Fibroblast growth factor homologous factors serve as a molecular rheostat in tuning arrhythmogenic cardiac late sodium current

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Voltage-gated sodium channels (Na\textsubscript{v} channels) support the genesis and brisk spatial propagation of action potentials in the heart. Disruption of Na\textsubscript{v,1.5} inactivation results in a small persistent sodium influx known as late sodium current (I\textsubscript{Na,L}), which has emerged as a common pathogenic mechanism in both congenital and acquired cardiac arrhythmogenic syndromes. In the present study, using low-noise multichannel recordings in heterologous systems, LQTS3 patient-derived pluripotent stem cell cardiomyocytes and mouse ventricular myocytes, we demonstrate that the intracellular fibroblast growth factor homologous factors (FHF1–4) tune cardiac I\textsubscript{Na,L} in an isoform-specific manner. This scheme suggests a complex orchestration of I\textsubscript{Na,L} in cardiomyocytes that may contribute to variable disease expressivity of Na\textsubscript{v,1.5} channelopathies. We further leverage these observations to engineer a peptide inhibitor of I\textsubscript{Na,L} with a higher efficacy compared with a well-established small-molecule inhibitor. Overall, these findings lend insights into molecular mechanisms underlying FHF regulation of I\textsubscript{Na,L} in pathophysiology and outline potential therapeutic avenues.
isofoms/variants inhibit \( I_{\text{Na,L}} \) (‘protective’ variants), whereas other variants have either no effect or may exacerbate \( I_{\text{Na,L}} \) (‘nonprotective’ variants). We hypothesize that the balance between endogenously expressed ‘protective’ and ‘nonprotective’ FHF variants in cardiomyocytes may ultimately dictate the arrhythmogenic phenotype. Armed with these insights, we engineered a minimal domain derived from protective FHFs as a selective and potent inhibitor of pathogenic \( I_{\text{Na,L}} \). Overall, these findings lend key insights into the molecular mechanism for FHF regulation of \( I_{\text{Na,L}} \) and potential pathophysiological changes that ultimately lead to cardiac instability.

**Results**

**Endogenous FHF tunes \( I_{\text{Na,L}} \) of mutant Na\(_{A},1.5\) in aMVMs.** As FHF2 is natively expressed in the murine heart\(^{33,34}\), we examined whether manipulation of ambient FHF levels in aMVMs tunes \( I_{\text{Na,L}} \). To do so, we utilized our recently published transgenic mouse models\(^{45}\) expressing (1) pseudo-wild-type Na\(_{A},1.5\) (pWT) and (2) isoleucine-glutamine/alanine-alanine (IQ/AA) mutant channels that abolish the interaction of CaM, a de facto channel subunit involved in the modulation of \( I_{\text{Na,L}} \). When recombiantly expressed, IQ/AA mutant channels exhibit elevated \( I_{\text{Na,L}} \) (refs. \(^{33,34}\)). However, our previous studies have shown that this mutation has surprisingly little effect on \( I_{\text{Na,L}} \) in murine myocytes\(^6\). Whether this discrepancy stems from \( I_{\text{Na,L}} \) modulation by endogenous FHFs in cardiomyocytes is not fully established. Accordingly, we undertook cell-attached multichannel recordings to measure changes in the magnitude of \( I_{\text{Na,L}} \) on downregulating FHF2 levels using short hairpin (sh)RNA\(^{34}\). Cultured myocytes from both pWT and IQ/AA mice (Fig. 1a) exhibited minimal \( I_{\text{Na,L}} \) at baseline, similar to freshly dissociated myocytes in our previous studies\(^{33,34}\) (Fig. 1b–c,h). Of note, the amplitude of nontransgenic mice was also insensitive to FHF2 manipulation (Fig. 1h and Extended Data Fig. 1). These results demonstrate that FHF2 is an endogenous inhibitor of \( I_{\text{Na,L}} \) which may counteract increased late sodium current due to Na\(_{A},1.5\) mutations.

**FHF modulation of \( I_{\text{Na,L}} \) is isofom/splice-variant specific.** Intracellular FHFs are encoded by four distinct genes (FHF1/FGF12, FHF2/FGF13, FHF3/FGF11, FHF4/FGF14) which are differentially expressed in various excitable cells\(^{27,28}\). Adding further complexity, all four FHF isofoms undergo alternative splicing that generates variants with distinct amino (N) termini\(^{33}\). In the murine heart, select FHF2 splice variants are predominant, whereas in the human heart FHF1\(_A\) is the dominant isoform, although certain FHF2 splice variants are also expressed\(^{33,34}\). Given this diversity, we sought to determine whether FHF regulation of \( I_{\text{Na,L}} \) is specific to certain variants by comparing the effect of multiple FHFs on \( I_{\text{Na,L}} \) triggered by structure-guided and pathogenic Na\(_{A},1.5\) mutants. As HEK293 cells have minimal endogenous FHFs, this system serves as a convenient platform to dissect mechanisms\(^{34}\). Consistent with previous studies\(^{33,34}\), wild-type Na\(_{A},1.5\) exhibits minimal \( I_{\text{Na,L}} \) (Fig. 2a,m). FHF isofoms evoked no further change in \( I_{\text{Na,L}} \) of wild-type channels (Fig. 2b–d,m). By comparison, both the IQ/AA mutation (Fig. 2e,m), designed to mimic channelopathie mutations in the Na\(_{A},1.5\) carboxy-terminus (CT), and the \( \Delta KQ \) mutation, linked...
to LQTS3, demonstrated elevated $I_{\text{Na,L}}$ (Fig. 2l,m). Co-expression of FHF1A resulted in a strong reduction in $I_{\text{Na,L}}$ for both mutations (Fig. 2f,j,m), reminiscent of the protective nature of endogenous FHF2 in mouse cardiomyocytes. By comparison, co-expression of FHF1B yielded an approximately three-fold increase in $I_{\text{Na,L}}$ for IQ/AA and ΔKPQ mutants (Extended Data Fig. 2), and are therefore termed protective variants. By contrast, FHF2A and FHF2B yielded minimal change in $I_{\text{Na,L}}$ (Extended Data Fig. 2) and are therefore classified as nonprotective variants.

The divergence in functional effects of FHF splice variants/isoforms suggests that the net magnitude of $I_{\text{Na,L}}$ for a given mutation may be tuned by the relative cytosolic concentration of protective and nonprotective variants.
versus nonprotective FHFs (Fig. 3a). To test this possibility, we co-expressed complementary (c)DNAs of FHFIb, a nonprotective variant, along with FHF2s, a protective variant, at varying ratios and quantified changes in \(I_{\text{Na,L}}\) for the Na\(_{\text{v}}\)1.5 IQ/AA mutant. Multichannel recordings revealed an increasing Hill–Langmuir relationship for \(R_{\text{perm}}\) when plotted against the FHFIb:FHF2s cDNA ratio (\(\rho\)), with the half-maximal effect observed at \(\rho \approx 0.4\), that is, with ~2.5-fold higher expression of FHF2s compared with FHFIb (Fig. 3b). To probe the functional relevance of this scheme in cardiomyocytes, we utilized cultured aMVMs from IQ/AA mice. As these cells exhibit minimal basal \(I_{\text{Na,L}}\) owing to the presence of endogenous FHF2, we reasoned that overexpression of FHFIb may compete away endogenous FHFIb, yielding an increase in \(I_{\text{Na,L}}\). Consistent with this expectation, adenoviral transduction of FHFIb into cultured aMVMs from IQ/AA mice resulted in an approximately ten-fold increase in \(I_{\text{Na,L}}\) (Fig. 3c,d), further corroborating the hypothesis that competition between the protective and nonprotective FHF variants may play an important role in tuning channelopathic \(I_{\text{Na,L}}\) in cardiomyocytes. Having identified an empirical relationship between \(I_{\text{Na,L}}\) and relative expression of FHFIb isoforms, we next considered its impact on cardiac AP morphology. We modified the late sodium current component of the ToR-ORD in silico human ventricular AP model\(^{10}\) to be dependent on the relative concentrations of FHFIb and FHF2s. As expected, simulations with wild-type Na\(_{\text{v}}\)1.5 showed no change in AP duration (APD) as a function of \(\rho\) (Fig. 3e–g). Simulations incorporating the Na\(_{\text{v}}\)1.5 IQ/AA mutant, however, showed AP prolongation (at low heart rates) and AP alternans (at high heart rates) with increasing \(\rho\), consistent with increased \(I_{\text{Na,L}}\) and arrhythmogenicity (Fig. 3e–g). In all, these results suggest that the relative expression of protective versus nonprotective FHF variants may constitute a continuously tunable rheostat for \(I_{\text{Na,L}}\) and APD in cardiomyocytes.

**Fig. 3 | Competition between protective and nonprotective FHFs continuously tunes \(I_{\text{Na,L}}\)**. a, Schematic illustrating a competitive scheme of \(I_{\text{Na,L}}\) regulation by protective versus nonprotective FHF variants. b, Transfection of varying DNA concentrations of FHFIb versus FHF2s, yielding an increasing relationship between \(I_{\text{Na,L}}\) and the ratio of cDNA transfected (\(\rho\)). Inset: exemplar traces. Data are presented as mean ± s.e.m. Sample size for each dot: \(\rho = 1\), \(n = 8\) cells (297 sweeps); \(\rho = 0.5\), \(n = 5\) cells (385 sweeps); \(\rho = 0.33\), \(n = 5\) cells (229 sweeps); \(\rho = 0.2\), \(n = 12\) cells (654 sweeps); \(\rho = 0.1\), \(n = 9\) cells (537 sweeps). c, Exemplar trace showing increased \(I_{\text{Na,L}}\) in cultured aMVMs from IQ/AA mice on adenoviral transduction of FHFIb. d, Population data showing increased \(I_{\text{Na,L}}\) in IQ/AA mice on overexpression of FHFIb (\(n = 8\) cells (422 sweeps) from 3 mice). Data are presented as mean ± s.e.m. Control data reproduced from Fig. 1h. Statistical analysis: \(P = 0.012\) by two-tailed Mann–Whitney U-test. e, ToR-Ord in silico model showing AP prolongation and emergence of AP alternans for the IQ/AA mutant depending on the relative expression of protective versus nonprotective FHF isoforms. bpm, beats min\(^{-1}\). f, g, The APD of IQ/AA mutant increases as a function of \(\rho\) at both 45 beats min\(^{-1}\) (f) and 80 beats min\(^{-1}\) (g) pacing. No change in APD is observed with the wild-type.
canonical FH-interacting interface. Of note, the Δ1810 truncation ablates the FH-binding interface in the Na\textsubscript{v}1.5 CT. In both cases, we observed a baseline increase in \(I_{\text{Na,L}}\) (Fig. 4b and Extended Data Fig. 3f). Surprisingly, FHF\textsubscript{1A} strongly diminished \(I_{\text{Na,L}}\) for both mutants (Fig. 4b and Extended Data Fig. 3f), suggesting that the FH interaction with the Na\textsubscript{v}1.5 CT is not necessary for functional \(I_{\text{Na,L}}\) regulation, although this interaction may enhance the overall FH-binding affinity. Beyond channelopathic mutations, \(I_{\text{Na,L}}\) is also upregulated by phosphorylation of Na\textsubscript{v}1.5, a critical factor for arrhythmogenesis in heart failure and myocardial ischemia\(^1,3\). As such, we sought to determine whether protective FH variants may inhibit phosphorylation-dependent \(I_{\text{Na,L}}\). To maximally upregulate \(I_{\text{Na,L}}\), we co-expressed wild-type Na\textsubscript{v}1.5 with either (1) the catalytic C\textsubscript{4} subunit of protein kinase A (PKA\textsubscript{cat}) or (2) a constitutively
active Ca\textsuperscript{2+}/CaM-dependent kinase II (CaMKII\textsubscript{25kD}) mutant in HEK293 cells. This maneuver triggers runaway phosphorylation of Na\textsubscript{a}1,5 and thereby furnishes a baseline to discern any potential FHF-dependent change. As expected\textsuperscript{25,38}, we observed an approximately five-fold increase in \(I_{\text{Na,L}}\) in the presence of the PKA C\textsubscript{b} subunit (Fig. 4d,e) and a three-fold increase with CaMKII\textsubscript{25kD} (Fig. 4e and Extended Data Fig. 4a,d). Co-expression of either FHF1\textsubscript{a} or FHF2\textsubscript{a} sufficed to largely reverse this increase (Fig. 4d,e and Extended Data Fig. 4b,e,f). By comparison, FHF1\textsubscript{a} failed to alter \(I_{\text{Na,L}}\) (Fig. 4e and Extended Data Fig. 4c). Encouraged by these findings, we dissected the functional role of this modulation in native cardiomyocytes (Fig. 4f,g). At baseline, application of 5 \(\mu\text{M}\) forskolin did not appreciably change \(I_{\text{Na,L}}\) in cultured AMVMs from nontransgenic mice (Fig. 4f,g). However, after shRNA-mediated silencing of FHF2, we found a striking 20-fold increase in \(I_{\text{Na,L}}\) (Fig. 4f,g) suggesting that endogenous FHF2 plays a vital role in tuning phosphorylation-dependent \(I_{\text{Na,L}}\). Furthermore, these findings establish the generality, potency and physiological relevance of FHF regulation of \(I_{\text{Na,L}}\) triggered by various mechanisms.

As such, we sought to examine biophysical mechanisms underlying \(I_{\text{Na,L}}\) inhibition by protective FHF variants/isofoms. In particular, Na\textsubscript{a} inactivation is driven by an allosteric obstruction of the channel pore by the isoleucine–phenylalanine–methionine (IFM) motif in the channel III–IV linker, whereas \(I_{\text{Na,L}}\) results from a disruption of this process\textsuperscript{36}. Protective FHFs may inhibit \(I_{\text{Na,L}}\) via three broad schemes: FHFs may (1) enhance translocation of the IFM motif to its receptor site, (2) mimic the IFM motif to interact with the receptor site or (3) act elsewhere on the channel (Fig. 4h). To distinguish between these possible mechanisms, we disabled fast inactivation of Na\textsubscript{a}1,5 by either substituting the central phenylalanine with glutamine (IFM/IQM) or by a triple mutation in domain I S6 (LILA/WICW) that allosterically occludes the IFM–receptor interface. Single-channel recordings of the IFM/IQM mutant showed burst-like openings with strongly disrupted inactivation, as in our previous study\textsuperscript{18} (Fig. 4i). Co-expression of FHF1\textsubscript{a}, however, increased inactivation as evident from exemplar traces and the ensemble average relationship (Fig. 4j), thus excluding possibility (3). Nevertheless, we undertook cell-attached multichannel recordings to quantify \(I_{\text{Na,L}}\) in both HD and LQTS3 (\(\Delta\)KPQ)–iPSC–CM lines. Unlike the HD line (Fig. 5a,i), the LQTS3 (\(\Delta\)KPQ) line showed appreciable late channel openings, consistent with elevated baseline \(I_{\text{Na,L}}\) (Fig. 5a-i). As FHF2 mRNA targets both murine and human sequences, we probed changes in \(I_{\text{Na,L}}\) after transduction of FHF2\textsubscript{a} mRNA or scrambled mRNA. Both maneuvers resulted in no change in \(I_{\text{Na,L}}\) in either the HD line or LQTS3 (\(\Delta\)KPQ) lines (Fig. 5c–i). Adenoviral transduction of FHF1\textsubscript{a} revealed no change in \(I_{\text{Na,L}}\) for the HD line (Fig. 5g,i). In contrast, overexpression of the most potent protective FHF isoform, FHF1\textsubscript{a}, resulted in an inhibition of \(I_{\text{Na,L}}\) for the LQTS3 (\(\Delta\)KPQ) line (Fig. 5h,i). These findings illustrate the exquisite capability of protective FHF variants to inhibit pathogenic \(I_{\text{Na,L}}\). As increased \(I_{\text{Na,L}}\) is a commonality for many sodium channelopathies, manipulating FHF modulation of Na\textsubscript{a}1,5 may furnish an avenue to counter this pathophysiological change.

FixR: a minimal FHF domain that inhibits \(I_{\text{Na,L}}\).

The efficacy and generality of FHF1\textsubscript{a} in diminishing pathogenic \(I_{\text{Na,L}}\) suggest that this isoform may provide an ideal substrate for developing mechanism-inspired selective inhibitors of late sodium current. To do so, we sought to localize a minimal FHF segment that mediates \(I_{\text{Na,L}}\) inhibition. Sequence comparison of the protective FHF1\textsubscript{a} versus the nonprotective FHF1\textsubscript{b} suggested that the key domains responsible for functional \(I_{\text{Na,L}}\) regulation reside in the divergent N-terminal segment (Extended Data Fig. 7a). Indeed, expression of the entire FHF1\textsubscript{a} N terminus as a peptide strongly inhibited \(I_{\text{Na,L}}\) of Na\textsubscript{a}1,5 IQ/AA mutant channels, akin to full-length FHF1\textsubscript{a} (Fig. 6a-c). To further localize key effector domains, we generated multiple truncated versions of this region and found that the N-terminal 39 residues sufficed for strong inhibition of \(I_{\text{Na,L}}\) (Fig. 6b,c). Further truncations\textsuperscript{42} diminished efficacy of \(I_{\text{Na,L}}\) inhibition (Fig. 6c and Extended Data Fig. 7b-f). This suggests that the 39-amino acid segment constitutes a minimal effector domain for \(I_{\text{Na,L}}\) inhibition. As such, we termed this segment the FHF-inhibiting x region (FixR). This segment also includes the previously identified long-term inactivation particle. To further probe the importance of the long-term inactivation particle in \(I_{\text{Na,L}}\) inhibition, we undertook alanine substitution of two conserved residues Leu9 and Arg11 in FHF1\textsubscript{a} which have been previously shown to be critical in mediating long-term inactivation of neuronal Na\textsubscript{a} channels\textsuperscript{40,41}. Both FHF1\textsubscript{a} Leu9Ala and Arg11Ala mutants retained their ability to inhibit \(I_{\text{Na,L}}\), similar to wild-type FHF1\textsubscript{a} (Extended Data Fig. 8). These results hint at potential differences in molecular mechanisms underlying FHF regulation of long-term inactivation and \(I_{\text{Na,L}}\) modulation. Thus, to enter the area of efficacy of FixR in inhibiting \(I_{\text{Na,L}}\), resulting from various disease-linked Na\textsubscript{a}1,5 channelopathic mutations and channel phosphorylation. Indeed, FixR evoked an approximately tenfold inhibition of \(I_{\text{Na,L}}\) in nearly all cases (Fig. 6d–h and Extended Data Fig. 7a-k). Importantly, ranolazine is a well-established \(I_{\text{Na,L}}\) inhibitor with a higher efficacy in blocking \(I_{\text{Na,L}}\) compared with FixR\textsubscript{peak}. Indeed, we observed that incubation with

**Protective FHF inhibits \(I_{\text{Na,L}}\) in LQTS3 iPSC–CMs.** To dissect the potential pathophysiological relevance of \(I_{\text{Na,L}}\) regulation by FHFs, we studied cardiomyocytes (CMs) differentiated from either LQTS3 patient-derived iPSCs bearing the heterozygous \(\Delta\)KPQ mutation in the SCN5A gene (LQTS3 (\(\Delta\)KPQ)) line) or those from healthy donors (HD line). After a 30-d differentiation of CMs, robust spontaneous contraction of myocytes was observed. Gene expression analysis of both iPSC–CM lines revealed expression of FHF1\textsubscript{a}, FHF1\textsubscript{b} and FHF2\textsubscript{a}, whereas other isoforms/splice variants were expressed at lower levels (Extended Data Fig. 6 and Supplementary Table 1). Previous studies of adult human ventricles showed that FHF1\textsubscript{a} is the predominant variant with some expression of FHF2\textsubscript{a} and/or FHF2\textsubscript{b} and negligible expression of FHF1\textsubscript{b}. This difference in expression profile may reflect incomplete maturation of iPSC–CMs. Nevertheless, we undertook cell-attached multichannel recordings to quantify \(I_{\text{Na,L}}\) in both HD and LQTS3 (\(\Delta\)KPQ)–iPSC–CM lines. As expected, we observed a shortening of the ODs in the presence of FHF1\textsubscript{a} for various channelopathic mutations, consistent with an open-state block mechanism (Extended Data Fig. 5e–i). However, the magnitude of change in ODs varied depending on the specific mutation.
Acute inhibition of \( I_{\text{Na,L}} \) by cell-permeable FixR. A key hurdle in the practical use of FixR as an \( I_{\text{Na,L}} \) inhibitor is that it acts by interacting with a channel cytosolic domain and therefore requires intracellular delivery for robust function. Various cell-penetrating peptides have been used to facilitate cellular uptake of small proteins, fluorescent markers and nanoparticles\(^{44-46} \). We reasoned that fusing a protein transduction domain from HIV-1 trans-activators of transcription\(^{17} \) to FixR (FixR-cpp; Fig. 7a) may permit intra-cellular delivery of the peptide. To examine this, we synthesized FixR-cpp with an N-terminal FITC tag to fluorescently monitor cellular entry (Fig. 7a). We incubated HEK293 cells with varying concentrations of FixR-cpp for 2 h and measured fluorescence intensity from individual cells using flow cytometry. We found a concentration-dependent increase in the uptake of FixR-cpp with 10 \( \mu \text{M} \) concentration yielding an ~100-fold enhancement in fluorescence intensity (Fig. 7b,e and Extended Data Fig. 10a). To probe the functional effect of FixR-cpp, multichannel recordings were performed on HEK293 cells transfected with a \( \text{Na}_{\text{r,1.5}} \Delta \text{KPOQ} \) mutant channel after a 2-h incubation with 10 \( \mu \text{M} \) FixR-cpp. In comparison to the control conditions, FixR-cpp incubation diminished \( I_{\text{Na,L}} \) (Fig. 7g,h). Encouraged by these effects in heterologous cells, we probed the effect of FixR-cpp on \( I_{\text{Na,L}} \) in cardiomyocytes. Epifluorescence imaging confirmed robust uptake of FixR-cpp into freshly dissociated cardiomyocytes as evident from increased cytosolic yellow fluorescence (Fig. 7d and Extended Data Fig. 10b). The dose dependence of peptide uptake into aMVMs was probed by incubating freshly isolated aMVMs with varying concentrations of FixR-cpp for 2 h and measuring fluorescence intensity using a flow cytometer. We found a concentration-dependent increase in the uptake of FixR-cpp, with 10 \( \mu \text{M} \) concentration yielding an ~150-fold enhancement in fluorescence intensity (Fig. 7c,f). To probe functional changes in \( I_{\text{Na,L}} \), we cultured ventricular myocytes from the IQ/AA\(^{10} \) mouse transduced with FHF2 shRNA to down-regulate endogenous protective FHF. As in Fig. 1e, this maneuver resulted in increased \( I_{\text{Na,L}} \) (Fig. 7j). Incubation of FixR-cpp for 2 h resulted in a ~10-fold reduction in \( R_{\text{exit}} \) (Fig. 7j). These findings confirm the functionality of FixR-cpp and suggest its potential utility as a powerful mechanism-inspired strategy to inhibit \( I_{\text{Na,L}} \) in cardiomyocytes.

**Discussion**

Increased late sodium current has emerged as a dominant contributor to diverse cardiac pathologies\(^{12} \) and is, therefore, a highly

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**Fig. 5 | FHF regulation of pathogenic \( I_{\text{Na,L}} \) in iPSC–CMs.** a. Multichannel recordings showing minimal \( I_{\text{Na,L}} \) in iPSC–CMs derived from healthy donors. b. LQT3 patient-derived iPSC–CMs with a heterozygous \( \text{Na}_{\text{r,1.5}} \Delta \text{KPOQ} \) mutation showing increased \( I_{\text{Na,L}} \). c,d. Adenoviral expression of FHF2 shRNA resulting in no appreciable change in \( I_{\text{Na,L}} \) for both iPSC–CM lines (\( P=0.10 \) using the Kruskal–Wallis test). e,f. Transduction of scrambled shRNA also yielded no change in \( I_{\text{Na,L}} \) for HD iPSC–CMs (e) and LQT3(\( \Delta \text{KPOQ} \)) iPSC–CMs (f). g. Overexpression of FHF1\(_{\text{r}} \) yielded no change in \( I_{\text{Na,L}} \) for iPSC–CMs from healthy donors (\( n=12 \) cells). h. FHF1\(_{\text{r}} \) diminishing \( I_{\text{Na,L}} \) in LQT3(\( \Delta \text{KPOQ} \)) iPSC–CMs. i. Bar graph summary of \( R_{\text{exit}} \) confirming strong inhibition of pathogenic \( I_{\text{Na,L}} \) by FHF1\(_{\text{r}} \), in LQT3(\( \Delta \text{KPOQ} \)) iPSC–CMs. Data are presented as mean ± s.e.m. for HD iPSC–CMs: control, \( n=11 \) cells (595 sweeps); + scrambled shRNA, \( n=10 \) cells (685 sweeps); + FHF2 shRNA, \( n=10 \) cells (499 sweeps); + FHF1\(_{\text{r}} \), \( n=12 \) cells (615 sweeps). For LQT3(\( \Delta \text{KPOQ} \)) iPSC–CMs: control, \( n=11 \) cells (526 sweeps); + scrambled shRNA, \( n=12 \) cells (704 sweeps); + FHF2 shRNA, \( n=15 \) cells (557 sweeps); + FHF1\(_{\text{r}} \), \( n=13 \) cells (663 sweeps). Statistical analysis using Kruskal–Wallis test followed by Dunn’s multiple comparison test: *\( P=0.0012 \) compared with baseline.
sought-after target for antiarrhythmic therapeutics. Although previous studies have shown that intracellular FHFs can tune I_{Na,L}, the precise contribution of this modulation to cardiac physiology and pathophysiology, as well as the underlying molecular mechanisms, has yet to be fully determined. This, the full potential of this modulatory scheme for engineering selective I_{Na,L} inhibitors remains untapped. By leveraging multiple synergistic model systems and in-depth biophysical analysis, the present study furnishes three key advances: first, systematic analysis of FHF1–3 variants revealed an unexpected bidirectional modulation of I_{Na,L} amplitude depending on the specific FHF isoform/splice variant. Second, ade

The four FHFs isoforms (FHF1–4) have emerged as versatile Na_{v} modulators in neurons and cardiomyocytes. Structurally, the primary FHF-binding site resides in the CT-binding domain (CTD), in close proximity to the CaM-binding IQ domain. FHFs regulate multiple facets of channel function including shifts in voltage-dependent fast inactivation, trigger long-term inactivation, promote Na_{v} trafficking, suppress Ca_{v}2.1/CaM-dependent inhibition and inhibit I_{Na,L}. For I_{Na,L} regulation, which is of high relevance pathophysiologically, the prevailing view is that the binding of the FHF core domain with the CTD allosterically alters the stability of the III–IV linker to prevent late channel openings, in essence mimicking the action of CaM. As such, FHF regulation of I_{Na,L} has been presumed to be limited to mutations in the CTD that alter the CaM interaction. Our present findings reveal a more nuanced scheme of channel modulation. First, we find that FHF inhibition of I_{Na,L} is isoform/splice-variant specific, that is, only certain protective FHF variants containing an N-terminal domain inhibit I_{Na,L}, whereas others either upregulate I_{Na,L} or yield no change. Thus, the net magnitude of I_{Na,L} can be continuously adjusted in the manner of a rheostat, depending on the relative abundance of distinct FHF isoforms/varients in the cell. Second, we find that the protective FHF variants are surprisingly general in inhibiting I_{Na,L} triggered by various mechanisms, including channelopath mutations in segments outside the CaM-binding domain, as well as from channel phosphorylation.

Third, functional regulation of I_{Na,L} by FHF does not depend on its interaction with the CTD, because deletion of a large swath of...
the CTD (Δ1810) preserved robust \(I_{\text{Na,L}}\) inhibition. Fourth, in-depth analysis of FHF domains responsible for \(I_{\text{Na,L}}\) inhibition revealed that the β-trefoil core domain is dispensable; instead, a sub-domain within the alternatively spliced FHF N terminus (that is, FixR) suffices to confer \(I_{\text{Na,L}}\) regulation, when overexpressed exogenously. In the native setting, it is possible that the FHF core domain may serve as a high-affinity anchor to the CTD that enhances the local concentration of the N-terminal domain. This may enhance the efficacy of \(I_{\text{Na,L}}\) inhibition even at low FHF concentrations. Although the N-terminal region overlaps with the previously identified long-term inactivation particle\(^{46,50}\), additional neighboring residues are also required for high-efficacy \(I_{\text{Na,L}}\) regulation. In addition, alanine substitution of critical residues responsible for long-term inactivation largely preserved \(I_{\text{Na,L}}\) regulation, hinting at potentially distinct structural requirements for \(I_{\text{Na,L}}\) modulation. The N-terminal region is largely conserved among several protective FHF isoforms (FHF1\(_A\), FHF2\(_A\), FHF3). However, it is noteworthy that FHF2\(_C\) and FHF2\(_A\) also modestly inhibit \(I_{\text{Na,L}}\), despite exhibiting considerable sequence divergence. It is currently unknown whether these isoforms interact with the same sodium channel interface or whether they utilize a distinct mechanism of late sodium current inhibition. Future studies may help resolve these possibilities. Fifth, FHF was able to enhance channel inactivation when conventional fast inactivation was disabled, either by mutating the IFM inactivation particle\(^{46,50}\) or through domain S6 mutations that occlude accessibility of the transmembrane receptor site for the IFM particle\(^{46,56}\). Taken together, our findings are consistent with FHF utilizing a previously reported ‘open-channel block’ mechanism to inhibit sodium channels, although there may be some differences in structural determinants for \(I_{\text{Na,L}}\) regulation of Na\(_{1.5}\) (refs. \(^{42,45}\)). These findings bear broad ramifications for physiological regulation of late sodium current and for understanding pathogenic mechanisms underlying both inherited and acquired arrhythmias.

Genetic studies have revealed a staggering number of mutations in the SCN5A gene in patients with electrocardiographic abnormalities and life-threatening cardiac diseases\(^4\). The associated cardiac syndromes, however, show phenotypic variability and reduced disease expressivity for reasons that are not fully understood\(^3\). This is particularly evident for mutations in the channel CTD whereby some patients present with a gain-of-function LQTS3 phenotype whereas others exhibit a BrS phenotype consistent with a loss-of-function effect. The traditional view is that multiple biophysical defects can trigger distinct phenotypes in an idiosyncratic manner\(^{32,33,36}\). Our recent analysis of Na\(_{1.5}\) CT channelopathies indicates that disrupted CaM binding showed two distinct effects, specifically a reduction in peak \(P_{\text{O}}\) and a concomitant increase in \(I_{\text{Na,L}}\)\(^{16}\). Although these differences appear to be coarsely consistent with LQTS3 and BrS phenotypes, respectively, in silico modeling...
suggested that these changes alone are insufficient and additional factors were required to switch arrhythmogenic mechanisms. Our present findings suggest that endogenous FHFs in cardiomyocytes may subserve this role. In particular, adult human ventricular myocytes endogenously express multiple FHF isoforms/splice variants including the nonprotective FHF1_δ and certain protective FHF2 variants, albeit at lower levels. One intriguing hypothesis is that the relative proportion of protective versus nonprotective variants may fluctuate in different individuals. Such variability may in turn result in differential manifestation of \( I_{\text{Na,L}} \) at the cellular level, ultimately contributing to reduced disease expressivity. Future studies that correlate expression levels of specific FHF splice variants/isooforms in different individuals with their cardiac phenotype may help discern this possibility.

Physiologically, \( I_{\text{Na,L}} \) plays a notable role in shaping the plateau phase of the AP and for ion homeostasis\(^1\). The basal level of \( I_{\text{Na,L}} \) is partially determined by CaMKII phosphorylation of Na\(_v\)1.5. \( \beta \)-Adrenergic stimulation further upregulates \( I_{\text{Na,L}} \) by increased Na\(_v\)1.5 phosphorylation via both the PKA and the CaMKII pathways\(^2\)\(^-\)\(^4\). During cardiac pathologies, including myocardial ischemia and heart failure, these pathways are chronically upregulated, resulting in increased \( I_{\text{Na,L}} \) that promotes vulnerability for arrhythmias\(^5\)\(^-\)\(^8\). Our work suggests that this overarching process is finely tuned by endogenous FHFs in cardiomyocytes, potentially furnishing an important feedback mechanism. It is interesting that FHF2 gene expression is upregulated in pathological cardiac hypertrophy in mice\(^9\). It is unknown how this change in gene expression impacts functional \( I_{\text{Na,L}} \) regulation, as well as whether this change is pathogenic or compensatory. An important caveat is that our present study focused exclusively on \( I_{\text{Na,L}} \) resulting from incomplete inactivation of Na\(_v\)1.5. Beyond this mechanism, increased \( I_{\text{Na,L}} \) may also be generated by increased window current corresponding to the alterations in the overlap between steady-state activation and steady-state inactivation, as well as a small population of neuronal Na\(_v\) isoforms. The role of FHF in tuning \( I_{\text{Na,L}} \), due to these mechanisms, has yet to be determined.

Given its broad relevance to the pathophysiology of acquired and inherited arrhythmias, selective blockade of \( I_{\text{Na,L}} \) has garnered considerable interest both for physiological studies and as a therapeutic strategy\(^9\)\(^-\)\(^11\). To date, the dominant strategy for \( I_{\text{Na,L}} \) inhibition is small-molecule modulators such as ranolazine, GS967 and elicelazine, which target a common transmembrane local anesthetic-binding site\(^12\). Beyond this, recent studies have developed acyl- and aryl-sulfonamide compounds that target the fourth voltage-sensing domain to confer subtype-selective and highly state-dependent inhibition of Na\(_v\) channels, providing a potential avenue to engineer modulators\(^13\). In this context, our results point to FixR as an orthogonal template for designing \( I_{\text{Na,L}} \) inhibitors. Compared with ranolazine, FixR exerts an almost complete reversal of \( I_{\text{Na,L}} \) triggered by either channelopathetic mutations or channel phosphorylation, thus confirming both its generality and its potency. Furthermore, robust intracellular delivery of FixR may be attained through either viral gene delivery or acute application of a synthetic peptide attached to a cell-penetrating moiety. As FixR is genetically encodable, it can be targeted using cell-type-specific promoters or could be localized to subcellular domains such as the cardiac dyad or the intercalated disk, potentially permitting unprecendented insights into both the molecular and the cell physiological consequences of \( I_{\text{Na,L}} \). From a therapeutic perspective, given its relatively small size, peptidomimetics that structurally resemble FixR may furnish an alternative strategy for developing small-molecule \( I_{\text{Na,L}} \) inhibitors. Of note, Na\(_v\)1.5 channelopathetic mutations may also impact various aspects of channel function beyond \( I_{\text{Na,L}} \), including, for example, activation, steady-state inactivation and channel trafficking. Given its minimal functionality, it is unlikely that FixR will be capable of reversing these defects.

Of broader relevance, increased \( I_{\text{Na,L}} \) in neurons and skeletal muscle is linked to epilepsy or neurodevelopmental delay\(^12\)\(^-\)\(^15\), and myotonia\(^16\), respectively. As various FHF isoforms/splice variants are differentially expressed in these tissues\(^16\)\(^-\)\(^18\), the general framework for FHF modulation developed in the present study may lend key insights in complex pathophysiological mechanisms. Furthermore, FixR may provide a convenient strategy to dissect the functional importance of \( I_{\text{Na,L}} \) in these settings with high precision.

**Methods**

**Ethical statement.** All animal experimental procedures were carried out in accordance with regulations and established guidelines and were reviewed and approved by the Institutional Animal Care and Use Committee at Columbia University.

**Molecular biology.** The human Na\(_v\)1.5 channel corresponds to the clone with GenBank accession no. M77235.1 and is subcloned into pGW1 vector with HindIII and SalI. To facilitate construction of channelopathetic mutations in the channel CT, we introduced silent mutations into Na\(_v\)1.5 to introduce an NruI-cutting site near the channel CT (5329-ggaggcagc-5337 into 5329-gtgggcagc-5337). Subsequently, we used overlap-extension PCR and ligated the PCR product into Na\(_v\)1.5/pGW1 using NruI and XbaI restriction sites (mutations: E[1784]K, S[1904]L, IQ/AA and Q[1909]R). For generating the AKPQ and IFM/IQM mutations, we used overlap-extension PCR and ligated in Na\(_v\)1.5/pGW1 after a restriction digest using KpnI and XbaI sites. For generation of Na\(_v\)1.5 truncations, we PCR amplified relevant segments of the primer upstream of the KpnI and XbaI sites, which was truncated at the appropriate location (Δ1810 and Δ1885), included an XbaI site and ligated into Na\(_v\)1.5/pW1 vector. For LIL/A/WICM mutations, we used overlap-extension and restriction sites AgeI and Nhel for subsequent ligation. All segments subjected to PCR amplification were verified by sequencing. For generating human FHF2 splice variants, we synthesized gene fragments and ligated into pcDNA3 using BamHII/EcoRI restriction sites. Human FHF1\(_\delta\) and FHF\(_\delta\) were subcloned into ECP-N3 vector, and FHF2 splice variants and FHF3 were subcloned into pcDNA3. Truncations of the FHF\(_1\) N terminus were generated as fusion proteins with Venus fluorescent protein in the C terminus by encoding the relevant segments as noted in Extended Data Fig. 7a in the forward PCR primer and the fluorophore as the reverse PCR primer. After PCR amplification, the fragments were ligated into pIRES2-EGFP, followed by restriction digestion using Nhel and Xhol enzymes. A plasmid-encoding PKA catalytic subunit was from a previous study where it was cloned into pcDNA3.1 using PCR amplification and restriction digestion with KpnI/XbaI.

**Adenovirus generation.** Adenovirus-encoding FHF1\(_\delta\)-P2A-Venus, FixR-P2A-Venus, FHF2 shRNA, scrambled shRNA, FHF1\(_\delta\)-P2A-Venus and enhanced GFP (catalog no. 1060) were obtained from Vector Bioslabs. Briefly, FHF1\(_\delta\)-P2A-Venus and FixR-P2A-Venus, were synthesized as gene fragments (Twist Biosciences) flanked by BamHI and EcoRI restriction sites. Subsequently, the gene fragments were subcloned into the dual CMV\(_\beta\) vector containing a BamHII and EcoRI restriction sites. After PCR amplification, the fragments were ligated into pIRES2-EGFP, followed by restriction digestion using Nhel and Xhol enzymes. A plasmid-encoding PKA catalytic subunit was from a previous study where it was cloned into pcDNA3.1 using PCR amplification and restriction digestion with KpnI/XbaI.

**Cell culture and transfection.** HEK293 cells (American Type Culture Collection, catalog no. CRL1575) were cultured on glass coverslips in 60-mm dishes and transfected using the calcium phosphate method\(^19\). For electrophysiology experiments, we co-transfected 4–8\(\mu\)g of cDNA encoding the desired channel variant with 4\(\mu\)g of yellow fluorescent protein and 1\(\mu\)g of Simian virus 40 T-antigen. For experiments evaluating the effect of FHF, we transfected the relevant FHF variant at a 1:1 ratio with the \( \psi \)-subunit. For experiments considering using a forward FHF1/2 isoforms, we co-transfected FHF1\(_\delta\) and FHF2\(_\delta\) variants at specified ratios (ranging from 1:1 to 1:10). The culture medium was replaced after 4 h of transfection. Electrophysiology recordings were then performed at room temperature 1–2 d after transfection.

**Multichannel analysis of the late sodium current.** Multichannel records were obtained in the on-cell configuration with either HEK293 cells or aMVMs as in our previous study\(^20\). The pipette contained: 140 mM NaCl, 10 mM Hepes, 0.5 mM CaCl\(_2\); at 300 mosmol, adjusted with tetraethylammonium methanesulfonate and pH 7.4 adjusted with tetraethylammonium hydroxide. To zero membrane potential, the bath contained: 132 mM potassium glutamate, 5 mM KCl, 5 mM NaCl, 3 mM MgCl\(_2\), 2 mM (either acetate or bicarbonate) and 5 mM glucose; 20 mM Hepes; at 300 mosmol, adjusted with glucose and pH 7.4 adjusted with NaOH. Data were acquired at room temperature using the integrating mode of

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an Axopatch 200a amplifier (Axion Instruments, Molecular Devices). Patch pipettes (3–10 MΩ) were pulled from ultra-thick-walled borosilicate glass (Sutter Instruments, catalog no. BF200-116-10) using a horizontal puller (Sutter Instruments, catalog no. P-97), fire polished with a microforge (Narishige) and coated with Silgard (Dow Corning). Elementary currents were low-pass filtered at 2 kHz using a 4-pole Bessel filter and digitized at 200 kHz with an ITC-18 unit (Instrutech), controlled by customized MATLAB software (Mathworks). For each pulse, we obtained P/8 leak pulses. Leak subtraction was performed using an automated algorithm that fit the kinetics of the leak current or for capacitive transient with convex optimization and L1 regularization39. After leak subtraction, the unitary current for each patch was estimated using an amplitude histogram. Each stochastic trace was subsequently idealized. The ensemble average from 50–100 stochastic traces was computed for each patch and normalized to the peak current. The average late current for each patch (R_average) was computed as the average normalized late current at 10 ms after the depolarization for single-channel recordings of IFM/IQM and LILa/WICW mutants, the Pᵢ was calculated by dividing the ensemble average current by the unitary current and the number of channels in a patch estimated by stacking and mean-variance analysis.

Whole-cell recordings. Whole-cell recordings were obtained at room temperature with an Axopatch 200B amplifier (Axion Instruments). Electrodes were made of borosilicate glass (World Precision Instruments, catalog no. MTW 150-F4), yielding a pipette of 1–2 MΩ resistance, which was compensated by >70%. Pipettes were fabricated with a horizontal micropipette puller (model P-97, Sutter Instruments) and fire polished with a microforge (Narishige). Data acquisition utilized an ITC-18 (Instrutech) data acquisition unit controlled by customized MATLAB software (Mathworks). Currents were low-pass filtered at 2 kHz before digitization at several times that frequency. P/8 leak subtraction was used. Cells were maintained at a holding potential of ~120 mV.

Mouse models. The Institutional Animal Care and Use Committee at Columbia University approved all animal experiments (nos. AC-AABI1550 and AC-AABL0569). The pWT and IQ/AA lines were generated previously60. In brief, the human heart sodium channel α-subunit cDNA (hH1; Na,1.5) was fused to a vector containing the modified murine myosin heavy-chain-α, tetracycline-inducible promoter, a gift of J. Robbins and J. Meik. (University of Cincinnati, Cincinnati, OH). The Na,1.5 channel was engineered to be tetradotoxin sensitive by inserting a Cys374Tyr mutation. A 3X-FLAG epitope was ligated in-frame to the N terminus. These mice, on a B6CBA/F2 hybrid background, were bred with cardiac-specific rtTA (reverse tet-transactivator) transgenic mice (model P-97, Sutter Instruments) and backcrossed with a Microforge (Narishige). Data acquisition utilized an ITC-18 (Instrutech) data acquisition unit controlled by customized MATLAB software (Mathworks). Currents were low-pass filtered at 2 kHz before digitization at several times that frequency. P/8 leak subtraction was used. Cells were maintained at a holding potential of ~120 mV.

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Isolation, culture and adenoviral transduction of aMVMs. Mice ventricular myocytes were isolated by enzymatic digestion using Langendorff’s perfusion apparatus63. CMs were isolated from 8- to 12-week-old nontransgenic (C57BL/6) and female mice were used in all experiments. For nontransgenic mice, we used mice on a FVB/N background, obtained via the Mutant Mouse Resource and background, were bred with cardiac-specific rtTA (reverse tet-transactivator) was ligated in-frame to the N terminus. These mice, on a B6CBA/F2 hybrid background, were bred with cardiac-specific rtTA (reverse tet-transactivator) transgenic mice (model P-97, Sutter Instruments) and backcrossed with a Microforge (Narishige). Data acquisition utilized an ITC-18 (Instrutech) data acquisition unit controlled by customized MATLAB software (Mathworks). Currents were low-pass filtered at 2 kHz before digitization at several times that frequency. P/8 leak subtraction was used. Cells were maintained at a holding potential of ~120 mV.

Computational ventricular AP modeling. Computational modeling to predict the effect of competition between HHF1/2 splice variants/isofoms on ventricular APs was performed with the ToR-Ord model simulated using MATLAB62. We tuned the amplitude of late sodium current based on the empirical relationship between R_average and the ratio HHF1/HHF2, in Fig. 3c. Specifically, for the IQ/ AA mutant, the late sodium current was modified by the following equation: \( G_{\text{Na}} = (7.254 - 7.0899)(1 + (p/0.4279)^{340})/0.17 \). As the level of \( G_{\text{Na}} \) of wild-type channels is similar in the presence of HHF1A or HHF2, we modified the above equation to: \( G_{\text{Na}} = (0.2 - 0.0449)/1 + (p/0.4279)^{340} \). AFs were simulated for 100 beats at various heart rates and only the final 2 beats were considered for measurement of APD.

Dissociation of iPSC–CMs. The LQT3S (\( \Delta {\text{K}^{{\text{QP}}}} \))–iPSCs were generated with patient consent according to the Johns Hopkins University institutional review board (protocol no. 0108761). The iPSCs were generated from peripheral blood mononuclear cells using Sendai virus containing Yamanaka factors for reprogramming67. The healthy donor iPSC line had been generated previously60. Differentiation into CMs was modified by performing a previously established protocol60. The human induced (hi)PSCs were maintained in mTeSR medium (STEM Cells Technologies) and passaged every 4–6 days on Matrigel (Corning)-coated plates before differentiation. On day 0 (start of differentiation), hiPSCs were treated with 1 mg/ml collagenase B (Roche, catalog no. 1108807001) for 1 h, or until cells detached from the plates, to generate embryoid bodies (EBs). Cells were then collected and centrifuged at 300g for 3 min, and resuspended as small clusters of 50–100 cells by gentle pipetting in CM differentiation medium, composed of RPMI 1640 (Thermo Fisher Scientific, catalog no. 118750858) containing 2 mM of t-glutamine (Thermo Fisher Scientific, catalog no. 253030149), 4 mM Museumofhyaluronic acid (Millipore Sigma, catalog no. M6145) and 50 μg/ml of ascorbic acid (Millipore Sigma, catalog no. A4403). Differentiation medium was supplemented with 2 ng/ml of BMP4 (R&D Systems) and 10 μM Rock inhibitor (Y-27632 dihydrochloride, Tocris Fisher, catalog no. 1254/50), and EBs were cultured in Ultra-Low attachment 6-well plates (Corning Costar, catalog no. 3471) in a humidified incubator at 37 °C in 5% CO2 and 5% O2. On day 1, medium was changed to differentiation medium supplemented with 20 ng/ml of BMP4 (R&D Systems), 20 ng/ml of Activin A (R&D Systems) and 5 mg/ml of basic fibroblast growth factor (R&D Systems). On day 3, EBs were harvested and washed once with RPMI 1640. Medium was changed to differentiation medium supplemented with 2 mM of vascular endothelial growth factor (VEGF, R&D Systems) and 5 μM XAV939 (Reprocell-Stemgent, catalog no. 04-0046). From this point on, every 2–3d the medium was replaced with medium supplemented with 5 ng/ml of VEGF (R&D Systems) only.

Quantitative PCR. To assess FHF isoform expression levels, reverse-transcriptase quantitative PCR (RT-qPCR) was performed on cDNA with primers (Integrated DNA Technologies) specific for \( \text{FGF1} \) and \( \text{FGF2} \) isoforms. Reactions were achieved using SYBR green Mix (Roche) and customized primers of interest was performed using gene-specific primers and performed in triplicate. Reactions were achieved using SYBR green Mix (Roche) and customized primers as in a previous study41. Synthesis kit (BioRad). RT-qPCR for the evaluation of the expression of the gene of interest was performed using Graphpad Prism (Graphpad Software).
Material availability. All transgenic mice are available from S.O.M. under a material agreement with Columbia University. All other biological materials are available from M.B.J. under a material agreement with Columbia University.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability All data are available in the main text or the supplementary materials. Source data are available for this paper.

Code availability Customized MATLAB scripts used for multichannel analysis are available at Github: https://github.com/manubenjohny/LateCurrent.

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Author contributions

N.C., S.O.M. and M.B.-J. conceptualized and designed the research. N.C., S.R., J.D., A.H., R.M., D.R., L.Y., B.-X.C., J.O.O., D.S., B.C., I.E.D. and M.B.-J. performed research and acquired the data. N.C., S.R., J.D. and M.B.-J. analyzed the data. N.C., S.R., J.D., D.R., L.Y., B.-X.C., J.O.O., D.S., B.C., I.E.D., G.F.T., S.O.M. and M.B.-J. contributed new reagents/analytic tools. M.B.-J., G.F.T. and S.O.M. acquired funds. N.C. and M.B.-J. created the figures and wrote the original draft. All the authors revised the manuscript.

Competing interests

N.C., S.O.M. and M.B.-J. (inventors) filed a provisional patent (attorney docket no. CuS1046P/CU22077; filed 14 February 2022) for application of FixR for inhibiting late sodium current. The remaining authors declare no competing interests.

Additional information

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Extended Data Fig. 1 | shRNA suppression of FHF2 has no effect on $I_{\text{Na,L}}$ in non-TG aMVM. **a,** Epifluorescence and brightfield images of cultured aMVMs from non-TG mice transduced with FHF2 shRNA. Scale bar, 100 μm. ($n=3$ mice) **b,** Exemplar multichannel Na, recordings from uninfected non-TG mice show minimal late channel openings. Format as in Fig. 1b. Population data are shown in Fig. 1h. **c–d,** Application of both FHF2 shRNA and scrambled shRNA revealed no change in $I_{\text{Na,L}}$ openings for cultured aMVMs from non-TG mice. Population data are shown in Fig. 1h.
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | $I_{\text{Na,L}}$ regulation by the FHF2-3 splice variants. a, Schematic shows various FHF2 splice variants generated by alternate start sites. Conserved exons that encode the core domain are shown in red boxes labeled E2 to E5. b–e, Exemplar recordings of LQTS3-linked ΔKPQ mutant heterologously expressed in HEK293 cells in the presence of various FHF2 splice variants. f–i, Exemplar recordings of IQ/AA mutant in the presence of FHF2 splice variants. j, Bar graph summarizes changes in $I_{\text{Na,L}}$ quantified as $R_{\text{max}}$ upon co-expression of FHF splice variants. FHF2$_S$ and FHF2$_V$ yielded a partial reduction in $I_{\text{Na,L}}$ for both the IQ/AA and the ΔKPQ mutant, while FHF2$_Y$ and FHF2$_VY$ resulted in no change. Data presented as mean ± SEM. For ΔKPQ mutant, + FHF2$_Y$, n = 9 cells (512 sweeps); + FHF2$_V$, n = 10 cells (549 sweeps); + FHF2$_V$, n = 9 cells (561 sweeps); + FHF2$_Y$, n = 9 cells (499 sweeps). For IQ/AA mutant, + FHF2$_Y$, n = 10 cells (547 sweeps); + FHF2$_V$, n = 10 cells (807 sweeps); + FHF2$_V$, n = 10 cells (564 sweeps); + FHF2$_Y$, n = 9 cells (547 sweeps). Statistical Analysis, Kruskal-Wallis test followed by Dunn’s Multiple comparisons test, **p < 0.01; ***p = 0.026(ΔKPQ + FHF2$_V$) and *p = 0.039(ΔKPQ + FHF2$_V$), *p = 0.034(IQ/AA + FHF2$_V$) and *p = 0.011 (IQ/AA + FHF2$_V$) when compared to no FHF control of each mutant channel. k, Exemplar multichannel recording from recombinant NaV1.5 wild-type in HEK293 cells shown minimal late channel openings in the presence of FHF3$_A$. Population data is shown in Fig. 2m. l, Exemplar recordings show a partial reduction in late channel openings for NaV1.5 IQ/AA mutant channel in the presence of FHF3$_A$. Population data are shown in Fig. 2m.
Extended Data Fig. 3 | Generality of \( I_{\text{Na,L}} \) regulation by FHF1\(_{\alpha}\).

**a.** Schematic illustrates the \( \alpha \)-subunit of Na\(_V\)1.5 channel showing missense (grey) and nonsense (red) mutations in disparate channel domains.

**b-f.** Exemplar multichannel recordings of Na\(_V\)1.5 F1759A in the absence (top) and presence of FHF1\(_{\alpha}\) (bottom). c-f. Exemplar recordings suggest that FHF1\(_{\alpha}\) inhibits E[1784]K, S[1904]L, Q[1909]R, and \( \Delta \)1885 mutant.
Extended Data Fig. 4 | Inhibition of phosphorylation-dependent \( I_{\text{Na,L}} \) by FHF1-2 isoforms. a, Schematic shows Na\(_V\)1.5 phosphorylation by PKA and CaMKII upregulates \( I_{\text{Na,L}} \). b, FHF2S inhibits PKA-dependent \( I_{\text{Na,L}} \) of wild-type Na\(_V\)1.5 (similar to the effects of FHF1A in Fig. 3d). e, FHF1B fails to inhibit PKA-dependent \( I_{\text{Na,L}} \) of wild-type Na\(_V\)1.5. d, Exemplar multichannel recordings from wild-type Na\(_V\)1.5 upregulated by co-expression of constitutively active CaMKII (CaMKIIT286D). e-f, Both FHF1A (panel e) and FHF2S (panel f) inhibits CaMKIIT286D-triggered \( I_{\text{Na,L}} \) of wild-type Na\(_V\)1.5.
Extended Data Fig. 5 | Elementary mechanisms underlying FHF1A regulation of INa,L. a, FL distributions for Na\(_{\alpha,1.5}\) IFM/IQM at baseline (black line and gray shaded area; \(n = 231\) sweeps from 3 one-channel recordings) and upon overexpression of FHF1A (red line and rose shaded area; \(n = 217\) sweeps from 5 one-channel recordings). FL denotes the probability that the first opening occurred at time < \(t\). FHF1A had minimal effect on the FL distribution for the IFM/IQM mutant. b, FL distributions for Na\(_{\alpha,1.5}\) LILA/WICW at baseline (gray shaded area; \(n = 176\) sweeps from 3 one-channel recordings) and under overexpression of FHF1A (rose shaded area; \(n = 270\) sweeps from 6 one-channel recordings). FHF1A decreased the pedestal value of FL for the LILA/WICW mutant suggesting that FHF1A increases closed-state inactivation. \(p < 0.001\) by KS-test. c, Open-duration (OD) distribution for Na\(_{\alpha,1.5}\) IFM/IQM tallies the durations of a single sojourn to the open state. FHF1A shortens the OD distribution for the IFM/IQM mutant, hinting at potential increase in the rate constant for inactivation from the open state. For IFM/IQM, \(n = 7597\) openings from 6 cells. For IFM/IQM + FHF1A, \(n = 1332\) openings from 9 cells. \(p < 0.001\) by KS-test. d, FHF1A has no effect on OD distribution of the LILA/WICW mutant. For LILA/WICW, \(n = 22272\) openings from 8 cells. For LILA/WICW + FHF1A, \(n = 1361\) openings from 6 cells. e, Conditional open-duration (OD) distribution for Na\(_{\alpha,1.5}\Delta KPQ\) mutants, for single-level channel openings in the late phase, after 50 ms depolarization, (\(n = 12278\) openings from 12 cells). FHF1A shortened the OD distribution (\(n = 5011\) openings from 12 cells), while FHF1B evoked a minor increase in OD distribution (\(n = 2269\) openings from 6 cells). f-k, Conditional OD distributions confirm variable shortening of the OD distribution in the presence of FHF1A for E[1784]K (panel f; control, \(n = 9355\) openings from 15 cells; +FHF1A, \(n = 1683\) openings from 14 cells), S[1904]L (panel g; control, \(n = 7097\) openings from 10 cells; +FHF1A, \(n = 1929\) openings), Q[1909]R (panel h; control, \(n = 4406\) openings from 13 cells; +FHF1A, \(n = 1658\) openings from 11 cells), IQ/AA (panel i; control, \(n = 5345\) openings from 13 cells; +FHF1A, \(n = 1266\) openings from 13 cells; +FHF1B, \(n = 576\) openings from 9 cells), and \(\Delta 1885\) (panel j; control, \(n = 1049\) openings from 10 cells; +FHF1A, \(n = 576\) openings from 9 cells), all consistent with the presence of open-state block. The \(\Delta 1885\) mutant failed to show any appreciable change in OD distribution (panel k; control, \(n = 3955\) openings from 10 cells; +FHF1A, \(n = 2195\) openings from 9 cells). As FHF1A inhibits \(I_{\text{Na}}\) for this mutant, this effect likely modifies the closed state. l, Bar graph summary of mean OD calculated from OD distribution function in panels e-k from sample sizes noted in each panel. Each bar, mean OD value. e-k, Statistical Analysis, \(p < 0.001\) for comparisons of OD distribution absent FHF to with FHF1A for \(\Delta KPQ\), E[1784]K, S[1904]L, Q[1909]R, IQ/AA, and \(\Delta 1810\) mutants by KS-test.
Extended Data Fig. 6 | Relative expression profiles of FHF1-4 isoforms/splice-variants in iPSC-CMs. a, Analysis of relative expression of FHF1-4 isoforms/splice-variants using RT-qPCR demonstrate FHF1A, FHF1B, and FHF2S as the major isoforms in healthy-donor iPSC-CMs. We observed that FHF2S expression was ~3-fold higher than FHF1B in the healthy-donor iPSC-CMs while FHF1A was ~45% lower than FHF1B. Each bar and error, mean ± SEM. n = 3 independent cultures. (see Supplementary Table 1). b, In the LQTS3 (ΔKPQ) iPSC-CMs, FHF1B is the major isoform/splice-variant with ~50% lower level of FHF1A and ~20% lower level of FHF2S. The relative prevalence of FHF1A in the LQTS3 (ΔKPQ) iPSC-CMs is consistent with the high baseline $I_{Na}$ obtained in functional studies in Fig. 5. Each bar and error, mean ± SEM. n = 5 independent cultures. (see Supplementary Table 1).
Extended Data Fig. 7 | Identifying a minimal FHF1a domain capable of inhibiting \( I_{Na,L} \). a, Sequence alignment of various FHF1a amino-terminal peptides utilized to identify a minimal effector domain. b, Schematic illustrates potential interaction between the peptides and Na\(_{1.5}\) channel. c, Exemplar multichannel recordings from Na\(_{1.5}\) IQ/AA mutant show increase late channel openings when co-expressed with 1-18 (panel c), 18-39 (panel d), 12-17 (panel e), or 9-32 (panel f) peptides. g, Ranolazine yields a modest inhibition of \( I_{Na,L} \) for Na\(_{1.5}\) IQ/AA mutant. h-i, Both FixR (panel h) and ranolazine (panel i) inhibits \( I_{Na,L} \) for Na\(_{1.5}\) S1904L mutant channel. j, Bar graph shows \( R_{persist} \) for Na\(_{1.5}\) S1904L mutant channel in the presence of FixR or ranolazine. Black dashed line, \( I_{Na,L} \) for Na\(_{1.5}\) S1904L at baseline. Blue dashed line, \( I_{Na,L} \) for Na\(_{1.5}\) S1904L co-expressed with FixR. Each bar and error, mean ± SEM. n=10 cells (557 sweeps). Statistical Analysis, one-way ANOVA followed by Dunnet’s multiple-comparisons test ***p < 0.001. k, FixR also strongly inhibits \( I_{Na,L} \) for Na\(_{1.5}\) Δ1885 mutant channel. l-m, Adenoviral expression of both GFP (panel l) and FixR (panel m) yielded no change in \( I_{Na,L} \) in iPSC-CMs derived from healthy donors (HD).
Extended Data Fig. 8 | Mutations in the long-term inactivation particle preserves FHF1\textsubscript{A} regulation of \(I_{\text{Na, L}}\). 

\(a\)-\(b\), Exemplar recordings of Na\textsubscript{v, 1.5} IQ/AA mutant channel heterologously expressed in HEK293 cells in the presence of either FHF1\textsubscript{A} L9A mutant (panel \(a\)), or FHF1\textsubscript{A} R11A mutant (panel \(b\)). These conserved residues have been previously shown to be critical for long-term inactivation.

\(c\), Bar graph summarizes changes in \(R_{\text{persist}}\) upon co-expression of the different FHF1\textsubscript{A} mutant variant. Each bar and error, mean ± SEM. For FHF1\textsubscript{A} L9A mutant, \(n = 8\) cells (503 sweeps) and for FHF1\textsubscript{A} R11A mutant, \(n = 8\) cells (494 sweeps). Statistical Analysis, Kruskal-Wallis test followed by Dunn’s multiple comparisons test: ***p < 0.001; for each mutant compared to no FHF1\textsubscript{A}. 
Extended Data Fig. 9 | Both FHF1A and FixR have minimal effect on peak current density and steady-state inactivation of mutant Na\textsubscript{v}1.5. a, Exemplar current recordings for Na\textsubscript{v}1.5 IQ/AA mutant channels elicited in response to a family of voltage steps from −60 to +50 mV from a holding potential of −120 mV. b, Population data shows average peak current density (\(J_{\text{peak}}\)) – voltage relationship for IQ/AA mutant. Each dot, mean ± SEM with \(n\) denoted in parenthesis. c, Steady-state inactivation curve (\(h_\infty(V)\) curve) elicited using step-depolarizations from a holding potential of −120 mV for IQ/AA mutant. Each dot, mean ± SEM with \(n\) denoted in parenthesis. d-f, Overexpression of FHF1A does not appreciably alter peak current density (panel e) and steady-state inactivation (panel f) compared to that measured at baseline. g-i, Overexpression of FixR does not appreciably alter peak current density (panel h) and steady-state inactivation (panel i).
Extended Data Fig. 10 | See next page for caption.
Extended Data Fig. 10 | Extended characterization of cell-permeable FixR. Representative flow cytometric data shows the percentage of cellular uptake of FixR (FITC positive) into HEK293 cells and effect on cytotoxicity determined using a far red (APC) DEAD cell stain (Invitrogen). Cells are incubated with varying concentrations of FixR-cpp for 2 hours. Appreciable cellular uptake is observed between 5 and 25 μM. Minimal cell death is observed in the presence of up to 10 μM FixR-cpp. Additional epifluorescence and brightfield images show FixR-cpp uptake into freshly-dissociated cardiomyocytes from IQ/AAΔm mice (n = 3 mice). Scale bar, 10 μm.
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Software and code

Policy Information about availability of computer code

Data collection
Custommade in MATLAB 2010b (Mathworks) was used for acquisition of electrophysiological data and is available from Github, SD, FACS6a, C for FACS measurements
MicroManager 2.0 for image acquisition

Data analysis
Custommade in MATLAB 2010b (Mathworks) was used for data analysis and is available from Github [https://github.com/manubenjohny/LateCurrent], Graphpad Prism 9.1.1 for statistical analysis
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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Sample Size was determined based on variance in previous studies (ref 25, 27). All electrophysiology experiments included a sample size of at least 5 cells. For multichannel recordings, we obtained at least 50 sweeps per patch to calculate ensemble averages. |
|--------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | For multichannel recordings, sweeps with significant membrane instability were excluded. |
| Replication | For multichannel electrophysiology experiments, for each patch we obtained >50 sweeps (technical measurements) and greater than 6 cells (replicates). For HEK293 cells, data was collected from at least 2 independent transfections. For iPSC-CM cultures, we performed at least 2 independent differentiations, and aMVM cultures were obtained from at least 3 mice. For whole-cell electrophysiology, we collected data from at least 5 cells and 2 independent transfections. For flow-cytometric analysis of FixR uptake in HEK293 cells and mVM, we performed two independent experiments probing concentration dependence. For qPCR experiments, FHF expression level was measured from at least 3 independent differentiations. Epifluorescence imaging experiments were repeated from 3 independent mice. All attempts at replication were successful. |
| Randomization | Experiments were not randomized based on the type of experiments. HEK, iPSC-CM, and aMVM cells were maintained using similar culture conditions and cultures were selected at random to be transfected or infected with relevant plasmid or adenovirus respectively. Test and control groups were assessed in parallel and isochronically. Electrophysiological experiments were collected in a random order to account for any potential time-dependent effects. |
| Blinding | The investigators were not blinded due to the nature of experiments. In most cases, a single experimenter prepared cells and collected data and, as such, were aware of the group designation. In addition, we expressed fluorescent reporters to identify cells that were transfected, transduced, or treated, as such blinding was not possible. Electrophysiology results were validated by multiple experimenters. |

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We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Involved in the study |
|----------------------------------|-----------------------|
| n/a                              | [x] Antibodies        |
| [ ] Eukaryotic cell lines        | [x] Paleontology and archaeology |
| [x] Palaeontology and archaeology| [x] Animals and other organisms |
| [x] Animals and other organisms  | [x] Human research participants |
| [x] Human research participants  | [x] Clinical data     |
| [x] Clinical data               | [ ] Dual use research of concern |

| Methods                          | Involved in the study |
|----------------------------------|-----------------------|
| n/a                              | [x] ChIP-seq          |
| [ ] Flow cytometry               | [x] MRI-based neuroimaging |
Eukaryotic cell lines

Policy Information about cell lines

Cell line source(s) HEK293 cells (ATCC CRL 1573); Healthy Donor iPSC lines from Huang et al. JIMCC:133:1-11 (ref 78); LQT3 iPSC-CMs generated in this study.

Authentication Cell lines were authenticated using STR profiling (ATCC). iPSC lines were validated by gene sequencing and karyotyping.

Mycoplasma contamination All cell lines tested negative for mycoplasma.

Commonly misidentified lines No commonly misidentified lines were used in this study.

Animals and other organisms

Policy Information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

Laboratory animals Mice: B6C3F1 hybrid background, cardiac-specific Tα mice in a FVB/N background, and C57BL/6 male and females, 6 weeks to 4 months of age.

Wild animals No wild animals were used.

Field-collected samples No field-collected samples were used.

Ethics oversight The Institutional Animal Care and Use Committee at Columbia University approved all animal experiments (AC-AAB1555 and AC-AAB6056).

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

- The axis labels state the marker and fluorochrome used (e.g., CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a group is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation HEK293 cells and freshly dissociated mouse ventricular myocytes.

Instrument LSR II (BD biosciences).

Software Data acquired using BD FACSDiva and analyzed using FlowJo v10.

Cell population abundance No sorting was performed.

Gating strategy Cells were gated initially with FSC-H and SSC-H, and single cells identified by gating using FSC-A/FSC-H.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary information.