECGs obtained in conscious mice by telemetry (A–D, Left) and in sedated mice (E–G, Right) from WT and KO\textsuperscript{\textalpha MHC-Cre} mice. WT ECGs show normal sinus rhythm (p indicate p waves) by telemetry (A) and with sedation (E). Sinus pauses are evident in KO\textsuperscript{\textalpha MHC-Cre} mice by both telemetry (B) and sedated ECG (F). Solid arrows indicate location of the sinus pause and expected p wave. (C) Episode of atrial bigeminy with sinus pause and (D) atrioventricular block in telemetered KO\textsuperscript{\textalpha MHC-Cre} mouse. (G) Sinus pauses with ectopic atrial foci. Note the change in p wave morphology indicative of change in atrial focus after the sinus pause (red circles, Inset) compared with normal p wave (black circle, Inset).

Fig. S4. Activation of native TRPM7 current in freshly isolated SAN cell. Representative example of whole-cell recordings from a freshly isolated SAN cell demonstrating the current on “break-in” (Initial Current) and after 7–10 min of cell dialysis (Post run-up). Note that TRPM7 current is nearly absent on break-in.
Fig. S5. Ca\(^{2+}\) transient characteristics in isolated SAN and in SAN within intact sinoatrial node tissue from WT and KO\(^{αMHC-Cre}\) mice. (A) Ca\(^{2+}\) transient frequency firing frequency of SAN cells within the intact sinoatrial node tissue in WT compared with KO\(^{αMHC-Cre}\). (B) Peak Ca\(^{2+}\) transients are unchanged in isolated WT and KO\(^{αMHC-Cre}\) SAN. (C) Peak Ca\(^{2+}\) transient (FF\(_{0}\)) in response to application of 10 mM caffeine to isolated SAN cells. **P < 0.01. NS, not statistically different.

Fig. S6. AP rate and duration of isolated WT SAN in the absence and presence of 500 nM FTY720. (A) AP firing rate. (B) AP duration at 50% repolarization (APD50) and 90% repolarization (APD90). NS, not statistically different.
<table>
<thead>
<tr>
<th>WTIKO&lt;sup&gt;MHC-Cre&lt;/sup&gt;</th>
<th>Tyrode’s</th>
<th>ISO 2nM</th>
<th>ISO 100nM</th>
<th>P, A vs. B</th>
<th>P, A vs. C</th>
<th>P, B vs. C</th>
<th>P, WT vs. KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rate (bpm)</td>
<td>127 ± 25</td>
<td>7</td>
<td>169 ± 22</td>
<td>7</td>
<td>226 ± 20</td>
<td>7</td>
<td>NS</td>
</tr>
<tr>
<td>MDP (mV)</td>
<td>−65 ± 2</td>
<td>7</td>
<td>−68 ± 2</td>
<td>7</td>
<td>−72 ± 1</td>
<td>7</td>
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</tr>
<tr>
<td>Eth (mV)</td>
<td>−43 ± 2</td>
<td>7</td>
<td>−43 ± 2</td>
<td>7</td>
<td>−48 ± 3</td>
<td>7</td>
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<tr>
<td>DDSL (mV/ms)</td>
<td>0.09 ± 0.01</td>
<td>7</td>
<td>0.10 ± 0.01</td>
<td>7</td>
<td>0.12 ± 0.03</td>
<td>7</td>
<td>NS</td>
</tr>
<tr>
<td>APA (mV)</td>
<td>79 ± 4</td>
<td>7</td>
<td>88 ± 5</td>
<td>7</td>
<td>98 ± 7</td>
<td>7</td>
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</tr>
<tr>
<td>dV/dt (mV/ms)</td>
<td>15 ± 6</td>
<td>7</td>
<td>33 ± 9</td>
<td>7</td>
<td>33 ± 10</td>
<td>7</td>
<td>NS</td>
</tr>
<tr>
<td>APD (90% repol, ms)</td>
<td>103 ± 15</td>
<td>7</td>
<td>99 ± 11</td>
<td>7</td>
<td>96 ± 8</td>
<td>7</td>
<td>NS</td>
</tr>
<tr>
<td>KO&lt;sup&gt;MHC-Cre&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rate (bpm)</td>
<td>39 ± 16</td>
<td>5</td>
<td>53 ± 15</td>
<td>5</td>
<td>161 ± 38</td>
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<tr>
<td>MDP (mV)</td>
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<td>−61 ± 2</td>
<td>5</td>
<td>−65 ± 2</td>
<td>5</td>
<td>NS</td>
</tr>
<tr>
<td>Eth (mV)</td>
<td>−37 ± 2</td>
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<td>−39 ± 2</td>
<td>5</td>
<td>−39 ± 2</td>
<td>5</td>
<td>NS</td>
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<tr>
<td>DDSL (mV/ms)</td>
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<td>0.11 ± 0.03</td>
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<tr>
<td>APA (mV)</td>
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<td>90 ± 7</td>
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<td>96 ± 6</td>
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<tr>
<td>dV/dt (mV/ms)</td>
<td>12 ± 5</td>
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<td>26 ± 14</td>
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<tr>
<td>APD (90% repol, ms)</td>
<td>172 ± 23</td>
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<td>156 ± 19</td>
<td>5</td>
<td>110 ± 7</td>
<td>5</td>
<td>NS</td>
</tr>
</tbody>
</table>

APA, action potential amplitude; APD, action potential duration at 90% repolarization; DDSL, diastolic depolarization slope; dV/dt, action potential upstroke velocity; Eth, action potential threshold; MDP, mean diastolic membrane potential; NS, not statistically different; Rate, action potential firing rate. *P < 0.05; **P < 0.01.