Augmentation of myocardial $I_f$ dysregulates calcium homeostasis and causes adverse cardiac remodeling

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HCN channels underlie the depolarizing funny current ($I_f$) that contributes importantly to cardiac pacemaking. $I_f$ is upregulated in failing and infarcted hearts, but its implication in disease mechanisms remained unresolved. We generated transgenic mice ($HCN4^{tg/wt}$) to assess functional consequences of $HCN4$ overexpression-mediated $I_f$ increase in cardiomyocytes to levels observed in human heart failure. $HCN4^{tg/wt}$ animals exhibit a dilated cardiomyopathy phenotype with increased cellular arrhythmogenicity but unchanged heart rate and conduction parameters. $I_f$ augmentation induces a diastolic Na$^+$ influx shifting the Na$^+/Ca^{2+}$ exchanger equilibrium towards ‘reverse mode’ leading to increased [Ca$^{2+}$].

Changed Ca$^{2+}$ homeostasis results in significantly higher systolic [Ca$^{2+}$], transients and stimulates apoptosis. Pharmacological inhibition of $I_f$ prevents the rise of [Ca$^{2+}$], and protects from ventricular remodeling. Here we report that augmented myocardial $I_f$ alters intracellular Ca$^{2+}$ homeostasis leading to structural cardiac changes and increased arrhythmogenicity. Inhibition of myocardial $I_f$ per se may constitute a therapeutic mechanism to prevent cardiomyopathy.
The hyperpolarization-activated cyclic nucleotide-gated channel 4 (HCN4) is the dominant HCN isoform in the sinoatrial node and is significantly involved in generation and regulation of heart rhythm. Apart from its abundant expression in the pacemaker and conduction system, the adult working myocardium is characterized by low HCN levels. At early embryonic stages, however, HCN4 is abundantly transcribed in the whole heart and contributes importantly to I_f triggered automaticity of ventricular myocytes. Moreover, HCN4 was identified as a cell marker for the cardiomyogenic progenitor pool of the first heart field, implicated in the earliest stage of cardiac mesoderm formation and morphogenesis. Toward birth, HCN4 transcription is downregulated in working-type cardiomyocytes and remains at low levels during adult stages, suggested to prevent pathological remodeling. In this context, increased I_f and HCN4 expression was reported in failing hearts and after myocardial infarction, but its pathophysiological role remains to be established. Notably, pharmacological blockade of I_f improves cardiovascular outcome in patients with chronic heart failure—results that were considered to be driven mainly by improved myocardial energy supply at lower heart rates. However, recent data demonstrated that I_f blockade in patients with coronary artery disease with preserved myocardial function did not improve outcome despite marked reduction of heart rate, pointing towards mechanisms of I_f blockade that may be particularly beneficial in failing hearts. In this regard, pleiotropic effects of I_f blockade beyond heart rate reduction have been suggested, but these mechanisms have not been specified yet.

To study the significance of increased I_f in the working myocardium, we generated transgenic mice that express human HCN4 (hHCN4) under control of the murine cardiac troponin I (cTNI) gene promoter. We here show that a moderate increase of I_f in cardiomyocytes to levels observed in heart failure leads to cardiac dilation and impaired cardiac function. HCN4 activity essentially is linked to calcium regulation of cardiomyocytes and augmented I_f results in dysregulated Ca^{2+} homeostasis driving cell death and cardiac remodeling.

### Results

**Generation of transgenic mice overexpressing hHCN4.** To study the significance of HCN4 channel overexpression-mediated I_f augmentation in the working myocardium, transgenic mice were generated that express hHCN4 under control of the murine cTNI gene promoter (Fig. 1a). HCN4^tg/wt offspring are born at a Mendelian frequency and exhibit no overt cardiac or general abnormalities. HCN4^tg/wt mice produce abundant hHCN4 transcripts (Fig. 1b) leading to increased HCN4 protein levels (Fig. 1c). Intense anti-HCN4 staining signals at the plasma membrane of HCN4^tg/wt cardiomyocytes (Fig. 1d) indicate efficient trafficking of hHCN4 channels to the cell surface, while wild type tissue yields only little HCN4 signals. Accordingly, patch-clamp recordings showed significantly increased I_f densities in HCN4^tg/wt cardiomyocytes. At physiological resting membrane potentials (RMP) of ventricular cardiomyocytes (−90 mV) we observed a two–threefold higher I_f density in HCN4^tg/wt cardiomyocytes compared to wild type (Fig. 1e, f), recapitulating the magnitude of current increase that was observed in cardiomyocytes of heart failure patients.

**HCN4^tg/wt mice develop structural heart disease.** While cardiac morphology was similar at birth, we uncovered cardiac dilation with significantly increased heart-to-body weight ratios in HCN4^tg/wt mice 2 months postpartum (Fig. 2a–g). Right ventricular diameters, in particular, were found markedly enlarged (Fig. 2d) and dilation was accompanied with reduced wall thickness (Fig. 2e). To assess cardiac in vivo phenotypes, echocardiographic evaluations were performed at 3 and 6 months of age. At 3-month of age left ventricular wall thickness of HCN4^tg/wt mice was lower compared to wild type (Fig. 2i). Similar to the histological findings at 2 months of age there was a trend toward higher left ventricular internal diameters in HCN4^tg/wt animals (Fig. 2j, k), although these changes did not reach statistical significance. Fractional area shortening did not differ between groups at this age (Fig. 2l). At 6 months of age, however, HCN4^tg/wt mice displayed a dilated cardiomyopathy phenotype (Fig. 2m, n) with lower left ventricular wall thickness, dilation of left ventricular internal diameters (Fig. 2n–p) and systolic dysfunction with reduced fractional shortening of 22.9 ± 1.4% compared to 27.5 ± 1.4% in controls (means ± s.e.m., P = 0.031, unpaired t-test) (Fig. 2i). Furthermore mitral valve Doppler imaging displayed increased E/A ratios in transgenic mice indicative for impaired left ventricular relaxation and diastolic dysfunction (2.16 ± 0.22 in HCN4^tg/wt mice compared to 1.62 ± 0.07 in controls, P = 0.021, unpaired t-test) (Fig. 2m, n).

In line with macroscopic alterations in HCN4^tg/wt hearts, we observed significantly increased diameters of cardiomyocytes (Fig. 2c) and elongated sarcomeres (Fig. 3a, b). However, HCN4 overexpression did not induce cardiac fibrosis (Fig. 3c, d), and we observed no myocyte disarray or cellular infiltrations (Fig. 3a, e). Early treatment of HCN4^tg/wt mice with the I_f blocker ivabradine significantly reduced chamber dilation and wall rarefaction (Fig. 2q–g), and normalized cardiomyocyte diameters (Fig. 2c), indicating that increased I_f causes the altered structural phenotype.

### Transcriptional alterations in HCN4^tg/wt hearts.

We next asked whether structural and functional remodeling in hearts of HCN4^tg/wt mice is associated with activation of the fetal gene program. Evaluation of mRNA, isolated from 6-months-old mice, revealed markedly increased levels of the myocardial cell growth markers GSK-3β and mTOR and slightly upregulated levels of the prototypical hypertrophic marker genes MYH7 (encoding βMHC), CnA (encoding calcineurin A) and NPPA (encoding ANP) (Fig. 4b). We further explored transcriptional profiles of ion channels and transporters, implicated in action potential generation, excitation-contraction coupling and intracellular calcium homeostasis (Fig. 4c, d). Interestingly, HCN4^tg/wt hearts displayed significant downregulation of the sodium–calcium exchanger gene NCX1 (Fig. 4c) and intrinsic murine HCN genes were slightly diminished (Fig. 4d).

Furthermore, levels of transcripts of the repolarizing channel genes KCNQ1 (encoding Kv7.1) and KCND2 (encoding Kv4.2) were decreased, while the repolarizing channel gene KCNH2 (encoding Kvl1.1) showed a trend toward higher levels (Fig. 4e). Other genes regulating calcium handling and cellular electrophysiology, however, showed transcript levels that were not significantly changed.

**HCN4 overexpression impacts calcium handling proteins.** Based on NCX1 downregulation in HCN4^tg/wt hearts, we hypothesized that dysregulated intracellular sodium–calcium homeostasis might be a pathomechanistic component in I_f overexpression-mediated cardiomyopathy. To evaluate a contribution of calcium handling proteins we performed immunoblotting of various key players. In line with the transcriptional analysis we observed significantly reduced levels of NCX1 protein in HCN4^tg/wt hearts, a finding that was paralleled by lower levels of SERCA2 and phospholamban (PLN) whole protein content (Fig. 4e, f). Of note, phosphorylation of PLN at its CaMKII-specific Thr-17 site was significantly increased, whereas the rise at
its protein kinase A (PKA)–site Ser-16 did not reach statistical significance. Moreover, the Ca2+–sensor protein μ-calpain showed higher levels in HCN4tg/wt hearts, while the L-type calcium channel protein Cav1.2 remained unchanged (Fig. 4e, f). These data document profound alterations in the regulation of calcium handling proteins under the in vivo conditions of cardiomyocytes from transgenic animals (Fig. 5a) and after incubation with 3 μM ivabradine (Fig. 5a). Remarkably, we observed a distinctive rise in [Ca2+]i baseline levels and significantly increased systolic [Ca2+]i transients under steady state field stimulation of cardiomyocytes isolated from HCN4tg/wt hearts (Fig. 5a–d). The efficiency of Ca2+ release, however, reflected by the time to peak, was almost unchanged (Fig. 5e). Of note, [Ca2+]i levels, high in HCN4tg/wt cardiomyocytes, dropped to significantly lower levels in ivabradine-treated HCN4tg/wt cardiomyocytes (Fig. 5b), suggesting that abundant HCN4 current mediates diastolic Ca2+ overload and altered Ca2+ homeostasis. Furthermore, the markedly increased slope of Ca2+ uptake (Fig. 5f) and shortened time to half decay (D50) of calcium transients (Fig. 5d) indicate that HCN4 overexpression influences uptake of increased [Ca2+]i to the SR mediated by SERCA. Consistently, Ii inhibition normalized the slope Ca2+ uptake and [Ca2+]i parameters in cardiomyocytes of transgenic animals (Fig. 5a–f). In addition, Ca2+–load experiments using caffeine 10 mM (Supplementary Fig. 1) showed that the caffeine-induced Ca2+ release peak was significantly higher in HCN4tg/wt cardiomyocytes, indicating that the SR Ca2+ content is increased, which is consistent with the experimental data of higher Ca2+ transients upon electrical stimulation (Fig. 5g). Of note, recordings of Ca2+ transients

**Fig. 1** Generation and characterization of transgenic HCN4tg/wt mice. a The transgene carries a 4.3 kb promoter fragment of the murine cTnI gene (cTnI) fused to the human HCN4 cDNA and the bovine growth hormone gene poly A signal (BGH-pA). b Profiling of the endogenous murine HCN1 (mHCN1), HCN2 (mHCN2), HCN4 (mHCN4), and transgenic human HCN4 (hHCN4) transcripts in the ventricular myocardium of 2-month-old HCN4tg/wt mice measured by quantitative real-time PCR, (qRT-PCR). Wild type = 1.0 (n = 6 animals; ***P < 0.001; unpaired t-test). c Western blot and quantitative analysis of HCN4 protein in the ventricular myocardium of HCN4tg/wt transgenic and wild type mice, normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for control of protein loading. For comparison samples with different protein load (10, 20, 30 μg) are shown (n = 6 animals/ groups; *P < 0.05, **P < 0.01, ***P < 0.001; unpaired t-test). d Immunohistochemistry for HCN4 (red) in ventricular myocardium of wild type (left) and transgenic (right) mice. Scale bars 20 μm. Representative Ii currents (e) and current-voltage relationship (f) recorded from ventricular cardiomyocytes isolated from HCN4tg/wt and wild type mice (n = 20 cells from six wild type mice and n = 16 cells from five HCN4tg/wt mice; *P < 0.05, **P < 0.01, ***P < 0.001; unpaired t-test). Data are expressed as mean ± s.e.m. Source data are provided as a Source data file.
showed abnormal prolongation of diastolic Ca\(^{2+}\) decay (D\(_{90}\) > 2000ms) in 39% of cardiomyocytes isolated from HCN4\(^{tg/wt}\) hearts (evaluated separately—please refer to Supplementary Fig. 2), changes that were reduced to wild type level after treatment of HCN4\(^{tg/wt}\) cardiomyocytes with ivabradine, underlining that increased I\(_F\) profoundly disorganizes cellular Ca\(^{2+}\) homeostasis.

Reverse mode NCX blockade ameliorates altered Ca\(^{2+}\) cycling. To elucidate the role of NCX in HCN4-mediated perturbation of Ca\(^{2+}\) cycling we recorded [Ca\(^{2+}\)]\(_i\) transients from wild type and HCN4\(^{tg/wt}\) cardiomyocytes at baseline and in the presence of the selective NCX inhibitor ORM-10103 20.

Interestingly, NCX inhibition by ORM-10103 (10 \(\mu\)M) reversed the changes in Ca\(^{2+}\) metabolism nearly to wild type

**Reverse mode NCX blockade ameliorates altered Ca\(^{2+}\) cycling.**
levels (Fig. 6a–f). Increased diastolic $[\text{Ca}^{2+}]_i$, declined remarkably (Fig. 6b) and the amounts of total cellular Ca$^{2+}$ movement, reflected by the peak area, returned to normal levels (Fig. 6c). Moreover, the increased slope Ca$^{2+}$ uptake (Fig. 6f) and shortened time to half decay ($D_{50}$) of calcium transients (Fig. 6d) were restored to levels not significantly different from wild type. Thus, our data demonstrate an important pathophysiological implication of the NCX current in the changed cardiac Ca$^{2+}$ homeostasis of HCN4tg/wt animals.

HCN4tg/wt hearts show increased apoptosis. We then asked whether increased $[\text{Ca}^{2+}]_i$ would provoke apoptosis in HCN4tg/wt hearts. Using TUNEL assay, we detected significantly higher proportions of nuclei with DNA fragmentation in ventricles of HCN4tg/wt hearts compared to wild type (Fig. 7a, b). In addition, screening of genes associated with apoptosis by qRT-PCR demonstrated a significant rise of the apoptosis effector caspase-3 in transgenic myocardium (Fig. 7c, d; Supplementary Fig. 3). Moreover, we observed higher transcript levels of the Ca$^{2+}$-sensor protease $\mu$-calpain and the tissue transglutaminase (Fig. 7d), both implicated in myocardial cell death and structural remodeling in association with [Ca$^{2+}]_i$ overload$^{21}$. Further analysis of caspase-3 tissue activity revealed a marked increase of caspase-3-positive cells in HCN4tg/wt ventricles (Fig. 7e, f), pointing to increased apoptotic cell death as an explanation for wall thinning and dilated cardiomyopathy.

Electrophysiological changes in HCN4tg/wt cardiomyocytes. Action potential (AP) recordings of HCN4tg/wt and wild type cardiomyocytes were performed using ruptured whole-cell patch clamp (Fig. 8a). The AP was significantly longer in the transgenic hearts (Fig. 8b). The repolarization time to a membrane potential of $-40\text{mV}$ was also prolonged (Fig. 8c). The recovery time to a membrane potential of $+20\text{mV}$ was prolonged in the HCN4tg/wt hearts (Fig. 8d).

**Fig. 2** Morphological and functional phenotype of transgenic HCN4tg/wt mice. **a** Representative HE-stained cryosections display morphology of 2-months-old wild type (left) and HCN4tg/wt (right) hearts showing cardiac dilation and significantly reduced wall thickness in particular of the right ventricle. Scale bars: 1 mm. **b–g** HE-stained cryosections illustrating quantitative effects of HCN4 transgene overexpression on the structural phenotype of hearts in the presence and absence of ivabradine at two month postpartum. Heart-to-body weight ratio (b), cross section of left ventricular cardiomyocytes (c), right ventricle diameter-to-body weight ratio (d), and wall thickness (e), left ventricle diameter-to-body weight ratio (f) and wall thickness (g) of wild type, HCN4tg/wt, and ivabradine-treated HCN4tg/wt mice at 2 months postpartum (n = 6 animals/group; *P < 0.05, **P < 0.01, ***P < 0.001; ANOVA).

**Fig. 3** Histological analysis of transgenic HCN4tg/wt hearts. **a** Electron microscopy analysis of left ventricular sarcomere structure of 2-months-old wild type and HCN4tg/wt hearts. Scale bars: 1250 nm. **b** Comparative percentile plot of sarcomere lengths’ distribution in cardiomyocytes of wild type (dashed line, n = 480 sarcomeres from six mice) and HCN4tg/wt (solid line, n = 480 sarcomeres from six mice) animals. **c** Masson’s trichrome staining of right ventricular paraffin sections from wild type and HCN4tg/wt hearts, 2 months postpartum. Scale bars: 100 μm. **d** Comparative analysis of fibrosis by semi-automatic quantification using ImageJ-based plug-in showed similar levels of fibrosis between wild type (gray column) and HCN4tg/wt (black column) tissue (n = 6 animals/group; *P < 0.05, **P < 0.01, ***P < 0.001; unpaired t-test). **e** HE staining of right ventricular cryosections from wild type and HCN4tg/wt hearts, 2 months postpartum. Scale bars: 50 μm. Data are expressed as mean ± s.e.m. Source data are provided as a Source data file.
clamped technique (Fig. 8a). In HCN4<sup>tg/wt</sup> cardiomyocytes RMP was more positive than in wild type cardiomyocytes (wild type: 

\[ P_{50} = -61.25 \text{ mV}, n = 14 \] vs. HCN4<sup>tg/wt</sup>: 

\[ P_{50} = -54.1 \text{ mV}, n = 12; p = 0.0013; \text{unpaired } t\text{-test} \] (Fig. 8c), consistent with diastolic depolarisation caused by HCN4-mediated sodium influx.

Likewise, the amplitude of overshoot was diminished in transgenic cardiomyocytes (Fig. 8b), pointing to an inactivation of voltage-gated Na<sup>+</sup>-current at more depolarized RMP. AP properties were evaluated at three different pacing frequencies (0.5, 1, 2 Hz) (Fig. 8d–f). AP duration at 20 (APD<sub>20</sub>) and 50% (APD<sub>50</sub>)
Fig. 4 Transcriptional changes and expression of Ca^{2+} handling proteins in HCN4^{tg/wt} hearts. qPCR and Immunoblots from heart samples of 6 months-old mice. qPCR measurements of gene transcription related to cell growth, the fetal gene program and hypertrophy, cardiac ion channels, and cardiac ion channels (6 months-old mice n = 4–8 animals/group). The bars show the transgenic gene expression normalized to wild type expression level = 1.0, which is indicated by the dashed line. *P < 0.05; **P < 0.01; ***P < 0.001 (unpaired t-test). Replicative immunoblot of Ca^{2+} handling proteins. Protein expression of transgenic animals compared to wildtype littermates obtained from immunoblots (6 months-old mice n = 3 animals/group). Two replicate immunobLOTS per animal, in pPln-S16 and pPln-T17 one replicate immunoblot per animal. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. *P < 0.05; **P < 0.01 (unpaired t-test). Data are shown as mean ± s.e.m. Source data are provided as a Source data file.

Fig. 5 HCN4 overexpression affects intracellular Ca^{2+} homeostasis. a Representative [Ca^{2+}], transient traces measured as change in the Fluo-4 fluorescence in electrically driven cardiomyocytes from wild type (baseline (gray) and ivabradine treated (green)) and HCN4^{tg/wt} (baseline (black) and ivabradine treated (magenta)) mice (3 months of age). b–f Quantitative analysis reveals a significant change of [Ca^{2+}], to higher diastolic baseline levels (b), and larger amounts of total cellular Ca^{2+} movement (reflected by the area under the curve) during the systole in HCN4^{tg/wt} cardiomyocytes (c). Similar time to peak among groups indicates unaffected Ca^{2+} release from sarcoplasmic reticulum (SR) by ryanodine receptor (RyR) activity (d). Quantification of time to half decay of Ca^{2+} transients (D_{50} (e), and slope Ca^{2+} uptake (f), show that removal of increased baseline [Ca^{2+}] to the SR, driven by SERCA, is augmented in HCN4^{tg/wt} cardiomyocytes. Changes of HCN4^{tg/wt} cardiomyocytes in Ca^{2+} homeostasis were mostly reversed by treatment with the I_f blocker ivabradine (3 μM) underlining that the overexpressed HCN4 current is the pivotal cause for the observed Ca^{2+} imbalance. Data are expressed as mean ± s.e.m. (wild type: n = 90 cells, HCN4^{tg/wt}: n = 42 cells, iva-treated wild type: n = 48 cells, iva-treated HCN4^{tg/wt}: n = 75 cells derived from six animals/group; *P < 0.05, **P < 0.01, ***P < 0.001 compared to wild type; ANOVA followed by Tukey test). Source data are provided as a Source data file.

repolarization were shorter in transgenic cells, with significant changes of APD_{90} at all pacing frequencies and of APD_{20} at 0.5 Hz. Evaluation of APD_{90}, by contrast, did not differ significantly between wild type and HCN4^{tg/wt} cardiomyocytes (Fig. 8d–f).

HCN4^{tg/wt} cardiomyocytes are prone to arrhythmias. Based on the overexpression of depolarizing I_f and consecutive Ca^{2+} overload we asked whether HCN4^{tg/wt} cardiomyocytes elicit increased cellular automaticity and/or arrhythmogenesis. Recordings of [Ca^{2+}], transients and APs (Fig. 9a–g) frequently revealed sustained trains of automaticity in HCN4^{tg/wt} cardiomyocytes (Fig. 9c), while such changes were only sparsely observed in wild type cells. Under steady state field stimulation the [Ca^{2+}], transients of wild type cardiomyocytes quickly decayed (Fig. 9a), while a subset of HCN4^{tg/wt} cardiomyocytes showed abnormal diastolic [Ca^{2+}], clearance (Fig. 9b, Supplementary Fig. 2). Correspondingly, transgenic cardiomyocytes were prone to afterdepolarizations (ADs), which resulted in premature APs and arrhythmic firing (Fig. 9e, f). Among all cells
recorded, the percentage of cells exhibiting ADs or virtually spontaneous firing, was significantly higher in transgenic (55.5 ± 7.6%; n = 5 hearts; 12–30 cells per heart) than in wild type cardiomyocytes (17.8 ± 3.4%; n = 5 hearts, 22–34 cells per heart; P = 0.0002; ANOVA followed by Tukey test) (Fig. 9g). To delineate the immediate contribution of I_{f} to arrhythmogenesis within our model we treated the cells with 3 μM ivabradine (iva). Remarkably, iva treatment diminished spontaneous firing and ADs, and percentage of HCN4^{tg/wt} cardiomyocytes showing arrhythmic behavior after treatment was not different from wild type (Fig. 9g), demonstrating an important contribution of increased I_{f} to cellular arrhythmogenicity.

**Electrophysiological phenotype of HCN4^{tg/wt} mice.** To evaluate ECG parameters and to seek for arrhythmias in HCN4^{tg/wt} mice we performed ECG recordings under anesthesia and telemetric recordings in freely roaming animals. Both approaches revealed similar heart rates and conduction parameters among transgenic (HCN4^{tg/wt}) and wild type mice and did not exhibit significant changes of additional ECG parameters (Supplementary Fig. 4, Supplementary Table 1). However, increased numbers of premature ventricular captures and few non-sustained ventricular tachycardias (mostly triplets) were recorded in transgenic but not in wild type animals (Fig. 9h, i), while sustained ventricular tachycardias were not observed. This indicates that HCN4^{tg/wt} mice have a predisposition to ventricular arrhythmogenesis, although high-grade arrhythmias are not part of the phenotype.

**Discussion**

We here report that augmentation of myocardial I_{f} leads to the development of a cardiomyopathy phenotype with biventricular chamber dilation, significantly reduced wall thickness and decreased ejection fraction. To study the myocardial effect of increased I_{f} under conditions that minimized its influence on heart rate, we have generated transgenic mice that express human HCN4 (HCN4^{tg/wt}) under control of the murine cTNI promoter. While abundantly expressed in the working myocardium, cTNI is downregulated in the SAN and the conduction system. This is phenotypically reflected by telemetric recordings revealing similar heart rates and conduction parameters in transgenic and wild type mice (Supplementary Fig. 4, Supplementary Table 1), thus...
providing the opportunity to selectively study the consequences of HCN4 overexpression in the working myocardium.

There is growing evidence that ion channel disorders not only cause cardiac arrhythmias, but also contribute to structural abnormalities of the heart as well. We and others reported that HCN4 loss-of-function mutations are associated with noncompaction cardiomyopathy, pointing to an involvement of HCN4 in ventricular wall maturation at embryonic stages. Accordingly, HCN4 has been identified as primary cell marker for the cardiomyogenic progenitor pool of the first heart field, implicated in the earliest stage of heart formation. During later development and adult stages, HCN4 is downregulated in the healthy myocardium, while abundant expression is restricted to the SAN and the conduction system. Remarkably, in this context, HCN2 was recently shown to be implicated in the earliest stage of heart formation during later right (RV) and left (LV) ventricular tissue from wild type and HCN4−/− ventricular cardiomyocytes (~hHCN4 transcription from the intermediate-late phase of fetal development). We and others reported that several early genes reappear in reverse order compared to their developmental brain morphology and function, as well.

It is well documented that the the expression of the main ventricular HCN isoforms 2 and 4 are significantly increased in ventricular myocytes of heart failure patients. These changes are considered reminiscent of the immature myocyte phenotype, based on the fact that during pathological remodeling several early genes reappear in reverse order compared to embryonic development. To mimic maladaptive upregulation of HCN4 in our model, the cTNI promoter was used to activate hHCN4 transcription from the intermediate-late phase of fetal development, leading to augmented levels in the myocardium throughout postnatal and adult stages, normally characterized by low HCN4 expression. At physiological RMP of ventricular cardiomyocytes (~90 mV) patch clamp recordings revealed a two–three-fold higher If density in HCN4−/− cardiomyocytes compared to wild type (Fig. 1e, f), reflecting the magnitude of current increase that was found in cardiomyocytes of heart failure patients. Accordingly, transgenic mice developed myocardial alterations within two months postpartum, supporting a primary maladaptive involvement of increased If. Likewise, we observed a rise of apoptosis and markers of the fetal gene program (Fig. 3a, b) with significantly upregulated cell growth markers e.g. glycogen synthase kinase-3 beta (GSK-3B) and mammalian target of rapamycin (mTOR) (Fig. 4b), indicating cardiac remodeling and activation of compensatory cellular responses. Thus, postnatal myocardium appears susceptible to augmented If levels, and carefully regulated HCN patterning may constitute a prerequisite for the maintenance of structural cardiac integrity. In line, chronic peri- and postnatal treatment of transgenic mice with the If channel blocker ivabradine antagonized the myocardial changes (Fig. 2), underlining that augmented If is key to structural alterations occurring in HCN4−/− animals. Noteworthy, the heart rate lowering effect of ivabradine may contribute to protection from adverse remodelling as well.

Given the primary Na+ conductance of HCN4 channels at cardiomyocyte RMP (~90 mV) and the close interrelation of intracellular Na+ and Ca2+ homeostasis, we asked how increased If might influence Ca2+ cycling. To elucidate the impact of increased If, we measured [Ca2+]i transients in isolated, electrically driven cardiomyocytes in the pre- and absence of ivabradine. Strikingly, we observed a marked rise in [Ca2+]i baseline levels and significantly augmented systolic [Ca2+]i transients in transgenic cardiomyocytes – changes that were fully recovered in

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**Fig. 7** Assessment of myocardial apoptosis. (a) Representative TUNEL assay for detection of fragmented nuclear DNA (green) and nuclear counterstaining (red) in cryosections of right ventricles from wild type (left) and HCN4−/− (right) mice (2 months of age). (b) Quantification of TUNEL-positive nuclei in right (RV) and left (LV) ventricular tissue from wild type and HCN4−/− mice revealed a significant increase in the number of cells with fragmented nuclear DNA in both ventricles of transgenic animals (n = 6 animals/group; ***P < 0.001; unpaired t-test). (c) Genes with transcriptional changes > 1.5 fold in HCN4−/− compared to wild type hearts (values are normalized to corresponding wild type n = 1.0) using the Mouse Apoptosis RT2 Profiler PCR Array (SABioscience). Pronounced transcriptional changes were observed in the apoptosis effector gene caspase-3 (for additional data, please refer to Supplementary Fig. 3). (d) qRT-PCR transcription analysis of the caspase 3 (Casp3), tissue transglutaminase (tTG), and calpain 1 (Capn1) genes in ventricles of HCN4−/− mice normalized to corresponding wild type littermates (wt = 1.0; n = 6 animals/group; ***P < 0.001; unpaired t-test). (e) Immunohistochemical detection of caspase-3 activity (brown) in representative sections of right ventricular tissue from wild type (left) and HCN4−/− (right) mice. (f) Quantitative analysis shows proportion of cells positive for active caspase-3 in right and left ventricular tissue from wild type and HCN4−/− mice (n = 6 animals/group; ***P < 0.001; unpaired t-test). Data are shown as mean ± s.e.m. Source data are provided as a Source data file.
the presence of ivabradine. Thus, in the myocardium chronic upregulation of I_f exerts a diastolic Ca^{2+} overload and importantly interferes with cellular Ca^{2+} homeostasis.

As the primary mechanism of Ca^{2+} efflux in cardiac myocytes is via electrogenic Na^{+}/Ca^{2+} -exchange (NCX), Ca^{2+} homeostasis is tightly linked to Na^{+} regulation\(^{32}\). Under physiological conditions, NCX primarily operates in (forward) Na^{+} -in/ Ca^{2+} -out mode, and reduces diastolic [Ca^{2+}]_i levels by extruding Ca^{2+}. In pathological states, however, when [Na^{+}] is increased, NCX exerts ‘reverse mode’ function, moving Ca^{2+} into the cell\(^{32}\). Notably, [Na^{+}] is known to rise in a rate dependent fashion\(^{25}\), which exacerbates [Ca^{2+}]_i overload at high heart rates. The impact of I_f on [Na^{+}]_i, in relation to membrane potential has been demonstrated in sheep Purkinje fibers by voltage-clamp recordings in early works\(^{33,34}\) after the I_f mixed Na^{+} and K^{+} ionic nature was originally described\(^{35}\). These studies showed that membrane hyperpolarization to below −60 mV significantly increased intracellular Na^{+} activity, and related this increase to Na^{+} influx through I_f, thus providing a direct link between [Na^{+}]_i and the size of I_f. Based on these data, we hypothesized that HCN4 overexpression-mediated accumulation of [Na^{+}] may raise Ca^{2+} influx via reverse mode NCX activity (Fig. 10). According to this assumption, instant application of ORM-10103, an agent that effectively inhibits reverse mode NCX\(^{29,36}\), abolished Ca^{2+} overload, demonstrating the pathophysiological relevance of dysregulated NCX. Likewise, inhibition of increased Na^{+} influx via HCN4 channels using ivabradine facilitated resetting of the NCX equilibrium towards the ‘forward mode’ and restored [Ca^{2+}]_i to wild type levels, similar to ORM-10103. Of note, depolarization of RMP by augmented I_f may lower the driving force of forward NCX a fortiori, thereby aggravating chronic accumulation of [Ca^{2+}]_i in HCN4\(^{tg/wt}\) hearts. Based on these data it is reasonable to assume that inhibition of upregulated I_f in clinical heart failure may attenuate Ca^{2+} overload of the ventricular myocardium. Synergistically, heart rate lowering decreases [Na^{+}]\(^{32}\) and therefore shifts the Na^{+}/Ca^{2+} exchanger equilibrium towards the forward mode, which will alleviate [Ca^{2+}]_i overload independently from ventricular I_f blockade. Thus, besides improved energy supply through heart rate reduction the delineated mechanisms may importantly contribute to positive outcome of patients with heart failure and increased heart rates treated by I_f blockade\(^{23}\).

We next addressed the effects of increased I_f and changed Ca^{2+} homeostasis on cellular electrophysiology. Action potential recordings of isolated HCN4\(^{tg/wt}\) cardiomyocytes showed depolarized RMP and reduced amplitude of overshoot in a rate-dependent fashion, consistent with HCN4-mediated diastolic Na^{+} influx. Furthermore, APD\(_{25}\) and APD\(_{50}\) were shorter compared to WT, while APD\(_{90}\) was similar. This in part is consistent with previous data obtained from cardiomyocytes overexpressing HCN2\(^{37}\), which showed that outward tail current of I_f shortened APD at membrane potentials more positive than reversal potential (~−35 mV for HCN4\(^{38}\)), while inward tail current of I_f prolonged APD at membrane potentials more negative than reversal potential. However, prolongation of APD\(_{90}\) was not observed in our model, most likely due to the depolarizing driving
force of the electrogenic ‘reverse mode’ NCX, known to effectively shorten AP duration when $[\text{Na}^+]_i$ is increased$^{39}$. Interestingly, we found that myocytes isolated from HCN4tg/wt hearts showed a higher incidence of spontaneous APs than wild type cells, and a subset of cells exerted virtual pacemaker function. The increased automaticity may originate from $I_f$ augmentation and not from spontaneous Ca$^{2+}$ cycling-mediated membrane depolarisation, as the NCX equilibrium is shifted toward ‘reverse mode’ leading to an outward, hyperpolarizing current. Moreover, HCN4tg/wt cardiomyocytes were
**Fig. 9** HCN4 overexpression leads to increased arrhythmogenicity.  

**a-c** Representative [Ca^{2+}]_{i} transients measured as change in the Fluo-4 fluorescence in cardiomyocytes from wild type **a**, and HCN4^{tg/wt} **b**, **c** mice (3 months of age).  

**a** Normal behavior, exhibited by cardiomyocytes isolated from wild type mice, characterized by lack of automaticity (defined as spontaneous beating rate < 0.2 Hz) and rapid decay of Ca^{2+} transients under field stimulation. In contrast, cardiomyocytes from HCN4^{tg/wt} mice frequently showed spontaneous pacemaker activity, characterized by periodic firing of [Ca^{2+}]_{i} transients (b), or [Ca^{2+}]_{i} transients with abnormal prolongation of diastolic [Ca^{2+}]_{i} decay (defined as \(D_{90}>2000\text{ms}\)) (c). Field stimulation in **a**, **b** is denoted by green arrows.  

**d-f** Recording of spontaneous action potentials (APs) in wild type (**d**), and HCN4^{tg/wt} (**e**, **f**) cardiomyocytes. While most wild type cells lacked relevant automaticity (defined as spontaneous beating rate < 0.2 Hz) (**d**), a subset of HCN4^{tg/wt} cells showed spontaneous pacemaker activity, with or without afterdepolarizations (ADs). ADs (denoted by dash in **e**, **f**) frequently induced spontaneous APs (denoted by arrow in **f**), leading to arrhythmic firing.  

**f** depicts a detail of **e, g**. Quantitative analysis of spontaneous activity of wild type (gray), HCN4^{tg/wt} (black) and ivabradine-treated HCN4^{tg/wt} (magenta) cardiomyocytes indicate a greater proportion of cells with pacemaker activity (Pace) or ADs in HCN4^{tg/wt} cardiomyocytes compared to wild type. Remarkably, spontaneous firing and ADs were diminished after treatment with ivabradine, and percentage of cells showing arrhythmic behavior was not different from wild type thereafter. Data are expressed as mean ± s.e.m. (n = 5 independent experiments [mice] per group, 12–34 cells evaluated per experiment; \(P<0.05\), \(**P<0.01\), \(***P<0.001\) compared to wild type; ANOVA followed by Tukey test).  

**h, i** Telemetry recording from a HCN4^{tg/wt} mouse showing an episode with premature ventricular contractions to occur singular and as triplet. **I** depicts a detail of **h**. Source data are provided as a Source data file.

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**Fig. 10** Proposed pathogenetic processes. Model of how augmented HCN4 expression in the myocardium may contribute to pathological remodeling. HCN4 upregulation mediates diastolic Na^{+} influx into the cell, leading to a rise of [Na^{+}]_{i}, Ca^{2+} homeostasis is tightly linked to Na^{+} regulation via the NCX activity. Under physiological conditions NCX primarily operates in “forward” Na^{+}-in/Ca^{2+}-out mode, reducing diastolic [Ca^{2+}]_{i}. High [Na^{+}]_{i} gives rise to a shift of the NCX equilibrium towards “reverse mode” thereby leading to increased [Ca^{2+}]_{i}. Changed cytoplasmic Ca^{2+} cycling, in turn, interferes with SR calcium uptake resulting in increased SR Ca^{2+} stores, driven by augmented phosphorylation of PLN. Sarcoplasmic Ca^{2+} overload causes afterdepolarizations (ADs) and cardiac arrhythmogenesis, and high diastolic [Ca^{2+}]_{i} activates μ-calpain Ca^{2+}-sensor- and caspase-3-related apoptosis leading to adverse remodeling. Brown arrows indicate regulation of protein expression; green arrows denote changes of ion concentration.
In summary, we show that a two–three-fold increase of I\textsubscript{f} in cardiac myocytes, which is comparable to levels observed in human heart failure, affects cardiac structure and promotes cellular arrhythmogenicity in HCN4\textsuperscript{tg/GL} mice. Activation of reverse-mode NCX by increased HCN4-mediated Na\textsuperscript{+} influx leads to augmented [Ca\textsuperscript{2+}], and dysregulated Ca\textsuperscript{2+} homeostasis driving apoptosis and adverse cardiac remodeling. This might have particular implications in disease states associated with increased I\textsubscript{f}, affecting calcium cycling as well as excitation-contraction coupling. Thus, direct cardioprotective mechanisms in addition to improved energy supply at lower heart rates may underlie the beneficial effects of I\textsubscript{f} inhibition in heart failure. Our findings provide insight in previously undescribed cardiac pathomechanisms, bridging a changed electrical state to a structural phenotype, which importantly could influence future treatment strategy of heart failure.

Methods

Generation of transgenic mice. Transgenic mice were generated in a C57BL/6Ncrl background (Charles River Laboratories, Wilmington, MA, USA) by pronuclear injection of a 8.8 kb DNA fragment carrying a 4.3 kb promoter fragment of the murine cTnl gene\textsuperscript{18,19} into the 5' end of the human HCN4 cDNA\textsuperscript{48} that was fused to the bovine growth hormone gene poly A signal (Fig. 1a). Tail DNA analysis revealed four different founders that were subsequently bred with C57BL/6 N wild type mice to generate progeny that appeared healthy and fertile.

Care and use of mice. Mixed genotype groups of each gender of no more than five animals were housed in standard mouse cages under specific pathogen-free conditions (12:12-h dark–light cycle, constant temperature, constant humidity and food and water ad libitum). All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication number 85–23, revised 1996), and with the European Community guidelines for the use of experimental animals as well as all relevant ethical regulations. Protocols were approved by the local regulatory authority (#35-9185.81/G-20/11 and #35-9185.81/G-226/16 Regierungsspräsidium, Karlsruhe, Germany). We used male mice for all experiments.

Surface and telemetric ECG recordings. For surface ECG recordings mice were anesthetized with isoflurane vapor titration to maintain the lightest anesthesia possible\textsuperscript{49}. On average, 1.5% vol/vol isoflurane was required to maintain adequate anesthesia. Mice were placed on a heating pad with continuous monitoring of body temperature (via a rectal probe) maintaining at 37°C. Surface ECGs were recorded using an 8-channel stimulator attached to each limb and chest in a midsternal position. The electrodes were connected to a Powerlab System (AD Instruments, Hastings, UK) and ECG recording were performed with a high and low frequency filter set to 1 kHz and 0.1kHz. ECG recordings were analysed by an investigator blinded to the experimental groups using the Chart 5 software (AD Instruments, Hastings, UK). In addition to average heart rate, at least 30 complexes of each trace were developed by sequential exposure to blocking reagent containing 3% bovine serum albumin and 5% dry milk. Primary antibodies were directed against HCN4 (1:200 diluted in 3% dry milk), NCX1 (1:250), Cavi1.2 (1:500), Serca2a (1:1000), Phospholamban (1:500), phosphorylated Phospholamban Thr-17 (1:2000), phosphophorylated Phospholamban Ser-16 (1:2000) and Calpain1 (1:2000). Appropriate secondary antibodies were developed by sequential exposure to horseradish peroxidase conjugating 3% bovine serum albumin and 5% dry milk. Primary antibodies were stained with 3% 3,3'-diaminobenzidine (DAB) and counterstained with hematoxylin.

Protein analysis. Protein immunodetection was performed by sodium dodecyl sulfate (SDS) gel electrophoresis. Tissue sections obtained from indicated mouse left and right ventricle samples were rinsed in phosphate buffered saline (PBS), rapidly frozen in liquid nitrogen and stored at −80°C. Tissue samples were homogenized (TissueRuptor, QIAGEN, Hilden, Germany) in a radioimmunoprecipitation (RIPA) lysis buffer containing 50 mM Tris–HCl (pH 7.4), 0.5% NP-40, 0.25% sodium deoxycholate, 1% RNase A, 1 mM RNase, 1 mM Na\textsubscript{2}VO\textsubscript{4}, 1 mM NaF, and protease inhibitors (Complete; Roche, Indianapolis, IN, USA). The protein concentration was determined using the bicinchoninic acid (BCA) protein assay (Thermo Scientific, Rockford, IL, USA), and equal amounts of protein were separated on SDS polyacrylamide gels. Nitrocellulose membranes were developed by sequential exposure to horseradish peroxidase conjugating 3% bovine serum albumin and 5% dry milk. Primary antibodies were directed against hCN4 (1:200 diluted in 3% dry milk), NCX1 (1:250), Cavi1.2 (1:500), Serca2a (1:1000), Phospholamban (1:500), phosphophorylated Phospholamban Thr-17 (1:2000), phosphophorylated Phospholamban Ser-16 (1:2000) and Calpain1 (1:2000). Appropriate HRP secondary antibodies (anti-Mouse IgG, 1:2000) were used. Signals were developed using an enhanced chemiluminescence assay (ECL Western Blotting Reagents, GE Healthcare, Buckinghamshire, UK) and quantified with ImageJ 1.41 Software (National Institutes of Health, Bethesda, MD, USA). Protein content was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using anti-GAPDH primary antibodies (1:40,000) and corresponding secondary antibodies for digital quantification of optical density. For detailed information on primary and secondary antibodies used please refer to Supplementary Table 2. Western blot images used to compose result panels are available in Supplementary Fig. 5.

Isolation of cardiomycocytes. Cardiomyocytes for intracellular calcium measurements were obtained following the Liao & Jain protocol\textsuperscript{52}. Briefly, the mice received 200 IU heparin i.p. prior to sacrifice. The thoracic cavity was opened and a cannula with perfusion solution was inserted from the atria. The heart was harvested and perfused in the Langendorf system with Perfusion Buffer (in mM: 135 NaCl, 4 KCl, 1 MgCl\textsubscript{2}, 10 HEPES, 0.33 NaH\textsubscript{2}PO\textsubscript{4}, 10 glucose, 20, 2,3-butanediones monoxime, 5 taurine, pH 7.2 at 37 °C for 5 min), followed by Digestion Buffer treatment (0.3 mg/mL body weight collagenase D (Roche, Indianapolis, IN, USA), 0.4 mg/mL body weight collagenase B (Roche, Indianapolis, IN, USA), 0.05 mg/mL body weight protease XIV (Sigma Aldrich, St. Louis, MO, USA) in 25 mL Perfusion Buffer) until the heart muscle was pale and some signs of extracellular matrix dissociation and for additional 5 min with Perfusion Buffer to complete cell dissociation. The cardiomyocytes of triple mechanically dissociated in Transfer Buffer A in mM: 135 NaCl, 4 KCl, 1 MgCl\textsubscript{2}, 10 HEPES, 0.33 NaH\textsubscript{2}PO\textsubscript{4}, 5.5 glucose, 10, 2,3-butanediones monoxime, 5 mg/mL bovine serum
Cellular electrophysiology. $I_v$ was recorded under voltage-clamp conditions (whole-cell configuration) in isolated mouse ventricular cardiomyocytes at room temperature (21–23 °C), essentially as published55. In detail, patch-clamp measures of $I_v$ ventricular cardiomyocytes were performed in a solution containing (in mM): 137 NaCl, 5 KCl, 1.8 CaCl$_2$, 2 MgCl$_2$, 2.4 CaCl$_2$, 5 glucose, and 10 Hepes, pH corrected to 7.4 using 1 M NaOH. The patch pipettes filled with borosilicate glass (GB-180-P, Science Products, Hofheim Germany) were generated on a DMZ Universal puller (Zeitz Instruments, Munich, Germany) and fire polished to give a final resistance of 2–4 MΩ. The pipette solution contained (in mM): 130 K-Aspartate, 5 NaATP, 5 CaCl$_2$, 2 MgCl$_2$, 11 EGTA and 10 Hepes, pH corrected to 7.3 using 1 M KOH. Pharmacological agents were added to the bath solution to block L- type calcium currents (10 μM nisoldipine), the slow component of delayed rectifier K$^+$$^*$ current ($I_{Ks}$, 10 μM 4-AP), the rapid component of delayed rectifier K$^+$$^*$ current ($I_{Ks}$, 10 μM E4031), ATP-dependent K$^+$$^*$ current ($I_{KATP}$, 1 μM glibenclamide) and inward rectifier K$^+$$^*$ currents ($I_{IK}$, 50 μM BaCl$_2$). $I_v$ currents were normalized to cell capacitance which was obtained from the time constant of a current transient evoked by a 5 mV potential step at the beginning of each sweep.

Recordings of cardiac action potentials were carried out in an extracellular solution containing (in mM): 137 NaCl, 4 KCl, 1.8 CaCl$_2$, 0.5 MgCl$_2$, 10 Hepes, 5.5 glucose, and a pipette solution containing (in mM): 120 K-Aspartate, 5 NaCl, 5 NaCl, 2 MgCl$_2$, 10 Hepes. For quantification of AP duration and amplitude, cells were held in current-clamp mode and current was injected to achieve a membrane potential of −90 mV. If injected currents exceeded 200pA, recordings were discarded and not included in analysis. APs that showed a clear delay with respect to the subtraction were not used for any analysis. APs were analyzed avoiding the time with no electrical stimulation of the Fluo-4 of 345 nM, according to the manufacturer (Supplementary Fig. 2).

Out of 8–10 Ca$^{2+}$ transients recorded, the five least were analyzed, avoiding the non-physiological Ca$^{2+}$ transients due to time with no electrical stimulation between cell recordings. Ca$^{2+}$ transients were eligible for regular analysis when lacking lateral oscillations showing regular Ca$^{2+}$ clearance under field stimulation at 0.2 Hz. Cells that exhibited pacemaker activity (defined as spontaneous beating rate < 0.2 Hz) or abnormal diastolic [Ca$^{2+}$i], decay (defined as $D_{950}$ > 2000ms) were excluded from regular analysis and evaluated in a separate record (please refer to Supplementary Fig. 2).

Calibration of calcium transients. The cardiomyocytes were loaded with 10 μM Fluo-4, AM (Molecular Probes, Leiden, The Netherlands) dissolved in Transfer Buffer (5.4 KCl, 1.8 CaCl$_2$, 10 Hepes, 5.5 glucose, pH 7.4 at 37 °C) for 30 min. For pharmacological treatments ivabradine (Molekula, Munich, Germany; cat. no. 89982651/155974-00-8) or ORM-10103 (Sigma-Aldrich, St. Louis, MO, USA) digitized at 20 kHz and with a 1401 Power3 Analog/Digital Converter (CED, Cambridge, UK) stored on a hard drive, and analysed off-line with custom MATLAB routines (Mathworks, Natick, USA) software. Recordings with less than 10% leak current were considered for data analysis. No leak subtraction was performed during the experiments. Recordings were discarded if access resistance exceeded 20 MOhm or changed for more than 20% during the recording period.

Recording of calcium transients. The cardiomyocytes were loaded with 10 μM Fluo-4, AM (Molecular Probes, Leiden, The Netherlands) dissolved in Transfer Buffer (5.4 KCl, 1.8 CaCl$_2$, 10 Hepes, 5.5 glucose, pH 7.4 at 37 °C) for 30 min. For pharmacological treatments ivabradine (Molekula, Munich, Germany; cat. no. 89982651/155974-00-8) or ORM-10103 (Sigma-Aldrich, St. Louis, MO, USA) digitized at 20 kHz and with a 1401 Power3 Analog/Digital Converter (CED, Cambridge, UK) stored on a hard drive, and analysed off-line with custom MATLAB routines (Mathworks, Natick, USA) software. Recordings with less than 10% leak current were considered for data analysis. No leak subtraction was performed during the experiments. Recordings were discarded if access resistance exceeded 20 MOhm or changed for more than 20% during the recording period.

**Statistical analysis.** All experiments and primary analyses were blinded. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Prism Inc. La Jolla, CA). Statistical significance and comparisons between samples were analyzed by a two-tailed unpaired Student’s t-test. Comparisons between multiple groups were performed using one-way ANOVA followed by Bonferroni (for calcium recordings), Tukey (for histochecmical analysis) or Dunn’s (for action potential amplitude) post tests. Differences were considered significant at a level $p$ < 0.05. Data are presented as arithmetic mean ± s.e.m. or median ± maximum/minimum.
References

1. DiFrancesco, D. & Tortora, P. Direct activation of cardiac pacemaker channels by intracellular cyclic AMP. Nature 351, 145–147 (1991).

2. Schulze-Bahr, E. et al. Pacemaker channel dysfunction in a patient with sinus node disease. J. Clin. Invest. 111, 1357–1354 (2003).

3. Schweizer, P. A. et al. cAMP sensitivity of HCN pacemaker channels determines basal heart rate but is not critical for autonomic rate control. Circ. Arrhythmia Electrophysiol. 3, 542–552 (2010).

4. Duhme, N. et al. Altered HCN4 channel C-linker interaction is associated with familial tachycardia-bradycardia syndrome and atrial fibrillation. Eur. Heart J. 34, 2768–2775 (2013).

5. Glitsch, H. G., Husch, H. & Verdonck, F. The contribution of Na and K ions to the pacemaker current in sheep cardiac Purkinje fibres. Pflügers Arch. Eur. J. Physiol. 406, 664–471 (1986).

6. Schweizer, P. A. et al. Transcription profiling of HCN-channel isoatypes throughout mouse cardiac development. Basic Res. Cardiol. 104, 621–629 (2009).

7. Yasui, K. et al. Icuroent and spontaneous activity in mouse embryonic ventricular myocytes. Circ. Res. 88, 536–542 (2001).

8. Liang, X. et al. HCN4 dynamically marks the first heart field and conduction system precursors. Circ. Res. 113, 399–407 (2013).

9. Später, D. et al. A HCN4+ cardiomyogenic progenitor derived from the first heart field and human pluripotent stem cells. Nat. Cell Biol. 15, 1098–1106 (2013).

10. Pitcairn, E. et al. Coordinating heart morphogenesis: a novel role for hyperpolarization-activated cyclic nucleotide-gated (HCN) channels during cardiogenesis in Xenopus laevis. Commun. Integr. Biol. https://doi.org/10.1080/19420889.2017.1309488 (2017).

11. Scholz, J. & Thum, T. Hallmarks of ion channel gene expression in end-stage heart failure. FASEB J. 17, 1992–1608 (2003).

12. Ophoff, R. A. et al. Scale analysis of ion channel gene expression in the mouse heart during development. Circ. Genomic Precis. Med. https://doi.org/10.1161/CIRCGEN.117.001980 (2018).

13. Schweizer, P. A. et al. The symptom complex of familial sinus node dysfunction and myocardial noncompaction is associated with mutations in the HCN4 channel. J. Am. Coll. Cardiol. 64, 757–767 (2014).

14. DiFrancesco, D. et al. The role of the funny current in pacemaker activity. Circ. Res. 86, 434–446 (2000).

15. Fox, K. et al. Ivabradine in stable coronary artery disease without clinical heart failure and atrial fibrillation. Circ. Res. 98, 405–429 (2006).

16. DiFrancesco, D. et al. A study of the ionic nature of the pacemaker current in calf Purkinje fibres. J. Physiol. 314, 373–393 (1981).

17. Ito, N. et al. ORM-10103, a novel specific inhibitor of the Na+Ca2+ exchanger, decreases early and delayed afterdepolarizations in the canine heart. Br. J. Pharmacol. 170, 768–778 (2013).

18. Armonda, A. A., Hobai, I. A., Tomasselli, G. F., Winslow, R. L. & O’Rourke, B. Role of sodium-calcium exchanger in modulating the action potential of ventricular myocytes from normal and failing hearts. Circ. Res. 93, 46–53 (2003).

19. Zhang, X. et al. Persistent increases in Ca2+ influx through Cav1.2 shortens action potential and causes Ca2+ overload-induced afterdepolarizations and arrhythmias. Basic Res. Cardiol. 111, 4 (2016).

20. Xie, Y., Sato, D., Farbinkel, A., Qu, Z. & Weiss, J. N. So little source, so much sink: Requirements for afterdepolarizations to propagate in tissue. Biophys. J. 99, 1408–1418 (2010).

21. Chen, K. R., Ross, J. & Hoshijima, M. Calcium and heart failure: the cycle game. Nat. Med. 9, 508–509 (2003).

22. Nakayama, H. et al. Ca2+- and mitochondrial-dependent cardiomyocyte necrosis as a primary mediator of heart failure. J. Clin. Invest. 117, 2431–2444 (2007).

23. Edmiston, M. et al. Myosin controls cardiac calcium cycling and contractility via regulation of L-type calcium channel surface expression. Nat. Commun. https://doi.org/10.1038/ncomms11835 (2016).

24. Foo, R. S. Y., Mani, K. & Kitis, N. R. Death begets failure in the heart. J. Clin. Investig. 115, 565–571 (2005).

25. Olivetti, G. et al. Apoptosis in the failing human heart. N. Engl. J. Med. 336, 1131–1141 (1997).

26. Muth, J. N., Bodi, I., Lewis, W., Varadi, G. & Schwartz, A. A Ca2+-dependent transgenic model of cardiac hypertrophy: a role for protein kinase Ca. Circulation 113, 140–147 (2003).

27. O’Rourke, B. et al. Functional adult myocardium in the absence of Na+Ca2+ exchange. Circ. Res. 95, 604–611 (2004).

28. Knollmann, B. C. et al. Casq2 deletion causes sarcoplasmic reticulum volume increase, premature Ca2+ release, and catecholaminergic polymorphic ventricular tachycardia. J. Clin. Investig. 116, 2510–2520 (2006).

29. Mitchell, G. F., Jeron, A. & Koren, G. Measurement of heart rate and Q-T interval in the conscious mouse. Am. J. Physiol. 274, H747–H751 (1998).

30. Barrett, R. B., Harbi, S., Hoffman, J. E., Zavadil, J. & Costee, W. A. Large-scale analysis of ion channel gene expression in the mouse heart during perinatal development. Physiol. Genom. 28, 273–283 (2006).
52. Liao, R. & Jain, M. Isolation, culture, and functional analysis of adult mouse cardiomyocytes. Methods Mol. Med. 139, 251–262 (2007).
53. Aff, J. et al. Control of heart rate by CAMP sensitivity of HCN. channels Proc. Natl. Acad. Sci. 106, 12189–12194 (2009).
54. Graf, E., Heubach, J. F. & Ravens, U. The hyperpolarization-activated current I_{hi} in ventricular myocytes of non-transgenic and β2-adrenoceptor overexpressing mice. Naunyn. Schmiede. Arch. Pharmacol. 364, 131–139 (2001).
55. Williams, I. A. & Allen, D. G. Intracellular calcium handling in ventricular myocytes from mdx mice. Am. J. Physiol. Circ. Physiol. 292, H846–H855 (2006).
56. Grynkiewicz, G., Poenie, M. & Tsien, R. Y. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. 260, 3440–3450 (1985).
57. Chevessier, F. et al. A new mouse model for the slow-channel congenital myasthenic syndrome induced by the AChR εL221F mutation. Neurobiol. Dis. 45, 851–861 (2012).

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Author contributions
P.Y., M.K.O. and P.A.S. conceived and designed the study and the experiments. P.Y. and M.K.O. generated the transgenic mouse model. P.Y., S.N. and P.A.S. performed the molecular biology experiments. P.Y. and P.A.S. performed the histopathology and immunohistochemistry experiments of mouse hearts. P.G., C.B., C.S., A.P.S. and H.E. performed and analyzed the patch-clamp experiments. M.M., M.W. and R.F. carried out the calcium recordings of isolated cardiomyocytes. S.N., M.K., T.F. and P.A.S. performed the telemetric ECGs of mice and data analysis. S.N. and P.A.S. performed the mouse echocardiography and data analysis. H.A.K. provided administrative support and final approval of the paper. A.D. and D.T. supervised data analysis and provided expertise with paper writing. The paper was written principally by P.A.S.

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