Fhf2 gene deletion causes temperature-sensitive cardiac conduction failure

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Fever is a highly conserved systemic response to infection dating back over 600 million years. Although conferring a survival benefit, fever can negatively impact the function of excitable tissues, such as the heart, producing cardiac arrhythmias. Here we show that mice lacking fibroblast growth factor homologous factor 2 (FHF2) have normal cardiac rhythm at baseline, but increasing core body temperature by as little as 3°C causes coved-type ST elevations and progressive conduction failure that is fully reversible upon return to normothermia. FHF2-deficient cardiomyocytes generate action potentials upon current injection at 25°C but are unexcitable at 40°C. The absence of FHF2 accelerates the rate of closed-state and open-state sodium channel inactivation, which synergizes with temperature-dependent enhancement of inactivation rate to severely suppress cardiac sodium currents at elevated temperatures. Our experimental and computational results identify an essential role for FHF2 in dictating myocardial excitability and conduction that safeguards against temperature-sensitive conduction failure.

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ever-induced arrhythmias\(^1\) and seizures\(^2\) are well documented, and are often associated with mutations in sodium channels, suggesting that deficient sodium current reserve is an important determinant for electrical instability during hyperthermic states. Indeed, elevation in core body temperature by fever or external heating\(^3\) is a known trigger for ventricular fibrillation/malignant syncpe in patients with Brugada syndrome (BrS)\(^3\), an inherited arrhythmia condition diagnosed by characteristic electrocardiographic (ECG) abnormalities in the right precordial leads. Loss of function mutations in SCN5A, which encodes the pore-forming subunit of the cardiac sodium channel Na\(_V\)1.5, have been identified in \(\sim 20\%\) of BrS patients\(^3\). Biophysical analyses of mutant sodium channels from febrile BrS patients have not identified a unifying mechanism for the temperature-sensitive phenotype\(^6\)\(^7\). These data suggest that factors in addition to SCN5A are playing important roles in regulating the sodium current that ultimately predispose BrS patients to fever-induced arrhythmias.

FHF2s, also termed iFGFs, are a family of proteins that bind to the cytoplasmic tails of voltage-gated sodium channels (VGSCs)\(^8\)\(^9\)\(^10\), modulating channel inactivation and cellular excitability\(^11\)\(^12\)\(^13\). We generated mice lacking fibroblast growth factor homologous factor 2 (\(Fhf2^{KO}\)) to study its role in regulating cardiac excitability under normal and pathological states. \(Fhf2^{KO}\) mice have normal cardiac rhythm at baseline, but exhibit temperature-sensitive electrocardiographic changes, including coved-type ST elevations and progressive conduction failure that is fully reversible upon return to normal body temperature. Optical mapping reveals severe conduction slowing in mutant hearts at 37 °C that is further exacerbated by temperature elevation. FHF2-deficient cardiomyocytes generate action potentials upon current injection at 25 °C but are unexcitable at 40 °C. Loss of FHF2 results in a hyperpolarizing shift of steady-state inactivation of the sodium current and accelerates the rate of closed-state and open-state sodium channel inactivation, which synergizes with temperature-dependent enhancement of inactivation rate to severely suppress cardiac sodium currents at elevated temperatures. Our experimental and computational results demonstrate that FHF2 is a key regulator of myocardial excitability, protecting the heart against conduction failure under hyperthermic conditions.

**Results**

**Derivation and validation of \(Fhf2^{KO}\) mice.** We engineered mice bearing a deletion within the X-linked \(Fhf2\) gene (Fig. 1a,b) in order to test for possible effects on cardiac rhythm. The absence of FHF2 protein in \(Fhf2^{KO}\) mice was confirmed in immunoblots of heart and brain tissue (Fig. 1c), and by ventricular myocyte immunofluorescence (Fig. 1d). Notably, there was no noticeable change in Na\(_V\)1.5 protein levels or subcellular localization in \(Fhf2^{KO}\) ventricular myocytes. The IRES-lacZ insertion within the \(Fhf2^{KO}\) allele (Fig. 1a) allowed for whole-mount X-Gal staining of the heart and demonstrated widespread \(Fhf2\) gene expression in atria, ventricles, and the His-Purkinje system (Fig. 1e).

**\(Fhf2^{KO}\) mice exhibit hyperthermia-induced conduction defects.** Female \(Fhf2^{KO/ KO}\) and male \(Fhf2^{KO/ Y}\) mice were viable and fertile. \(Fhf2^{KO/ Y}\) mice exhibited normal baseline ECG parameters at 37 °C (Fig. 2a and Supplementary Table 1). Cardiac structural

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**Figure 1 | Derivation and validation of \(Fhf2^{KO}\) mice.** (a) Schematic of \(Fhf2^{WT}\) and \(Fhf2^{KO}\) alleles. The \(Fhf2^{KO}\) allele differs from the \(Fhf2^{WT}\) allele by the replacement of a 570-base genomic segment spanning coding exon 3 with a cassette containing splice acceptor site, internal ribosome entry site, a β-galactosidase coding sequence and residual loxP site. Positions and orientations of three PCR primers are indicated (blue arrowheads). (b) PCR genotyping of \(Fhf2^{WT}\) and \(Fhf2^{KO}\) alleles. Simultaneous amplification of wild-type 350 bp and mutant 430 bp allelic segments were resolved on agarose gel. (c) Immunoblotting of \(Fhf2^{WT/Y}\) and \(Fhf2^{KO/Y}\) tissue extracts. 40 mcg protein from Triton X-100-soluble fractions of brain and heart lysates were electrophoresed and immunoblotted to detect FHF2 (top), Na\(_V\)1.5 (middle) and GAPDH (bottom). The prominent \(~30\) kDa species in \(Fhf2^{WT/Y}\) hearts corresponding to the FHF2\_Y isoform are not detected in \(Fhf2^{KO/Y}\) samples. (d) Immunofluorescence detection of FHF2 and Na\(_V\)1.5 in \(Fhf2^{WT/Y}\) and \(Fhf2^{KO/Y}\) cardiomyocytes. Dissociated cells were fixed and probed with antibodies to FHF2 (red), Na\(_V\)1.5 (red) and N-cadherin (green) along with DAPI nuclear stain (blue). FHF2 and Na\(_V\)1.5 colocalize to \(Fhf2^{WT/Y}\) striated T-tubules. Na\(_V\)1.5 is comparably localized in the \(Fhf2^{KO/Y}\) myocyte, while FHF2 is not detected. (e) Whole-mount X-Gal staining of a \(Fhf2^{lacZ/lacZ}\) heart. LacZ at native sites of \(Fhf2\) expression in targeted alleles (a) was detectable throughout the heart, including atria (A), ventricles (V) and bundle of His (His). Higher magnification views of His bundle (black dashed box) and the left ventricular free wall (red dashed box). Scale, 25 μm (d); 1 mm (e, left); 250 μm (e, right).
and functional assessments by transthoracic echocardiography were also normal under euthermic conditions (Supplementary Table 2). However, Fhf2KO/Y mice were highly temperature-sensitive. Elevation of core body temperature by external heat source resulted in marked conduction slowing as evidenced by progressive P and QRS wave prolongation and atrioventricular (AV) block (Fig. 2a and Supplementary Table 1). Above 40°C, all mutant mice developed coved-type ST elevations with T wave inversions, reminiscent of the Brugada pattern ECG (Fig. 2a). Mutant mice did not tolerate sustained temperature elevation due to high-grade AV block and progressive conduction failure. With subsequent cooling to 37°C, all ECG conduction parameters returned to baseline. In contrast, male wild type (Fhf2WT/Y) mice displayed normal ECG parameters throughout the temperature ramp up to 43°C (Fig. 2a and Supplementary Table 1). Measurement of intracardiac intervals including atrial-His (AH) and His-ventricular (HV) intervals, which are measures of AV nodal conduction time and His-Purkinje-mediated ventricular activation time, respectively, were similar to Fhf2WT/Y mice at 37°C but were significantly prolonged in mutant mice at elevated temperatures (Fig. 2b and Supplementary Table 1).

**Fhf2KO mice display increased sensitivity to flecainide.** Fhf2KO/Y animals were highly sensitive to the sodium channel blocker flecainide (15 mg·kg⁻¹), which caused marked P and QRS wave prolongation and AV block at 37°C (Fig. 2c and Supplementary Table 3). In contrast, Fhf2WT/Y mice were able to tolerate high dose flecainide (30 mg·kg⁻¹), and despite having marked prolongation of P, PR and QRS durations, the conduction parameters remained stable during temperature ramp up to 43°C (Fig. 2d and Supplementary Fig. 1).

**Isolated Fhf2KO hearts retain temperature-sensitive defects.** To rule out extra-cardiac contributions to the temperature-sensitive phenotype, including autonomic effects, explanted Fhf2KO/Y and Fhf2WT/Y hearts were Langendorff-perfused and subjected to a temperature ramp protocol with a maximum perfusion temperature of 43°C (Fig. 2e). Similar to the in vivo findings, Fhf2KO/Y hearts exhibited progressive P and QRS wave prolongation, and AV block on volume-conducted ECG with temperature elevation. Coved-type ST elevations with T wave inversions were again seen in mutant hearts at temperatures greater than 40°C. Fhf2WT/Y hearts displayed stable conduction parameters throughout the temperature recordings.

Optical mapping of Fhf2KO/Y and Fhf2WT/Y hearts using a voltage-sensitive dye was performed to evaluate differences in epicardial conduction velocities (CV) at 37 and 39°C. Activation maps of the anterior wall were obtained at a pacing cycle length of 200 ms (Fig. 2f). Epicardial CV were markedly slower in Fhf2KO/Y compared with Fhf2WT/Y hearts at 37°C (0.25 ± 0.0063 m·s⁻¹ versus 0.56 ± 0.01 m·s⁻¹), KO versus WT, respectively (P = 1.35E-5, Student’s t-test). CV slowing was global and not confined to the right ventricular outflow tract, as has been reported in some patients with BrS (refs 14–16). Fhf2KO/Y hearts showed further reduction in epicardial CV at 39°C, while Fhf2WT/Y hearts maintained stable epicardial CV measurements at higher temperature.

**Fhf2KO cardiomyocytes have reduced excitability at 40°C.** To study the effects of FHF2 on cellular excitability, we acutely dissociated ventricular myocytes prepared from Fhf2WT/Y and Fhf2KO/Y mice and tested their ability to generate action potentials (AP) at 25 and 40°C in response to current injection through whole-cell patch. Fhf2WT/Y cells could generate action potentials at either temperature, although AP waveforms were significantly attenuated at 40°C (Fig. 3a and Supplementary Table 4). By contrast, Fhf2KO/Y cells had reduced amplitude AP waveforms at 25°C and failed to fire at 40°C (Fig. 3a and Supplementary Table 4). We conclude that the Fhf2KO temperature-dependent conduction defect reflects, at least in part, an underlying deficit in excitability of individual cardiomyocytes.

**Altered sodium channel inactivation in Fhf2KO cardiomyocytes.** The basis of Fhf2KO cellular excitation deficit was probed by recording sodium currents from voltage clamped Fhf2WT/Y and Fhf2KO/Y cardiomyocytes. Voltage step commands elicited similar transient sodium current densities in Fhf2WT/Y and Fhf2KO/Y cells at 25°C, although peak currents in Fhf2KO/Y cells fell by 37% when recorded at 30°C (Fig. 3b and Supplementary Table 4). Loss of Fhf2 altered sodium channel inactivation in several ways. Channels in Fhf2KO/Y cells underwent steady-state inactivation at more hyperpolarized potential than did channels in Fhf2WT/Y cells at 25°C (Fig. 3c and Supplementary Table 4). Fhf2 deficiency also accelerated the rate of sodium current decay when recorded at either 25 or 30°C, with mutation and elevated temperature combining to produce the fastest rate of open-state inactivation (Fig. 3d and Supplementary Table 4). Sodium channel closed-state inactivation rates were assayed by comparing peak sodium conductance generated by voltage ramps to conductance generated by instantaneous voltage step. While longer ramp durations reduced peak sodium conductance under all conditions due to closed-state channel inactivation, higher temperature and Fhf2 mutation each enhanced the effect of ramp duration on channel availability, with Fhf2 mutation and elevated temperature combining to yield the most severe reduction in availability (Fig. 3e, Supplementary Fig. 2 and Supplementary Table 4).

Sodium channel inactivation kinetics were further studied in human embryonic kidney (HEK) cells transfected to express cardiac sodium channel Nav1.5 with or without FHF2VY, the principal protein isoform of FHF2 expressed in mouse heart13. Patched HEK cells more easily survived temperature ramping than did cardiomyocytes allowing assessment of Nav1.5 inactivation parameters at 25, 35 and 40°C often within the same cell. Steady-state voltage dependent Nav1.5 inactivation at 25°C occurred with V1/2 = −82.2 ± 1.6 mV (n = 7) in the presence of FHF2VY and V1/2 = −93.5 ± 0.9 mV (n = 9) in the absence of FHF2 (P < 0.0002, Student's t-test) (Supplementary Table 5). The absence of FHF2VY also increased the rate of Nav1.5 closed-state and open-state inactivation at all temperatures (Fig. 3f, Supplementary Fig. 3 and Supplementary Table 5). Most notably, even a 45 mV ms⁻¹ voltage ramp at 40°C caused a greater than 50% reduction in Nav1.5 peak conductance in the absence of FHF2VY, while more than 80% of channels remained available under these conditions in the presence of FHF2VY (Fig. 3f and Supplementary Fig. 3).

**Computational models of Fhf2WT and Fhf2KO cardiomyocytes.** To establish whether hyperthermic excitation failure is attributable to altered cardiac sodium channel inactivation gating, we modified a previously generated mouse ventricular cardiomyocyte computational model17 to reflect the differential Nav gating properties in the presence and absence of FHF2. The Fhf2WT and Fhf2KO myocyte models differed only in the open- and closed-state inactivation rate constants of the embedded Nav 12-state Markov model. Both models generated the same peak Nav conductance in response to step depolarization and the same voltage dependence of Nav activation (Supplementary Fig. 4). But in comparison to the Fhf2WT model, Nav conductance in the Fhf2KO model displayed a hyperpolarizing shift in the voltage dependence of steady state inactivation (Supplementary
Fig. 4), exhibited faster closed-state inactivation in voltage ramp simulations (Fig. 4a), and faster open-state inactivation (Fig. 4b). Analogous to recorded channels, inactivation rates were temperature-dependent, such that \( Fhf2 \) mutation and temperature elevation combined generated the fastest \( Na^+ \) inactivation rates (Fig. 4a,b, Supplementary Fig. 4). Current injection simulations (Fig. 4c) demonstrated the consequences of altered \( Na^+ \) inactivation gating. The \( Fhf2^{WT} \) model fired action potentials at both 25 and 40 °C, although AP waveforms were attenuated at higher temperature, while the \( Fhf2^{KO} \) model exhibited complete excitation failure at elevated temperature.

**Discussion**

Preservation of cardiac sodium current density is critical for survival. Here we demonstrate that \( FHF2 \) acts to dampen the temperature-dependent acceleration of \( Na^+ \)1.5 open and closed-state inactivation and thereby maintain cardiac excitability and conduction...
throughout a range of physiologically relevant body temperatures. The importance of FHF2 becomes most apparent during hyperthermia when the intrinsic inactivation kinetics of Na\textsubscript{v}1.5, if left unchecked, can be brisk enough to severely impair sodium current density, leading to excitation block and conduction failure. Although the ECG parameters of Fhf2\textsuperscript{KO} mice were normal at 37 °C, the degree of conduction slowing measured by optical mapping was substantial. This difference may reflect that in vivo ECGs, unlike the optical maps, were measured during sinus rhythm, where near simultaneous multi-site activation of the ventricular myocardium by the His-Purkinje system can mask myocardial conduction slowing. Purkinje myocytes express additional FHF isoforms\textsuperscript{18}, which may confer some degree of protection against conduction slowing within the specialized conduction system in Fhf2\textsuperscript{KO} mice. The full extent of conduction slowing observed in the isolated heart preparations may also reflect the increased dependence of sodium current density on the slope of initial membrane depolarization in Fhf2\textsuperscript{KO} myocytes. In the intact heart, action potentials are triggered following non-instantaneous depolarization mediated by gap junctional currents passing from upstream cells\textsuperscript{19}. Therefore, sodium current density in Fhf2\textsuperscript{KO} hearts should be exquisitely sensitive to conditions that increase intercellular resistivity between cardiomyocytes, such as the increase in intristitial volume that may be seen with Langendorff perfusion\textsuperscript{20}. Indeed, the observation that FHF2 may modulate the dependency of sodium current density on junctional conductance has important implications for arrhythmia mechanisms associated with conditions that produce intercellular uncoupling, such as acute ischaemia\textsuperscript{21} or pathologic gap junction remodelling\textsuperscript{22}.

The dynamic nature of the ECG abnormalities in FHF2KO hearts is highly reminiscent of Brugada syndrome, where ECG parameters change from normal to coved-type ST elevations in the presence of fever or sodium channel blocking drugs. While the electrophysiological basis of the Brugada pattern ECG is controversial, some have reported focal conduction slowing in the right ventricular outflow tract as a potential mechanism\textsuperscript{14-16}. Although optical mapping of Fhf2\textsuperscript{KO} hearts demonstrated global conduction slowing, additional analysis may be revealing. Furthermore, exploring the interplay between FHF2-dependent alterations in sodium current physiology and other ionic currents that have been implicated in the Brugada pattern ECG will certainly be of significant interest. It should also be noted that in contrast to our results, Puranam \textit{et al.}\textsuperscript{23} recently reported embryonic lethality in their line of Fhf2\textsuperscript{KO} mice. While the basis for this discrepancy is unclear, one possibility is that this other reported mutant allele was maintained on a C57Bl/6 background, with background strain influencing phenotype. Whether this lineage of Fhf2\textsuperscript{KO} mice survives to adulthood in other background strains should be evaluated.

In summary, our results identify the critical role of FHF2 in maintaining adequate sodium current reserve in response to hyperthermic stress. These results have direct implications for fever-induced Brugada syndrome, where loss of function sodium channel mutations may conspire with FHF-dependent mechanisms, such as allelic expression levels, to produce temperature-sensitive effects. It will be of interest to extend our studies in Fhf2 mutant mice to additional fever models, such as challenge with lipopolysaccharide\textsuperscript{24} or cytokines\textsuperscript{25,26}. It is also worth noting that the co-expression of FHFs and Na\textsubscript{v}1.5 in the heart and brain may point to a unifying theory for both febrile arrhythmias and seizures\textsuperscript{23}.

\textbf{Methods}

\textbf{Fhf2\textsuperscript{KO} mouse derivation.} All protocols conformed to the Association for the Assessment and Accreditation of Laboratory Animal Care and the NYU School of Medicine Animal Care and Use Committee. A murine embryonic stem cell line bearing a ‘knockout first with conditional potential’ cassette integrated into the Fhf2 locus located on chromosome Xq28 (International Mouse Phenotype Consortium, clone EPD03394 F09) was injected into blastocysts to derive chimeric mice, which were outbred to establish viable mice bearing the targeted allele. Pronuclear injection of Cre recombinase-expressing plasmid into fertilized eggs of this lineage yielded progeny bearing Cre-mediated excision of the Fhf2\textsuperscript{KO} allele. Therefore, no additional excision of Cre-excised allele specifies bicistronic transcripts encoding truncated FHFs and Na\textsubscript{v}1.5. A 3-primer polymerase chain reaction reaction with Phyre DNA polymerase allows simultaneous detection of wild-type (WT) and knockout (KO) alleles (Fig. 1a). Mice carrying the KO allele were backcrossed three generations to the 129svPas strain before use in all cardiac physiology experiments.  

\textbf{Antibody reagents.} Immunofluorescence antibodies \textit{[target, dilution, \textit{species, company}]}: Primary antibodies: FHF2 (ref. 27) 1:500 (Epitope GGGMSHHNEST, Rabbit), Na\textsubscript{v}1.5 1:500 (mouse, Alomone, ASC-005), Sarcomeric Actinin 1:100 (mouse, Millipore: mab374). Secondary antibodies: Goat anti-Rabbit 1:15,000 (mouse, BD Biosciences, 610921). Mounting medium with DAPI (Vectashield, H-1200).

\textbf{Western blot antibodies.} Primary antibodies: FHF2 1:1000 (rabbit, Sigma, HPA002809); Na\textsubscript{v}1.5 1:5000 (rabbit, Alomone Labs. ASC-005); GAPDH 1:750 (mouse, Millipore: ab3774). Secondary antibodies: Goat anti-Rabbit 1:15,000 (Li-Cor 926-32211); Goat anti-Mouse 1:15,000 (Li-Cor 926-32220).

\textbf{Immunoblotting.} Brain and exsanguinated heart tissues were homogenized in cold detergent-free buffer, after which membranes were solubilized by adding 1% Triton X-100. Clarified lysates were run on 4–20% precast polyacrylamide gradient gels.
Figure 3 | Temperature-dependent excitation failure and altered sodium channel gating in the absence of FHF2. (a) Ventricular cardiomyocyte excitability. Superimposed voltage traces of representative patched Fhf2WT/Y and Fhf2KO/Y cardiomyocytes injected with depolarizing current (0–480 pA in 40 pA steps) at 25°C and in the same cells after raising temperature to 40°C. All tested Fhf2WT/Y cells were inexcitable at 40°C. (b) Cardiomyocyte NaV peak conductance (gNaV-peak). gNaV-peak following −120 mV to −35 mV depolarization step was measured for Fhf2WT/Y and Fhf2KO/Y cells at 25°C and 30°C. (c) Cardiomyocyte NaV steady-state inactivation. Available gNaV at 25°C after 60 ms conditioning at −140 to −40 mV is expressed as fraction maximal gNaV. Vertical dashed lines indicate V1/2 inactivation in Fhf2WT/Y and Fhf2KO/Y cells. (d) Cardiomyocyte NaV open-state inactivation rate. Superimposed representative traces (left) from Fhf2WT/Y (black line) and Fhf2KO/Y (grey line) cardiomyocytes at 25°C (top) and 30°C (bottom) following depolarization to −35 mV. Fast exponential time constant (τ) for decay of sodium current in response to voltage step to −35 mV are plotted for Fhf2WT/Y and Fhf2KO/Y cells at 25 and 30°C (right). (e) Voltage ramp NaV inactivation in cardiomyocytes. gNaV-peak in response to voltage ramps to −50 mV at different rates was expressed as percentage of gNaV-peak elicited by step depolarization (∞ ramp rate). Slower ramp rates decrease gNaV-peak through closed-state inactivation, which is sensitized by both elevated temperature and Fhf2 mutation (histogram). See Supplementary Fig. 2 for representative conductance traces. (f) Voltage ramp NaV,1.5 inactivation with and without FHF2VY. HEK cells transfected with NaV,1.5 ± FHF2VY subjected to voltage ramps to −30 mV at different rates at 25, 35 and 40°C. NaV,1.5 closed-state inactivation is increased by slowed voltage ramp, temperature elevation and the absence of FHF2VY. See Supplementary Fig. 3 for representative conductance traces. (g) NaV,1.5 open-state inactivation. Fast τ for NaV,1.5 current decay was measured in cells ± FHF2VY at 25, 35 and 40°C. Elevated temperature and absence of FHF2VY each accelerate NaV,1.5 open-state inactivation. Data represent mean ± s.e.m. *significant P values (all P values in Supplementary Tables 4 and 5); ns, not significant; Student’s t-test.
Cardiomyocyte enzymatic dissociation experiments. Cardiomyocytes were dissociated from adult hearts that were Langendorff perfused and enzymatically digested according to AFCS Procedure Protocols P990000125. Mice were heparinized (500 U kg\(^{-1}\)) and killed with 100% carbon dioxide. Hearts were surgically removed and transferred to nitrocellulose (Bio-Rad) overnight at 4 °C. Nitrocellulose membranes were incubated in blocking buffer consisting of KCl, 0.6 potassium phosphate monobasic (KH\(_2\)PO\(_4\)), 0.6 sodium phosphate dibasic (Na\(_2\)HPO\(_4\)), 1.2 magnesium sulphate heptahydrate (MgSO\(_4\).7H\(_2\)O), 12 sodium bicarbonate (NaHCO\(_3\)), 10 potassium bicarbonate (KHCO\(_3\)), 10 HEPES (composition (mmol l\(^{-1}\)): 113 sodium chloride (NaCl), 4.7 potassium chloride (KCl), 0.6 potassium phosphate monobasic (KH\(_2\)PO\(_4\)), 0.6 sodium phosphate dibasic (Na\(_2\)HPO\(_4\)), 1.2 magnesium sulphate heptahydrate (MgSO\(_4\).7H\(_2\)O), 12 sodium bicarbonate (NaHCO\(_3\)), 10 potassium bicarbonate (KHC\(_2\)).10 HEPES buffer solution, 30 mM tauroine, 5.5 mM glucose and 10 2.3-Butanediol cinnamoxime (BDI).

Cardiomyocytes were re-suspended in 10 ml of stop buffer 2 (composition: 1 x perfusion buffer, 10 mg Liberase TM and 12.5 μM calcium chloride (CaCl\(_2\)) added to increasing concentration of Ca\(_{2+}\) in 5 min intervals (5 μl 10 mM Ca\(_{2+}\) (62 μM), 50 μl 10 mM Ca\(_{2+}\). (112 μM), 100 μl 10 mM Ca\(_{2+}\) (212 μM), 30 μl 100 mM Ca\(_{2+}\) (500 μM) and 50 μl 100 mM Ca\(_{2+}\) (1 mM)). Viability of myocytes was reassessed using the above quality control guidelines. Only cell preparations that passed these criteria were used for experimentation.

Immunocytochemistry. Dissociated ventricular myocytes were fixed in 4%PFA for 5 min. Cells in suspension were spun onto slides using Shandon Cytospin II (Speed 250 r.p.m., 2 min). Cells were blocked with 10% serum and 0.01% Triton in PBS. Immunocytochemistry was performed in 5 min intervals (112 μM). Viability of myocytes was reassessed using the above quality control guidelines. Only cell preparations that passed these criteria were used for experimentation.

Mouse in vivo electrophysiology and flecainide drug challenge. ECGs were obtained using subcutaneous electrodes attached at the four limbs (MP100, BIOPAC Systems). 8–12 week-old, male mice were anaesthetised with inhaled (2% v/v) isoflurane. Heart rate and core body temperature (rectal temperature probe) were continuously recorded. Flecainide (15 mg kg\(^{-1}\) or 30 mg kg\(^{-1}\) was administered intravenously and body temperature, heart rate and RR interval were monitored every 10 min.
administered via intraperitoneal injection. ECG analysis was performed in an unbiased fashion where 100 beats at each temperature endpoint were analysed using LabChart 7 Pro version 7.2.1 (AD Instruments, Inc.). Intracardiac and body surface ECGs were recorded with a 12-lead ECG machine (INRAD, Model 8200) using a 6-channel recorder. Intracardiac recording intervals were measured from the His bundle catheter when the atrial, His, and ventricular electrograms were stable over >25 beats. Measurement of the AH interval was taken from the His bundle recording from the onset of deflection from baseline of the local atrial electrogram to the onset of deflection of the His bundle electrogram. Measurement of the HV interval was taken from the onset of deflection from baseline of the His bundle electrogram to the onset of deflection from baseline of the earliest ventricular electrogram, whether on surface ECG or intracardiac recordings. Core body temperature was initially maintained at 37 °C and gradually elevated up to 43 °C at a rate of 1 °C min⁻¹ using an external heat lamp.

**Transtracheal echocardiography.** Echocardiography was performed using the Vevo 2100 high-resolution ultrasound imaging system with a real-time 30 MHz linear array scanhead (MS400) at a frame rate of 235 fps, a focal length of 8 mm, and a 10 x 10 mm field of view (Visualsonics; Toronto, Canada). 8–12 week-old, male mice were anesthetised with 2% isoflurane, and hair was removed from the chest using a depilatory cream (Nair; Church & Dwight Co, Inc; Princeton, NJ). Warmed ultrasound transmission gel was placed on the chest and used to obtain left ventricular endpoints of cardiac function. B-mode cardiac imaging was conducted on transverse (short axis) plane. The papillary muscles were used for the short axis imaging landmark. M-mode recordings of the left ventricle were also recorded at the short axis B-mode imaging plane to obtain left ventricular function and dimension changes through the cardiac cycle. Heart rate was monitored and core body temperature was maintained at 37.5 °C using a heated platform and a hair dryer throughout the procedure. Data analysis was performed on VisualSonics Vevo 2100 V1.5.0 software (Visualsonics; Toronto, Canada). The following parameters were measured using three cardiac cycles from short axis M-mode images: diastolic and systolic left ventricular internal diameter, anterior wall thickness, and posterior wall thickness. From these measurements, left ventricular ejection fraction, percent fractional shortening, stroke volume, and cardiac output were calculated within the Vevo software.

**Heart isolation and Langendorff perfusion.** Male mice of 8–12 week-old were heparinized (500 U kg⁻¹) and killed with 100% carbon dioxide. Hearts were surgically removed via a thoracotomy. While fully immersed in oxygenated (95% O₂, 5% CO₂) Tyrode’s (composition (mmol L⁻¹): NaCl 114, NaHCO₃ 25, dextrose 10, KCl 4.6, CaCl₂ 1.5, NaPO₄ 1.2, MgCl₂ 0.7), the aorta was cannulated and Langendorff perfused at a constant pressure of 70 mm Hg. Perfusate temperature was initially maintained at 37 °C and increased by increments of 1 °C to a maximum temperature of 41 °C (at 5 °C intervals or 43 °C (voltage-clamped ECG). Perfusion temperature was allowed to reach steady state between temperature ramps.

**Optical mapping.** High-resolution optical mapping experiments were performed as follows: excited hearts from 8 to 12 week-old, male mice were initially perfused with Tyrode’s solution to clear blood and stabilize the heart, followed by Tyrode’s solution containing 10 μM blebbistatin. Hearts were allowed to recover for 20 min and then exposed to voltage-sensitive dye Di-4-ANEPPS (Molecular Probes Inc., Eugene, OR, USA). Light from green LEDs (530 nm; ThorLabs) was used as an excitation source and the emitted light (620 nm long pass) was detected with 1 pm detection (voltage-clamped ECG). Perfusion temperature was allowed to reach steady state between temperature ramps.

**Action potential recordings from adult cardiomyocytes.** Excitability of acutely dissociated ventricular cardiomyocytes from FHF2WT/VY and FHF2KO/VY mice were conducted using a MultiClamp700 Amplifier, Digidata 1.40 analogue/digital converter and Clampex10 software (Molecular Devices). Cells were placed in the recording chamber under a Nikon Eclipse microscope and perfused with carbogen-bubbled bath solution (115 mM NaCl, 26 mM NaHCO₃, 3 mM KCl, 1.2 mM KH₂PO₄, 3 mM glucose, 2 mM myo-inositol, 2 mM Na pyruvate, 7 mM NaOH-buffered HEPES pH 7.2 at 25 °C and green fluorescent cells were whole-cell patched with pipettes filled with 104 mM CsF, 50 mM tetrathyamine chloride, 10 mM HEPES pH 7.2, 5 mM glucose, 2 mM MgCl₂, 10 mM EGTA, 2 mM ATP, 0.2 mM GTP and having a 1–2 MΩ resistance. For all recording protocols, sodium current was isolated during data acquisition by P/N activation of leak and capacitive currents (N = 6). To ensure voltage clamping during sodium channel activation was adequate, cells were subjected to a 19-sweep series of voltage steps from a hold of −120 mV to −80 mV with 10 mV increments. As a measure of adequate clamp, transient current peaks for all voltage command were nested within the larger current trace of a following or preceding voltage step command.

**Steady-state channel inactivation protocol.** To determine the voltage dependence of steady state channel inactivation, a 19-sweep protocol used −120 mV holding command, a 60 s variable test voltage step (−120 + 5 mV) step) was run at −25 mV reporting pulse.

**Voltage ramp protocol.** As a measure of closed state channel inactivation rate, a 10-sweep protocol used −120 mV hold command followed by depolarization to −10 mV (either instantaneous voltage step) after a ramp ranging in time from 2 ms (≥ 45 mV ms⁻¹) to 18 ms (≥ 5 mV ms⁻¹).

**Temperature elevation.** After recording at 25 °C, temperature was ramped to 35 °C and later to 40 °C. At elevated temperatures, the cell was first tested as above to ensure maintenance of tight clamp, after which the voltage ramp protocol was conducted. For many cells, voltage ramp protocols could be successfully run at all three temperatures (examples in Supplementary Fig. 3).

**Sodium current recordings in adult cardiomyocytes.** All iSOD recordings in isolated cardiomyocytes were conducted in whole-cell configuration at 25 or 30 °C. Recording pipettes were filled with a solution containing (in mM): NaCl 5, CsCl 135, EGTA 10, MgATP 5, HEPES 15, pH 7.2 with CsOH. Cells were maintained in a solution containing (in mM): NaCl 5, CsCl 112.5, TEACl 20, CdCl₂ 0.1, MgCl₂ 1, CaCl₂ 1, HEPES 20, Glucose 11, pH 7.4 with CsOH. To determine the peak current voltage relation, 200 ms voltage pulses were applied to VT to −90 mV and then 30 mV voltage steps, from a holding potential of VT = −120 mV. Interval between voltage steps was 3 s. Steady state inactivation was determined by stepping VT to conditioning voltages of between −130 mV and −20 mV for 60 ms, followed by a 30 ms test pulse to VT = −20 mV to elicit iSOD. The steady state voltage-dependent inactivation curves were fitted to Boltzmann’s functions. As a measure of closed state channel inactivation rate, a 10-sweep protocol used −140 mV hold command followed by depolarization to −50 mV either instantaneously (voltage step) or as a ramp ranging in time from 2 ms (≥ 45 mV ms⁻¹) to 18 ms (≥ 5 mV ms⁻¹). All recordings were conducted utilizing an Axon multiclamp 700B Amplifier coupled to a pClamp system (versions 10.2, Axon Instruments, Foster City, CA).

**Cardiomyocyte excitation parameters.** Spike threshold was defined as the point of accelerated upward voltage deflection at the base of a spike, mathematically defined as the voltage when d²V/dt² > 0 as determined using Clampfit software (Molecular Devices).

\[ V_{\text{threshold}} = \frac{1}{2} \left( V_{\text{spike}} - V_{\text{rest}} \right) \]

**Values for FHF2WT/VY versus FHF2KO/VY** were assessed for significant difference by two-tailed Student’s t test. The number of FHF2WT/VY versus FHF2KO/VY cells that contributed to each data point ranged from 3 to 8, with 40°C was used to assess significant difference by two-tailed Fisher Exact Probability test using an online calculator (http://vassarstats.net/odds2x2.html).
Cardiomyocyte passive properties. Capacitance of each cell was measured as
\[ \text{Cap} = \frac{\Delta V}{\Delta q} \] (2)

where \( \Delta V \) is the current transient time integral to a negative voltage step command \( \Delta V \). Leak conductance was measured as
\[ g_{\text{leak}} = \frac{I_{\text{leak}}}{\Delta V_m} \] (3)

where \( \Delta V_m \) is the steady-state change in membrane voltage in response to a negative current clamp command \( I_{\text{leak}} \).

Cardiomyocyte sodium conductance density.
\[ g_{\text{Na,peak}} = \frac{\text{g}_{\text{Na,peak}}}{(V_m - \text{E}_{\text{Na}})} \] (4)

where \( \text{E}_{\text{Na}} \) is the reversal potential and \( \text{g}_{\text{Na,peak}} \) is the peak inward current in response to a voltage command \( V_m \) within the linear ohmic range of the current/voltage relationship.

Voltage dependence of steady state sodium channel inactivation. Peak sodium current accompanying the −25 mV reporting pulse was measured at each test voltage, and the fraction of channels available (not inactivated) at test voltage equalled \( I_{\text{Na,peak}}(V_m)/I_{\text{Na,peak}}(\text{Maximum}) \). To obtain \( V_{1/2} \) and \( k \) values for voltage dependence of steady state inactivation, current data were fitted to Boltzmann equation:
\[ f(V_{1/2}) = \frac{1}{1 + e^{(V_m-V_{1/2})/k}} + C \] (5)

Open-state sodium channel inactivation kinetics. Transient sodium current decay from 90% current peak to baseline was fitted to a two-term exponential decay function
\[ I(t) = I_1 \times e^{-V_{1/2}/k} + I_2 \times e^{-V_{1/2}/k} + C \] (6)

where \( I_1 \) and \( I_2 \) are the fast and slow decaying components of the transient current. The fast component \( I_1 \) associated with decay constant \( \tau_{\text{fast}} \) constituted between 70 and 100% of the total current in all analysed cells.

Closed-state sodium channel inactivation kinetics. Data from voltage ramp protocols provided an estimate of closed state inactivation rate as a function of voltage ramp rate. For each ramp command at each time point, we calculated
\[ g_{\text{Na}} = \frac{I_{\text{Na}}}{(V_m - \text{E}_{\text{Na}})} \] (7)
yielding sodium conductance traces as shown in Supplementary Fig. 3. Similarly,
\[ g_{\text{Na,peak}} = \frac{I_{\text{Na,peak}}}{(V_m(\text{at peak}) - \text{E}_{\text{Na}})} \] (8)

from which was calculated
\[ \text{percent maximal } g_{\text{Na,peak}}(\text{ramp}) = 100 \times \frac{g_{\text{Na,peak}}(\text{ramp})}{g_{\text{Na,peak}}(\text{step})} \] (9)

Computational modelling. The FlhQWT and FlhQKO cardiomyocyte models were generated by introducing modifications to the murine ventricular cardiomyocyte model from Bondarenko et al.\(^{35}\). These changes included (1) adjusting membrane capacitance to 70 pF to more closely match our recorded measurements, (2) recalibrating the leak conductances by dividing the inward rectifying postassium conductance two-fold and replacing with an equal density of potassium conductance to achieve nonspinking responses to current injections similar to cardiomyocyte recordings (Fig. 4c), (3) changing the densities of ultrarapid-activating delayed rectifier and rapid-activating delayed rectifier potassium conductances to 0.112 mS m\(^{-2}\) and 0.234 mS m\(^{-2}\), respectively, in order to obtain more realistic post-spike repolarization and (4) replacing the fast sodium conductance with a 12-state Markov model sodium conductance:

\[
\begin{align*}
\text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \gamma \\
\text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \gamma \\
\text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \gamma \\
\text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \gamma \\
\text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \gamma \\
\text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \gamma \\
\text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \gamma \\
\text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \gamma \\
\text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \gamma \\
\end{align*}
\]

This \( \text{Nav} \) model is an adaptation of the 13-state model of Raman and Bean\(^{31}\), with the blocked open state and consequent resurgent current omitted. All rate constants were scaled by a thermodynamic factor
\[ Q_1 = 3!^{(-\text{degC} - 32.76)/10} \] (10)
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**Acknowledgements**

Supported by National Institutes of Health grants (R01GM098540 to M.G., R01HL105983, R01HL82727 and NCRR S10 RR026881 to G.I.F. and T32 GM066704 and F31 HL132438 to A.S.). We thank Mario Delmar for helpful discussions as well as Fang-Yu Liu and Jie Zhang for their technical assistance.

**Author contributions**

D.S.P., A.S., C.V. and G.M. designed and performed all in vivo and ex vivo cardiac recordings. C.M., X.L. and M.G. designed and performed all single cell recordings, K.K. performed ES cell and DNA injections and embryo reimplantation to establish the *Fhf2* KO mouse. D.S.P., A.S., C.M. and G.M. designed and performed all molecular/cellular biology experiments and genotyping. D.S.P., M.G. and G.I.F. directed the research and co-wrote the paper, with the assistance of A.S.

**Additional information**

**Supplementary Information** accompanies this paper at http://www.nature.com/naturecommunications

**Competing financial interests:** The authors declare no competing financial interests.

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**How to cite this article:** Park, D. S.* et al.* Fhl2 gene deletion causes temperature-sensitive cardiac conduction failure. *Nat. Commun.* 7, 12966 doi: 10.1038/ncomms12966 (2016).

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