Fibroblast Growth Factor Homologous Factors are Important Modulators of Cardiac Ion Channels

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pharmacology and Cancer Biology in the Graduate School of Duke University

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ABSTRACT

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Abstract

Fibroblast growth factor (FGF) homologous factors (FHFs, FGF11-14) are a family of FGFs that are not secreted, nor activate FGF receptors. Instead, they remain intracellular and bind to the voltage-gated Na$^+$ channel C-terminus and modulate function. Several lines of evidence, including data from heterologous expression systems and the distribution of FGF13 within the ventricular cardiomyocyte suggested that FHFs also modulate the Ca$_{v}$.1.2 voltage-gated Ca$^{2+}$ channel. The central hypothesis to this study is that FHFs modulate both voltage-gated Na$^+$ and Ca$^{2+}$ channels in the ventricular cardiomyocyte and therefore are loci for cardiac arrhythmia. Using an adult ventricular cardiomyocyte system with adenoviral gene transfer, we manipulated the levels of FGF13 in the cell and performed electrophysiology, biochemistry and immunocytochemistry to analyze the effects on voltage-gated Ca$^{2+}$ channel localization and function. We showed that FGF13 is in complex with Junctophilin-2 and modulates Ca$_{v}$.1.2 current density and localization to the T-tubule, leading to changes in Ca$^{2+}$-induced Ca$^{2+}$ release and ultimately a shortened ventricular action potential. Through collaboration with the Mayo Clinic, a mutation in FGF12, the most highly expressed FHF in human ventricle was found in a patient with Brugada syndrome. Using similar methodology, we determined that this mutation results specifically in a Na$_{v}$.1.5 loss of function with differential effects on Ca$_{v}$.1.2 function, resulting in a Brugada-like ventricular action potential. This data shows that FHFs are potent modulators of multiple ion channels and novel arrhythmogenic loci.
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List of Abbreviations

SA – sinoatrial
AV – atrioventricular
ECG – electrocardiogram
Na\(^+\) – sodium
Ca\(^{2+}\) – calcium
K\(^+\) – potassium
V – voltage
I – current
I\(_{Kr}\) – rapid K\(^+\) current
I\(_{Ks}\) – slow K\(^+\) current
K\(_{Ca}\) – Ca\(^{2+}\) -activated K\(^+\) channel
CNG – cyclic nucleotide-gated
HCN – hyperpolarization-activated cyclic nucleotide modulated
TRP – transient receptor potential
K\(_{ir}\) – inward rectifying K\(^+\) channel
Tetrodotoxin – TTX
SCA – spinocerebellar ataxia
FGF – fibroblast growth factor
FHF – fibroblast growth factor homologous factor
LVA – low-voltage activated
HVA – high-voltage activated
AID – α-interaction domain
CICR – Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release

RyR – ryanodine receptor

SERCA – sarcoplasmic-endoplasmic reticulum Ca\textsuperscript{2+} ATPase

NCX – Na\textsuperscript{+} /Ca\textsuperscript{2+} exchanger

SR – sarcoplasmic reticulum

T-tubules – transverse tubules

JPH2 – Junctophilin-2

LQTS – long QT syndrome

SCD – sudden cardiac death

BrS – Brugada syndrome

AIS – axon initial segment

NKA – Na\textsuperscript{+} /K\textsuperscript{+} ATPase

A – amperes

C – capacitance

Q – charge

R\textsubscript{S} – series resistance

R\textsubscript{M} – membrane resistance

F – Farad

EGTA – ethylene glycol tetraacetic acid

NMDG – N-methyl-D-glucamine

g – conductance

MEM – minimal essential medium

DMP – dimethyl pimelimidate
CON – control

FGF13 KD – FGF13 knockdown

SCR – scrambled shRNA

FFT – fast fourier transform

MORN – membrane occupation recognition nexus

WT-FGF12 – wild-type FGF12

Q7R-FGF12 – FGF12 Q7R mutant

ITC – isothermal titration calorimetry
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1. Introduction

1.1 Sudden cardiac death: clinical significance

Sudden cardiac death (SCD) is the leading cause of death in the developed world. Accounting for the deaths of more than 1 million people worldwide annually, it surpasses the total from lung and breast cancer, stroke, and acquired immune deficiency syndrome combined.\(^1\) SCD is also the leading cause of death in the United States of America, accounting for greater than 500,000 deaths per year.\(^2\) While the majority of cases of SCD are due to ischemic heart disease, SCD in the absence of structural heart disease typically occurs in a younger population of patients and is due to inherited or genetic disease.\(^3\) These electrical disturbances in the absence of heart disease point to an intrinsic defect in the electrical activity of the heart as driven by ion channels and have been termed “ion channelopathies.” Inherited/genetic arrhythmias will be the focus of this thesis.

1.2 The electrical activity of the heart

Ion channels are integral membrane proteins that allow the conduction of ions across the cell membrane. Ion channels drive the electrical activity of the heart, reflected in the sequential activation of cells in specialized, pacemaker regions of the heart and the propagation of electrical activity through the ventricles (Figure 1). Intrinsic activity of the heart is initiated at the sinoatrial (SA) node and propagated through the atria to the atrioventricular (AV) node, where there is a brief pause. Electrical activity then spreads through the bundle of His to the Purkinje fibers, through the septum, to the apex and working myocardium of the ventricles.
The gross electrical activity of the heart can be recorded non-invasively using the electrocardiogram (ECG, Figure 1, bottom). The P-wave reflects SA node activity (yellow) leading atrial depolarization (green). The PR interval (time between P and R wave) is the pause time within the AV node (orange). Following this pause, ventricular depolarization occurs from the septum, to the apex and up to the ventricular walls, portrayed on the ECG as the QRS complex (blue). Repolarization of the ventricle then occurs in the opposite direction to depolarization (T wave). As shown in Figure 1, cells in each of these specialized regions excite through generation of action potentials. SA and AV nodal action potentials have a slow upstroke, whereas atrial and ventricular...
action potentials have a fast upstroke and a plateau phase prior to returning to resting membrane potential. This heterogeneity is a reflection of the different ion channels expressed in the different regions; SA and AV nodal action potentials are initiated by slow calcium (Ca\(^{2+}\)) channels, whereas atrial and ventricular action potentials are initiated by fast sodium (Na\(^{+}\)) channels (the details of which will be discussed in Sections 1.2 and 1.3). Because the plasma membrane, made up of a phospholipid bilayer, does not permit the movement of ions, ion channels are necessary to allow the conduction of ions between the inside and outside of the cell. These ion channels also possess the property of voltage-dependence, allowing them to open in a particular order within the cell. The phospholipid bilayer acts to create a capacitor (a difference of charge separated by an insulator) because composition of the cytoplasm compared to the serum creates an electrical gradient in which the inside of the cell is negative compared to the outside. Additionally, the concentrations of ions inside and outside the cell create chemical gradients. For example, the positively charged cations, Na\(^{+}\) and Ca\(^{2+}\) are at very low concentrations inside the cell compared to the outside, in contrast to potassium ions (K\(^{+}\)), which are highly concentrated inside the cell. Using the Nernst equation, where \(S\) is an arbitrary ion with charge \(z_s\), \(R\) is the gas constant (8.3145 V C mol\(^{-1}\) K\(^{-1}\)), \(F\) is the Faraday constant (\(N_qe=9.6486 \times 10^4\) C mol\(^{-1}\)), and the intracellular and extracellular concentration of \(S\) ([\(S\)]\(_i\) and [\(S\)]\(_e\), respectively) is known, one can predict the reversal potential (\(V_s\)) of the ion and thus the direction of flow (into or out of the cell) at a given membrane potential.
This complex integration of these ionic currents results in the cardiac action potential. Here we will focus on the intricacies of the ventricular action potential, which has a different action potential waveform and different ion channel expression compared to the pacemaker SA and AV nodal cells (Figure 1).

1.2.1 The ventricular action potential

A more detailed view of the ventricular action potential can be seen in Figure 2.

Equation 1: Nernst Equation

\[ V_S = \frac{RT}{zF} \ln \frac{[S]_0}{[S]_i} \]

The complex integration of these ionic currents results in the cardiac action potential. Here we will focus on the intricacies of the ventricular action potential, which has a different action potential waveform and different ion channel expression compared to the pacemaker SA and AV nodal cells (Figure 1).

1.2.1 The ventricular action potential

A more detailed view of the ventricular action potential can be seen in Figure 2.
Figure 2: The key ion channels in cardiac cells and their contribution to the cardiac action potential phases. Reprinted by permission from Macmillan Publishers Ltd: Nature. Marbán E., Cardiac channelopathies, 415;213-218, copyright 2002.
The cardiac ventricular action potential is divided into five phases, each of which is controlled by specific ion channels. By convention, movement of positive current (I) into the cell is depicted as a negative deflection; outward movement of positive current is depicted as a positive deflection. Phase 0 is the rapid upstroke of the action potential mediated almost entirely by voltage-gated Na⁺ channels (purple, SCN5A, Naᵥ1.5), which allow Na⁺ to enter the cell down its electrochemical gradient, represented by a downward deflection. Phase 1 represents initial repolarization which occurs upon activation of rapidly activating and inactivating A-type K⁺ channels (green, KCND, Kᵥ4). The driving force for K⁺ is from the inside of the cell, to the outside, represented by a positive deflection in the current trace. The plateau phase, phase 2, occurs due to the slower activation and inactivation of inward Ca²⁺ current (orange, CACNA1C, Caᵥ1.2) in response to depolarization, relative to the Na⁺ channel. Repolarization (phase 3) is mediated by multiple K⁺ channels, including those encoded by KCNH2 and KCNE2 (HERG channel) and KCNQ1 forming the rapid (Iᵥr) and slow (Iᵥs) K⁺ currents respectively (green). The cell is maintained at its resting membrane potential (~ -85 mV) by the inward rectifying K⁺ channel encoded by KCNJ2 (green), represented as phase 4.

1.2.2 Ca²⁺ is the ultimate transducer of electrical signals into physiologic function

The function of excitable cells such as neurons and cardiomyocytes is not just to produce electrical activity. The electricity produced by ion channel activation needs to be translated into physiologic function. This is accomplished through the activation of voltage-sensitive Ca²⁺ channels that allow the entrance of Ca²⁺ into the cell such that Ca²⁺ may act as a second messenger.
Changes in intracellular Ca\textsuperscript{2+} concentration in response to activation of voltage-gated Ca\textsuperscript{2+} channels leads to many processes such as muscle contraction, neurotransmitter and hormone secretion, changes in gene expression and activation of signal transduction pathways.\textsuperscript{4} In the ventricular cardiomyocyte, activation of voltage-gated Ca\textsuperscript{2+} channels does not only create the prolonged plateau phase. Additionally, Ca\textsuperscript{2+} influx through voltage-gated Ca\textsuperscript{2+} channels increases Ca\textsuperscript{2+} inside the cell such that it can bind to and activate Ca\textsuperscript{2+} release channels in the sarcoplasmic reticulum (SR, a Ca\textsuperscript{2+} storage organelle). This leads to an even greater release of Ca\textsuperscript{2+} from the SR, allowing Ca\textsuperscript{2+} to bind to troponin, leading to the activation of the myosin-ATPase in the contractile machinery of the muscle and the creation of tension, leading to contraction. This is termed excitation-contraction coupling and will be discussed in greater detail in Section 1.3.

1.3 Voltage-gated ion channels

1.3.1 Voltage-gated ion channel structure

Voltage-gated ion channels are pore-forming structures within the plasma membrane. The principle subunits, so called α subunits (Figure 3), are composed of four homologous domains (I-IV).\textsuperscript{5,6} Within each homologous domain there are six transmembrane α-helices (S1-S6) with intracellular N- and C- termini.\textsuperscript{4}
Figure 3: Schematic of the voltage-gated ion channel structure. It has four homologous domains (I-IV), each made up of six transmembrane segments. S4 represents the voltage sensors. The channels have intracellular N- and C- termini.

While the α subunits of voltage-gated Na$^+$ channels and voltage-gated Ca$^{2+}$ channels are composed of one single peptide, encoding all 24 transmembrane segments, voltage-gated K$^+$ channels are comprised of tetramers of α subunits that are homologous to an individual domain in a Na$^+$ or Ca$^{2+}$ channel. While not the focus of this thesis, Ca$^{2+}$-activated K$^+$ channels (K$_{Ca}$), cyclic nucleotide-gated channels (CNG), hyperpolarization-activated cyclic nucleotide-modulated (HCN) channels, and transient receptor potential (TRP) channels also share a similar structure to the voltage-gated K$^+$ channels. The simplest structural motif is within the inwardly rectifying K+ channel (K$_{ir}$) in which the four subunits only have two transmembrane segments, M1 and M2, analogous to S5 and S6 in the other voltage-gated channels. When two of the pore motifs of K$_{ir}$ channels are linked together, they form the two-pore K+ channels.
The S5 and S6 segments along with the membrane re-entrant loop in between act as the selectivity filter and the pore. One of the first mammalian voltage-gated ion channels to be crystallized was the $K_r$ channel, of which the M1 and M2 segments (equivalent to S5 and S6) form an “inverted teepee” arrangement around the central pore, with the outer mouth formed by the membrane re-entrant loop. Other channels that have been crystallized show a similar structure.

The S1 through S4 segments comprise the voltage-sensor of the channels. In particular, the S4 segments have repeated motifs of one positively charged amino acid, followed by two hydrophobic residues. These are thought to serve as the primary voltage sensors as mutations in the charged amino acids in particular have tremendous effects on gating. It is still unclear how exactly this segment moves in response to voltage. However, it is known that the positive charges in these S4 segments respond to voltage change by movement that can be measured electrophysiologically as ~12 positive charges translocated outwardly (a small positive deflection).

While the transmembrane segments are critical for pore-formation, voltage-sensing and selectivity, the intracellular N- and C- termini and loops between the domains confer modulation of channel function by post-translational modifications such as phosphorylation and by binding of small intracellular ligands such as β subunits, $Ca^{2+}$, the $Ca^{2+}$-sensing protein, calmodulin, and other modulators of trafficking and gating. The function and modulation of voltage-gated $Na^+$ and $Ca^{2+}$ channels will be covered specifically in the two following sections.

### 1.3.2 Voltage-gated $Na^+$ channels

The basic properties of voltage-gated $Na^+$ channels, were described by Hodgkin and Huxley, beginning in 1952, when they were analyzing the action potential of the
squid giant axon using voltage clamp.\textsuperscript{24-27} They showed that nerve impulses are initiated by an inward current carried by Na\textsuperscript{+} in a voltage-dependent manner. This Na\textsuperscript{+} current inactivates rapidly, in 1-2 milliseconds, and outward K\textsuperscript{+} current re-establishes the resting membrane potential. Soon after, Hille developed a model of the ion selectivity filter and how it specifically selects for Na\textsuperscript{+} through a dehydration of the ion through interaction with carboxyl side chains of the channel followed by rehydration in the pore lumen and exit into the cell.\textsuperscript{28} Additionally, Hille discovered that certain Na\textsuperscript{+} channels are the targets for local anesthetics such as lidocaine and that they bind the channel through the open activation gate.\textsuperscript{29}

The Na\textsuperscript{+} channel protein was discovered through purification with a scorpion toxin found to block the channel, which revealed a large, 260 kDa \( \alpha \) subunit and smaller, 30-40 kDa \( \beta \) subunits (Figure 4). The topology of the Na\textsuperscript{+} channel is similar to other voltage-gated ion channels. Although the \( \alpha \) subunit is sufficient to traffic to the membrane and generate current, the channel is typically associated with two \( \beta \) subunits. Each have a transmembrane domain and an immunoglobulin-like extracellular domain and can modulate trafficking and gating of the channel.\textsuperscript{30}

There are nine isoforms (\( \text{Na}_v1.1-\text{Na}_v1.9 \)) of voltage-gated Na\textsuperscript{+} channels, which upon membrane depolarization, initiate action potentials in neurons and skeletal and cardiac muscle. They are divided generally into two groups based on their sensitivity to tetrodotoxin (TTX), first found in the puffer fish, Tetraodontidae, but later discovered in octopus, some frogs and newts.\textsuperscript{31} \( \text{Na}_v1.5 \) (in cardiac muscle), and \( \text{Na}_v1.8 \) and \( \text{Na}_v1.9 \) (found in dorsal root ganglion neurons) are all TTX-resistant channels and found on chromosome 3p21-24.\textsuperscript{32-34} These channels require micromolar concentrations of TTX.
(compared to nanomolar for the TTX-sensitive) for blockade, however can be fully blocked by cadmium.\textsuperscript{35, 36}

In neurons, voltage-gated Na\textsuperscript{+} channels are concentrated in the axon initial segment.\textsuperscript{37} They are required to generate an action potential in the axon in response to neural input.\textsuperscript{38} Mutations in voltage-gated Na\textsuperscript{+} channels can lead to hyperexcitability (typically gain of function) or conduction failure (typically loss of function). A paradoxical example of the effects of mutation on voltage-gated Na\textsuperscript{+} channels is a seizure disorder, called Dravet syndrome, which is due to a loss of function in Na\textsubscript{v}1.1. Na\textsubscript{v}1.1 is found primarily in inhibitory neurons, therefore a loss of inhibitory neuron excitability leads to hyperexcitability of the post-synaptic neuron and seizures.

In the heart, Na\textsubscript{v}1.5, encoded by SCN5A, predominates. Localized to the sarcolemma and intercalated discs of the cardiomyocyte,\textsuperscript{39} Na\textsubscript{v}1.5 is responsible for the initiation of the action potential and propagation through the cardiac myocardium. The "channelsome" of Na\textsubscript{v}1.5, reveals that Na\textsuperscript{+} channels reside in subdomains that are defined by the presence of Ankyrin-G, a scaffolding protein, originally discovered in red blood cells, but found to be required for proper neuronal and cardiac ion channel localization.\textsuperscript{40, 41} Ankyrin-G binds to the Na\textsuperscript{+} channel on the intracellular loop linking domains II and III (Figure 4, blue). Additionally, the C-terminal domain (CTD) of Na\textsubscript{v}1.5 interacts with multiple proteins that modulate its function and targeting. The crystal structure of the proximal CTD shows that calmodulin (CaM), which mediates the Ca\textsuperscript{2+} sensitivity of Na\textsubscript{v}1.5, binds to the Na\textsubscript{v}1.5 CTD distal to FGF13, a fibroblast growth factor (FGF) homologous factor (FHF).\textsuperscript{42-44} FHFs are intracellular FGFs (FGF11-14), bind to and modulate voltage-gated Na\textsuperscript{+} channels in brain, and we have shown how they
modulate $\text{Na}_\text{V}1.5$ specifically in heart. The role of FHFs in modulating cardiac $\text{Na}^+$ channel function will be discussed specifically in section 1.4.

Figure 4: Schematic of the $\text{Na}_\text{V}1.5$, the predominant cardiac $\text{Na}^+$ channel. It is typically associated with two $\beta$ subunits that interact with the intracellular N- and C-termini. Two important proteins that interact with $\text{Na}_\text{V}1.5$ are depicted at their interaction domains: Ankyrin-G (blue) at the II-III linker and FGF13 (red) at the C-terminus. Reprinted from Trends Cardiovasc Med, 211, Wei EQ, Barnett AS, Pitt GS, Hennessey JA, Fibroblast growth factor homologous factors in the heart: A potential locus for cardiac arrhythmias, 199-203, Copyright 2011.

### 1.3.3 Voltage-gated $\text{Ca}^{2+}$ channels

Voltage-gated $\text{Ca}^{2+}$ channels are the sine qua non of excitable cells-capable of translating electrical activity into cytoplasmic $\text{Ca}^{2+}$ changes that regulate cellular responses such as neuronal activity, muscle contraction and hormone release. The electrical activity they create can shape and form action potentials. For example, the pacemaker cells of the cardiac SA and AV node mentioned above only have HCN and
Ca\(^{2+}\) channels to generate the action potential.\(^{46,47}\) In contrast, the ventricular cardiomyocyte utilizes the fast inward Na\(^+\) current to generate the rapid phase 0 upstroke of the action potential and the Ca\(^{2+}\) current to form the plateau phase because Ca\(^{2+}\) channels have a much slower onset of activation and inactivation – hundreds of milliseconds versus one millisecond for Na\(^+\) channels\(^4\) (see Figure 2 for schematic showing their relative contributions to the cardiac action potential). Voltage-gated Ca\(^{2+}\) channels are permeant to divalent cations such as Ba\(^{2+}\) and Sr\(^{2+}\), which are used in experimental settings.

1.3.3.1 The voltage-gated Ca\(^{2+}\) channel superfamily

Voltage-gated Ca\(^{2+}\) channels were first classified by the amount of depolarization required for activation and then further classified by their pharmacological sensitivities and genetic analysis (Figure 5). In terms of their voltage-dependence of activation, one class only requires a small amount of depolarization to become activated, the low-voltage activated (LVA) Ca\(^{2+}\) channels. These channels also rapidly inactivate. In contrast, the second group, termed high-voltage activated (HVA) Ca\(^{2+}\) channels requires a large depolarization. These channels tend to inactivate more slowly.\(^{48,49}\) This contrast in activation allows one to hold the cell at depolarized potentials and isolate the HVA Ca\(^{2+}\) currents. In Bean, 1985,\(^{50}\) cardiac atrial cells were used to decipher these two currents. The cells were bathed in micromolar concentrations of TTX and 115 mM Ba\(^{2+}\). If the cells were held at -30 mV (which inactivates LVA channels), no current was recorded with a step to -20 mV (not depolarized enough to activate HVA channels). In contrast, with cells held at -30 mV and stepped to +10 mV, a large, inward, persistent current flowed, isolating the HVA currents. The LVA currents could be elicited by
holding the cell at -80 mV (hyperpolarized such that neither currents are inactivated) and stepping to -20 mV.

Further classification of HVA channels was done with pharmacologic differences between the channels. Cardiac, skeletal and smooth muscle, contain HVA channels that are dihydropyridine-sensitive. They additionally have a very slow rate of inactivation. These dihydropyridine-sensitive HVA Ca\(^{2+}\) channels were termed L-type for large and long-lasting, of which there are four genes that encode them. Muscle also contains LVA Ca\(^{2+}\) channels, which were termed T-type for tiny and transient, for which there are three genes. Neurons contain other HVA Ca\(^{2+}\) channels that have much faster inactivation than L-type. These are termed P/Q, N, and R type based on their pharmacology, with one gene for each, respectively.\(^5\) Figure 5 summarizes the homology, nomenclature and pharmacologic differences between the voltage-gated Ca\(^{2+}\) family.
Figure 5: Phylogeny of voltage-gated Ca\textsuperscript{2+} channel α subunits comparing the membrane-spanning segments and pore-loops (~350 amino acids). They are divided by high-voltage activated (HVA) or low-voltage activated (LVA), then by their alphabetical names as in Tsien et. al., 1988\textsuperscript{51}. Further classification was done using pharmacology. The α subunit protein name given is first its structural nomenclature and then the Snutch gene class.\textsuperscript{52} Pharmacologic information from Hille, 2001.\textsuperscript{4} Modified and reprinted from Neuron, Vol 25, Ertel EA, Campbell KP, Harpold MM, Hofmann F, Mori Y, Perez-Reyes E, Schwartz A, Snutch TP, Tanabe T, Birnbaumer L, Tsien RW, Catterall WA, Nomenclature of voltage-gated calcium channels, 533-535, Copyright 2000 with permission from Elsevier.

HVA Ca\textsuperscript{2+} channels associate with subunits that are necessary for proper function (Figure 6). The first HVA Ca\textsuperscript{2+} channel purified using H\textsuperscript{3}-dihydropyridine was the skeletal muscle Ca\textsubscript{V}1.1, from which a total of five subunits were co-purified.\textsuperscript{53} They were the α\textsubscript{1} (≈175 kDa, pore-forming subunit), α\textsubscript{2} (≈150 kDa), β (≈54 kDa), δ (17-25 kDa), and γ (≈32 kDa). It is now generally accepted that γ subunits play roles other than modulating Ca\textsuperscript{2+} channels.\textsuperscript{54} All subunits have been shown to enhance current density and affect kinetics in heterologous expression systems. The α\textsubscript{2} and δ subunits actually are one gene, which is cleaved and re-ligated with disulfide bonds. Thus, a reducing gel would show two separate bands.\textsuperscript{55, 56} β subunits are globular proteins that bind to an 18-amino acid binding motif on the intracellular loop between domains I and II, termed the α-interaction domain (AID).\textsuperscript{57} They enhance functional expression of HVA Ca\textsuperscript{2+} channels.
and have a major influence on the biophysical properties of the channel. Generally speaking, they hyperpolarize the voltage-dependence of activation, and increase the maximum open probability resulting in increased macroscopic current.\textsuperscript{58, 59} There is debate as to whether the β subunit enhances current density through masking an endoplasmic reticulum signal in the α subunit or inhibiting proteosomal degradation, of which there is evidence for both.\textsuperscript{60, 61} Nonetheless, the β subunit is required for functional expression of HVA Ca\textsuperscript{2+} channels, in both heterologous expression systems and primary cells. This thesis will focus primarily on the HVA, dihydropyridine-sensitive, L-type, Ca\textsubscript{v}1.2 Ca\textsuperscript{2+} channel, encoded by \textit{CACNA1C}, and most highly expressed in the heart.

\textbf{Figure 6:} A schematic of the voltage-gated Ca\textsuperscript{2+} channel and its associated subunits. The β subunit binds the II-III linker. The α\textsubscript{2}δ subunit has a small transmembrane segment and a large extracellular domain.
1.4 Voltage-gated Ca\(^{2+}\) channels translate electrical activity into physiologic function: focus on cardiac excitation-contraction coupling

1.4.1 Physiology of Ca\(^{2+}\)-induced Ca\(^{2+}\) release

Excitable cells translate their electrical activity into action by Ca\(^{2+}\) fluxes modulated by voltage-sensitive, Ca\(^{2+}\)-permeant channels. Therefore, Ca\(^{2+}\) acts as a second messenger to activate many cell functions.\(^4\) The intracellular Ca\(^{2+}\) concentration \([\text{Ca}^{2+}]_i\) is quite low, ranging from 30 to 200 nM such that when a Ca\(^{2+}\) channel opens, there is a dramatic increase in \([\text{Ca}^{2+}]_i\) (typically 10 μM).\(^6\) This increase immediately activates those mechanisms to bring \([\text{Ca}^{2+}]_i\) back to basal levels, such as pumps and buffers.

In the heart, Ca\(^{2+}\) cycling is crucial for each beat, and the L-type Ca\(_V\)1.2 Ca\(^{2+}\) channel is the primary mediator of excitation-contraction coupling. Reviewed in Bers, 2002\(^6\) (Figure 7), upon depolarization, Ca\(^{2+}\) enters the cell in the form of Ca\(^{2+}\) current \((I_{\text{Ca}})\) from the outside through the Ca\(_V\)1.2, L-type Ca\(^{2+}\) channel, creating the plateau phase of the action potential. This leads to Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) from the SR through the juxtaposed ryanodine receptor (RyR). This raises the \([\text{Ca}^{2+}]_i\), allowing Ca\(^{2+}\) to bind to troponin C on the myofilaments, activating the contractile machinery.

Equally important is cardiac relaxation which requires machinery to decrease the \([\text{Ca}^{2+}]_i\) back to baseline, leading to the dissociation of Ca\(^{2+}\) from the troponin C. There are four pathways that remove Ca\(^{2+}\) from the cell: the sarcoplasmic-endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA), the sarcolemmal Na\(^+\)/Ca\(^{2+}\) exchanger (NCX), sarcolemmal Ca\(^{2+}\) ATPase and the mitochondrial Ca\(^{2+}\) uniporter.
1.4.2 Subcellular architecture: the dyad

The juxtaposition of the Ca$_V$1.2 L-type Ca$^{2+}$ channel and RyR (about 12 μm) forms a dyad (see Figure 8) and is crucial for CICR from the SR. The opening of one local L-type Ca$^{2+}$ channel in each couplon (a single Ca$_V$1.2 juxtaposed to multiple RyR), leading to two to four Ca$^{2+}$ ions binding to RyR, is sufficient to activate full Ca$^{2+}$ release at that couplon. Coupled gating between RyRs or high local Ca$^{2+}$ (> 10 μM) leads to the activation of neighboring RyRs.$^{62}$

Confirmed by electron microscopy,$^{63}$ ventricular cardiomyocytes contain transverse tubules (T-tubules), which are invaginations of the sarcolemmal membrane in the transverse direction. These invaginations to the center of the cell explained how it
takes less than 40 ms for excitation to travel from the external membrane to the center of
the cell, about 50 μm.\textsuperscript{64} Ca\textsuperscript{2+} channels are enriched in the T-tubules – about three to
nine fold higher than the surface sarcolemma.\textsuperscript{65} The T-tubules bring the Ca\textsubscript{v}1.2, L-type
Ca\textsuperscript{2+} channels in direct juxtaposition with RyR in the SR to form the dyad.

\textbf{Figure 8:} A schematic depicting the cardiac dyad composed of the ryanodine
receptor (RyR) in sarcoplasmic reticulum (SR) juxtaposed to the Ca\textsubscript{v}1.2 Ca\textsuperscript{2+}
channel in the T-tubule. Junctophilin-2 (JPH2) mediates the distance between the
two membranous structures.\textsuperscript{66} JPH2 contains four domains, the most N-terminal
contains eight membrane occupation recognition nexuses (MORN), followed by an
α-helix, the divergent region and a transmembrane domain (TMD) through the SR.
Reprinted with permission from Wolters Kluwer, \textit{Circ Res}, Hennessey JA, Wei EQ,
Pitt GS. Fibroblast growth factor homologous factors modulate cardiac calcium

Interestingly, T-tubules are absent in neonatal cardiomyocytes, and appear to be
regulated by the load on the heart, as well as biochemical factors. Two molecules
responsible for some regulation of the T-tubules that I would like to focus on are BIN1
(amphiphysin 2) and junctophilin-2 (JPH2).
BIN1, a member of the BAR domain family of proteins, invaginates membranes into narrow tubules.\textsuperscript{67} Even when expressed ectopically in non-muscle cells, it causes tubular formation and has been shown to be concentrated at the sites of developing T-tubules.\textsuperscript{68} Additionally, BIN1 has been shown to be crucial for trafficking the Ca\textsubscript{v}1.2 L-type Ca\textsuperscript{2+} channel to the T-tubule\textsuperscript{69} and decreases in BIN1 have been associated with detubulation, decreased Ca\textsubscript{v}1.2 trafficking, and heart failure.\textsuperscript{70}

JPH2 promotes junction formation between the T-tubule and SR.\textsuperscript{71} As shown in Figure 8, JPH2 is capable of binding to the T-tubule plasma membrane via membrane occupation recognition nexus of which there are eight, and has a transmembrane domain through the SR. In between the two membrane binding motifs, there is an α helix thought to be responsible for the proper spacing between the T-tubule and SR, and a divergent region which varies among the four isoforms of JPH and is thought to mediate intracellular protein-protein interactions.\textsuperscript{66} Loss of JPH2 leads to disruption of the dyad architecture, leading to deficiencies in CICR, and dilated cardiomyopathy.\textsuperscript{72} Additionally, changes in JPH2 levels have been associated with heart failure,\textsuperscript{73} and mutations in JPH2 cause hypertrophic cardiomyopathy.\textsuperscript{74}

1.5 Fibroblast growth factor homologous factors\textsuperscript{1}

Fibroblast growth factor (FGF) homologous factors (FHF\textsubscript{s}) are a subset of the FGF family.\textsuperscript{75} Composed of four genes (FGF11-FGF14), FHF\textsubscript{s} were cloned by

\textsuperscript{1} Following an article published in Circulation Research (Wang \textit{et. al.}, 2011) in which we were the first to describe the endogenous role of fibroblast growth factor homologous factors in the heart, we were invited by Elizabeth G. Nabel, Editor-in-Chief for Trends in Cardiovascular Medicine to write a review of the current state of knowledge of FHFs in the heart and the questions that still surround them. This section is largely based on this review for which I was the supervising author.

homology after FHF1 (FGF12) was first identified in a screen for novel genes expressed in the retina. For each FHF, multiple isoforms with distinct N-terminal sequences are produced through alternative promoter usage and alternative splicing, yielding 10 FHF isoforms in humans. Unlike canonical FGFs, FHFs lack signal sequences, are not secreted, and do not bind to or activate FGF receptors. Rather, these intracellular proteins bind to and modulate voltage-gated Na\(^+\) channels.

The four mammalian FHFs bind directly to the CTD of voltage-gated Na\(^+\) channels, and different FHF isoforms differentially modulate Na\(^+\) channel function. In neurons, FHFs are concentrated at the AIS with voltage-gated Na\(^+\) channels and play a role in Na\(^+\) channel localization to the AIS and neuronal excitability. FHF dysfunction alters Na\(^+\) channel behavior in the nervous system and has been implicated in neurologic disease. Spinocerebellar ataxia 27 (SCA 27) is caused by a missense mutation in FGF14 due to decreased Na\(^+\) channels at the AIS leading to decreased neuronal excitability. In addition, nonspecific forms of X-linked mental retardation have been mapped to the FGF13 locus, suggesting that the FGF13 gene may be a candidate for these disorders.

### 1.5.1 FGF13 is the predominant cardiac FHF in mice and rats

Although all FHFs are highly expressed in the nervous system and developing heart, the expression profile of FHFs in adult cardiomyocytes remained unclear until recently. Quantitative reverse transcriptase-polymerase chain reaction with isoform-specific primers showed that FGF13 is the most abundant FHF in adult mouse and rat cardiomyocytes and that other FHF genes are expressed at relatively low levels. Specifically, FGF13-VY is the most highly expressed of all the FGF13 splice variants. Furthermore, only FGF13 could be detected by Western blot of mouse cardiomyocyte
lysate. These results are consistent with a recently published expression atlas of the FGF system in adult mouse showing high levels of FGF13 in the heart and low levels of other FHFs.\textsuperscript{88} The expression patterns of FHFs in human heart have not been reported and may be different from those in mouse.

### 1.5.2 FGF13 binds to Na\textsubscript{V}1.5 directly in murine cardiomyocytes

FGF13, in addition to being the predominant cardiac FHF, directly binds Na\textsubscript{V}1.5, the principal cardiac Na\textsuperscript{+} channel α subunit.\textsuperscript{78} FGF13 coimmunoprecipitated with Na\textsubscript{V}1.5 from mouse cardiomyocyte lysates, suggesting that FGF13 and Na\textsubscript{V}1.5 are in the same complex. An in vitro binding assay utilizing the Na\textsubscript{V}1.5 CTD and lysates expressing specific FGF13 isoforms indicates that this interaction is likely direct and that the CTD of the Na\textsubscript{V}1.5 binds to the core of FGF13. Data from surface plasmon resonance spectroscopy also support the hypothesis that the Na\textsuperscript{+} channel α subunit CTD and FGF13 core region interact directly.\textsuperscript{43, 79, 81} The crystal structure of a ternary complex of the Na\textsubscript{V}1.5 CTD, FGF13, and calmodulin confirmed this direct interaction and demonstrates that FGF13 binds to the globular domain of the Na\textsubscript{V}1.5 CTD, and residues distal to Ser1885 on Na\textsubscript{V}1.5 contribute significantly to the FHF interaction site on Na\textsubscript{V}1.5.\textsuperscript{43} Furthermore, immunocytochemistry shows that a portion of FGF13 colocalizes with Na\textsubscript{V}1.5 at the lateral membrane and intercalated disks in cardiomyocytes, two regions with distinct Na\textsubscript{V}1.5 populations.\textsuperscript{78, 89}

### 1.5.3 FGF13 modulates Na\textsuperscript{+} channel current density and kinetics in cardiomyocytes

The effect of FHF-Na\textsuperscript{+} channel α subunit binding on channel properties has been widely studied in heterologous expression systems and neurons. In these settings, FHF binding modulates Na\textsuperscript{+} current density, channel availability, and channel expression and
localization, the degree to which varies depending on the FHF isoform and Na\(^+\) channel α subunit.\(^{82-84}\)

The function of FHFs in cardiomyocytes and their role in the modulation of endogenous Na\(^+\) currents have not been studied until recently.\(^78\) Knockdown of FGF13 by infection of adult mouse ventricular cardiomyocytes with an adenovirus expressing a shRNA to all FGF13 splice variants reduced peak Na\(^+\) current density by 49%. Knockdown of FGF13 did not alter Na\(_V\)1.5 total protein or mRNA levels. However, surface Na\(_V\)1.5, measured by surface biotinylation, was reduced by 45%. In addition, FGF13 knockdown hyperpolarized the steady-state inactivation curve and delayed channel recovery from inactivation, further reducing channel availability (Figure 9). Together, these data suggest that FGF13-Na\(_V\)1.5 binding is necessary for proper Na\(^+\) channel function in cardiomyocytes.
Figure 9: Illustration of FGF13 regulation of Na\textsubscript{v}1.5 gating kinetics and membrane trafficking in wild-type (WT) cardiomyocyte (left) and FGF13 knockdown cardiomyocyte (right). Binding of FGF13 to the C-terminus of Na\textsubscript{v}1.5 modulates the voltage dependence of channel inactivation. FGF13 knockdown reduces the number of channels available to be activated: In the patch of sarcolemma illustrated, one of three channels is activated in the knockdown cardiomyocyte compared to four of five channels in WT cardiomyocyte. FGF13 is also required for proper trafficking of channels to the membrane: In the knockdown, only three of five channels are expressed at the membrane. Collectively, the reduced channel availability and surface expression lead to reduced current density. Furthermore, in the knockdown, delayed recovery from inactivation slows conduction velocity through cardiac tissue, providing a possible substrate for re-entrant arrhythmias. Reprinted with permission from *Trends Cardiovasc Med*, 211, Wei EQ, Barnett AS, Pitt GS, Hennessey JA, Fibroblast growth factor homologous factors in the heart: A potential locus for cardiac arrhythmias, 199-203, Copyright 2011.

1.6 Mutations in ion channels and their modulators lead to cardiac arrhythmia

Action potentials are shaped by the ion channels that contribute to them. Channelopathies can result from genetic mutation or malfunction of one or many ion channels within the cell. Genetic channelopathies can be a result of mutations in the
channel pore-forming subunits or a modulatory protein. The first cardiac channelopathies were identified in patients with congenital long QT syndrome (LQTS), in which mutations were found in the pore-forming subunits of voltage-gated K\(^+\) channels.\(^9\)

Channelopathies are responsible for about 50% of the sudden arrhythmic death (SAD) cases.\(^\)\(^9\) The two most prevalent congenital channelopathies are LQTS and Brugada Syndrome (BrS).\(^\)\(^9\)

1.6.1 Long QT Syndrome

Long QT syndrome is so named because it results in a prolonged QT interval on the surface ECG (Figure 10A, B). The QT interval is primarily determined by the action potential duration in the ventricular cardiomyocyte, and a prolonged QT interval typically occurs due to a reduction in repolarizing (typically K\(^+\)) currents or gain of function mutations in depolarizing (typically Na\(^+\) and Ca\(^{2+}\)) currents.\(^9\)

1.6.1.1 Genetics

The three most common mutations in LQTS are found in K\(^+\) channels, KCNQ1 (encoding KvLQT1, I\(_{Ks}\)) and KCNH2 (encoding HERG, I\(_{Kr}\)), and the voltage-gated Na\(^+\) channel, SCN5A (encoding Na\(_{V}1.5\)). Additionally, Timothy syndrome is a rare form of congenital LQTS caused by mutations in CACNA1C (encoding Ca\(_{V}1.2\)). Auxiliary subunits to the ion channels listed above such as β subunits, as well as proteins responsible for structure and trafficking such as ANK2, encoding Ankyrin-B and SNTA1, encoding α\(_{1}\)-syntrophin have also been shown to be loci for LQTS.\(^9\)

1.6.1.2 Pathophysiology

The mechanism through which these mutations might lead to disease is quite diverse, ranging from changes in intrinsic channel function (increased channel
availability, for example in the depolarizing currents), to proteosomal degradation or retention in the endoplasmic reticulum for the $K^+$ channels leading to decreased cell surface expression and decreased repolarization force.

For example, Timothy syndrome is characterized by a mutation in $\text{CACNA1C}$ which leads to a loss of channel voltage-dependence of inactivation. A prolonged $\text{Ca}^{2+}$ current leads to a longer phase 2 of the action potential (Figure 10A and B), leading to a prolonged QT interval.94

Clinically, a prolonged QT interval, predisposes patients to early after depolarizations (so called R on T phenomenon on ECG, Figure 10C), which can lead to a type of polymorphic ventricular tachycardia called torsades de pointes, ventricular fibrillation and SCD.92

**Figure 10:** Mechanism of prolonged QT interval in LQTS. A prolonged action potential (A) results in an increased time necessary to repolarize the cell back to resting membrane potential, leading to a prolonged QT interval on ECG, B. Reprinted from *Gene*, Vol 517, Abriel H, Zaklyazminskaya EV. Cardiac channelopathies: Genetic and molecular mechanisms, 1-11, Copyright 2013, with permission from Elsevier. C, a prolonged QT interval creates a susceptibility window during which an extrasystole can lead to torsades de pointes. Reprinted and with permission from John Wiley and Sons, *J Cardiovasc Electrophysiol*, Molecular and electrophysiological bases of catecholaminergic polymorphic ventricular tachycardia, Mohamed U, Napolitano C, Priori SG. 18:791-797, Copyright 2007.
1.6.2 Brugada syndrome

BrS is characterized by ST-segment elevation in the right precordial leads > 2 mm, pseudo right bundle branch block and T-wave inversion with an increased risk of SCD due to ventricular tachycardia.95

1.6.2.1 Genetics

BrS is typically inherited in an autosomal dominant manner, but many patients do not have a family history of BrS or SCD, making de novo mutations or incomplete penetrance possible.96 Mutations in the SCN5A gene account for 15-30% of all BrS cases.97 More recently, mutations in CACNA1C and its auxiliary subunits have also been implicated for a small portion of BrS.98 Other proteins known to traffic voltage-gated Na⁺ channels, such as MOG1 have also recently been implicated.99

1.6.2.2 Pathophysiology

Mutations in SCN5A or its trafficking proteins that lead to BrS are due to a loss of function of the Naᵥ1.5 Na⁺ channel. This can be due to a decrease in channel availability or a decrease in the amount of channel at the cell surface. Similar loss of function is observed in BrS patients due to Caᵥ1.2 loss of function. It is believed that this decrease in depolarizing currents leads to ST-segment elevation due to the higher expression of the repolarizing Iᵰ, Kᵥ4 K⁺ channel in the right ventricular epicardium compared to the endocardium. In a normal heart, there is little to no transmural (between epicardium and endocardium) voltage gradient (Figure 11A), even with higher epicardial Kᵥ4 expression. However, accentuation of the epicardial action potential notch due to loss of depolarizing Na⁺ or Ca²⁺ currents creates a larger transmural voltage gradient between the epicardium and endocardium, leading to ST-segment elevation in the form of a J-wave (Figure 11B). Additionally, this is typically heterogeneous within the
epicardium itself, leading to some cells with a complete loss of their dome and others that have delayed activation of the channels responsible for phase 2 and 3. This leads to dispersion of repolarization within the epicardium as well as a dispersion of repolarization between the epicardium and endocardium (transmural dispersion). This creates a vulnerable window during which an extrasystole can induce a re-entrant arrhythmia leading to ventricular tachycardia.

Because of this potential for re-entrant arrhythmia, patients with BrS have an increased risk for SCD. The majority of life-threatening arrhythmias develop during rest or sleep, or during routine daily activities. Additionally, fever and some medications can also provoke syncopes and cardiac arrest.  

![Diagram of action potentials](image)

**Figure 11:** Schematic representation of the right ventricular epicardial and endocardial action potential and the changes in the epicardial action potential that are thought to underly the electrocardiographic findings of BrS. Reprinted and with permission from John Wiley and Sons, *J Cardiovasc Electrophysiol*, The Brugada syndrome: ionic basis and arrhythmia mechanisms, Antzelevitch C, Feb;12(2):268-72, copyright 2001.
1.6.3 FHF may be unidentified loci for cardiac arrhythmias

The alterations in Na\textsubscript{v} 1.5 sarcolemmal expression and gating kinetics induced by FGF13 knockdown suggest that FHF loss-of-function mutations may be unrecognized causes of cardiac arrhythmias associated with Na\textsuperscript{+} channel loss of function. In addition, mutations in the gene encoding SCN5A that disrupt FHF interaction may produce equivalent effects as FHF loss-of-function mutations. FGF13 knockdown in mouse provides a model for studying potential loss-of-function mutations in cardiac FHFs, which may promote arrhythmogenesis via a combination of several mechanisms: (1) reduced trafficking of Na\textsubscript{v} 1.5 to the plasma membrane; (2) reduced channel availability; and (3) delayed recovery from inactivation, leading to (4) decreased conduction velocity (Figure 9).

Na\textsuperscript{+} channel dysfunction is central to LQTS and BrS. LQTS type 3 arises from mutations in SCN5A that disrupt fast inactivation of Na\textsubscript{v} 1.5, increasing inward sodium current and thereby prolonging the QT interval.\textsuperscript{102} Moreover, in recent years, mutations in genes encoding Na\textsubscript{v} 1.5 regulatory proteins have been discovered as new loci for cardiac arrhythmias. For instance, congenital LQTS types 9, 10, and 12 are linked to mutations in CAV3 (caveolin-3), SCN4B (β subunit 4 of sodium channel), and SNTA1 (α\textsubscript{1}-syntrophin), respectively, all of which lead to an increase in Na\textsubscript{v} 1.5 persistent current.\textsuperscript{103-105}

\textsuperscript{2} Following an article published in Circulation Research (Wang et al., 2011) in which we were the first to describe the endogenous role of fibroblast growth factor homologous factors in the heart, we were invited by Elizabeth G. Nabel, Editor-in-Chief for Trends in Cardiovascular Medicine to write a review of the current state of knowledge of FHFs in the heart and the questions that still surround them. This section is largely based on this review for which I was the supervising author.

Although no mutations in FHFs have yet been associated with inherited arrhythmias, it is possible that FHF dysfunction in cardiomyocytes may disrupt fast inactivation of cardiac Na⁺ channels, serving as an additional, yet unidentified locus for LQTS. It has been speculated that overexpression of FHFs above their endogenous low levels in the heart may produce physiological changes similar to those of LQTS.¹⁰⁶ The recent crystal structure of the FGF13 binding sites on Naᵥ1.5 suggests that FGF13 binds in a region of the CTD that contains a D1839G mutation associated with LQTS.⁴³,¹⁰⁷ This mutation may alter interaction with a knob formed by a conserved asparagine on FHFs, possibly affecting the affinity of FHF binding. Thus, it is possible that mutations at sites on either the FHF or Naᵥ1.5 CTD binding surfaces may produce a LQTS phenotype.

Of note, Liu et al. previously reported that a D1790G LQTS mutation abolished binding of FGF12B to the Naᵥ1.5 CTD.¹⁰⁶,¹⁰⁸ However, according to our recent crystal structure, the aspartate at 1790 does not contribute to the critical residues that form the FHF binding surface. Given that the interaction motif is conserved among Naᵥ1.x CTD-FHF pairs,⁴³ it is unlikely that FGF12B binds at a site different from FGF13; the identification of aspartate 1790 as a contributor to FHF binding may have been due to the use of a sensitive but not specific yeast two-hybrid method that used indirect assessment of interaction to perform the binding site mapping experiment.

BrS is often associated with a decrease in peak Na⁺ current due primarily to Naᵥ1.5 biophysical defects.¹⁰⁹ Given that only approximately 20% of BrS cases to date have defined genetic defects,¹⁰⁹ FHF mutations may be unexplored loci for the loss of Naᵥ1.5 channel function. Mechanisms for the decrease in current density include (1) reduced expression of the channel; (2) a shift in voltage or time dependence of activation, inactivation, or reactivation; and (3) accelerated or prolonged recovery from
inactivation. FHF dysregulation may contribute to the pathogenesis of BrS through many of these mechanisms. The FGF13 knockdown data suggest that FHF loss-of-function mutations may reduce current density by decreasing channel surface expression and causing a hyperpolarizing shift in inactivation. Furthermore, one of the hallmarks of Brugada syndrome—slowed conduction—could be explained by delayed Na⁺ channel recovery from inactivation. FHF loss-of-function mutations may lower the threshold of heart rate for which voltage-gated Na⁺ channels can fully recover, decreasing conduction velocity and maximum capture rate and providing a substrate for ventricular tachycardia/ventricular fibrillation and re-entrant arrhythmias.

In addition to modulating channel gating kinetics, FHFs play an important role in proper trafficking of Na⁺ channels to the cell membrane. In neurons, FGF14 appears to participate in targeting voltage-gated Na⁺ channels to the AIS: Expression of an FGF14 SCA 27-causing dominant negative mutant abolished binding of wild-type FGF14 to Naᵥ1.2 and reduced targeting of Na⁺ channels to the AIS. In the heart, FGF13 appears to play an analogous role in targeting sodium channels to the sarcolemma because knockdown of FGF13 is associated with reduced surface expression of Naᵥ1.5. Just as FHF mutations in the central nervous system impair neuronal excitability, mutations of FHFs in the heart, in addition to altering channel biophysical properties, may reduce cardiac excitability by disrupting channel membrane trafficking. It is unclear whether FGF13-mediated trafficking requires direct binding between FGF13 and the Naᵥ1.5 CTD or whether FGF13 acts in an integrated manner with other channel modulators. Because numerous modulatory proteins as well as the Naᵥ β subunits also bind to the Naᵥ1.x CTD and regulate channel trafficking, it is conceivable that FHFs may work in concert
with those other trafficking proteins. Laezza et al. (2007) speculate, for instance, that FHF s may modulate interaction between the Na\textsuperscript{+} channel \(\alpha\) and \(\beta\) subunits.

FHF s appear to be similar in function to ankyrins, which are adapter proteins responsible for proper expression and localization of ion channels and transporters in excitable and nonexcitable cells.\textsuperscript{112} Ankyrin-B (\textit{ANK2}) and Ankyrin-G (\textit{ANK3}) are the two predominantly expressed isoforms in the heart. Ankyrin-G binds to \(\text{Na}_V1.5\) at a motif in the linker loop between domains II and III (Figure 4);\textsuperscript{113} no direct interaction has been found between Ankyrin-B and \(\text{Na}_V1.5\). Genetic variants in \textit{ANK2} have been linked to LQTS type 4, also known as “Ankyrin-B syndrome,” an atypical arrhythmia with risk of sudden cardiac death.\textsuperscript{112} In addition, a mutation in the consensus sequence in \(\text{Na}_V1.5\) for binding to Ankyrin-G has been associated with BrS.\textsuperscript{114} This mutation reduced surface expression of \(\text{Na}_V1.5\) in cardiomyocytes and affected channel gating behavior in a heterologous expression system, suggesting that Ankyrin-G may modulate gating properties, in addition to its role in trafficking Na\textsuperscript{+} channels to the cell membrane.\textsuperscript{114} In a similar manner, mutations in the sequences encoding the CTD of \(\text{Na}_V1.5\) may abolish FHF binding, leading to reduced channel surface expression and abnormal gating properties. Whether FHFs and ankyrins interact to regulate channel activity remains unclear and merits further investigation.

It is likely that FHFs play other important roles in cardiomyocytes that have yet to be explored. Immunocytochemistry shows that FGF13 exhibits a widespread expression pattern in ventricular cardiomyocytes, including a punctate distribution in the cytoplasm and prominent nuclear localization.\textsuperscript{78} Of particular interest for future investigation are potential interactions between FHFs and other cardiac ion channels.
2. Exploring the role of FHF in cardiac physiology in the native ventricular cardiomyocyte: Methodology

In this section, I will explain the methodology of isolation of ventricular cardiomyocytes, adenoviral production and expression, and electrophysiology – the central experiments behind the data I will be describing in Chapters 3 and 4. For much of the optimization of these protocols, I worked with Chuan Wang, a postdoctoral fellow in the laboratory of Geoffrey Pitt. With this methodology, we published a paper on FGF13 modulation of Na\textsubscript{v}1.5 function in ventricular cardiomyocytes. Some of the data shown in this Chapter to validate the methods derive from this paper.

2.1 Introduction

Ion channels are not just single protein moieties, stuck in the membrane in isolation. Instead, as more data emerges, it is clear these channels are surrounded by a large complex of modulatory and scaffolding proteins, termed the “channelsome.” Indeed, an interaction proteomics screen of the neuronal pre-synaptic Ca\(^{2+}\) channels using affinity purification and mass spectroscopy showed there are ~200 proteins within the nanoenvironment of these channels. Additionally, using quantitative mass spectroscopy, they showed that these proteins vary in abundance, stability of assembly and preference for a specific pre-synaptic Ca\(^{2+}\) channel.\(^{54}\) Furthermore, many modulatory proteins are part of multiple ion channels’ nanoenvironment. Take for example the Ca\(^{2+}\) sensor, calmodulin, that binds to and modulates Ca\(^{2+}\) sensitivity of Na\(^+\), Ca\(^{2+}\), and K\(^+\) channels.\(^{43, 115, 116}\)

The cardiomyocyte consists of multiple nanoenvironments. The intercalated disc is defined by the presence of gap junctions made up on Connexin 43 and voltage-gated Na\(^+\) channels so that electrical activity can conduct through the myocardium.\(^{117}\) The sarcomere is its own nanoenvironment, which houses the contractile filaments and a whole host of modulators.\(^{118}\) The SR is responsible for storing Ca\(^{2+}\). Proteins in the SR are capable of releasing that Ca\(^{2+}\) as well as sensing the levels within that compartment.\(^{118}\) Finally, the T-tubules, a necessary structure for efficient CICR, house Ca\(_{\nu}1.2\) Ca\(^{2+}\) channels, NCX, and the Na\(^+\)/K\(^+\) ATPase.\(^{119}\) Within each of these nanoenvironments, there are proteins specific to the region and required for proper function of that region.

Investigators in the past when identifying ion channel modulators have ignored the other components of the cardiomyocyte and performed experiments of channel
function in a heterologous expression system – one channel with one modulator. What is becoming clearer as more ion channel modulators are identified as loci for inherited arrhythmias is that they are not portraying the whole picture in using those systems. Indeed, even twenty years after the discovery of the beta-adrenergic modulation of voltage-gated Ca\textsuperscript{2+} channels, it was only recently that one could recapitulate this mechanism in a heterologous expression system,\textsuperscript{120} and still with some controversy.\textsuperscript{121} This is one of many examples of data suggesting that heterologous expression systems do not provide a sufficient environment for all cellular physiologic processes.

There are many examples of channel modulators that play multiple roles, providing further evidence that the transfection of one type of channel with one type of modulator into a HEK293T cell does not accurately replicate the physiology of an excitable cell. For the FHFs, this has been a theme throughout the studies of their role in modulating voltage-gated Na\textsuperscript{+} channels in neurons; investigators have reported opposing effects when comparing heterologous expression systems to primary neuronal culture (Figure 12).\textsuperscript{82} Lou et. al.\textsuperscript{82} compared the current densities elicited either in a heterologous expression system with FGF14 overexpressed with Nav1.1 to that of a primary hippocampal neuron with FGF14 overexpression. Specifically, FGF14-B (labeled as FGF14-1b in Figure 12), decreases current density when co-expressed with Nav1.1 (Figure 12, top) and with Na\textsubscript{V}1.5 (not shown) and in Neuro-2A cells, a neuronal cell line.\textsuperscript{82} In contrast, when FGF14-B is overexpressed in a primary hippocampal neuron, it increases current density above the basal level (Figure 12, bottom). This epitomizes the opposing effects on Na\textsuperscript{+} channel function observed between heterologous expression systems and primary cells.
Figure 12: Comparison of effects of FHF on Na⁺ channel function in heterologous expression system versus neurons. Note that in a heterologous expression system, FGF14-1b decreases current density (top) while in hippocampal neurons, it increases current density compared to control. Modified and reprinted with permission from John Wiley and Sons, *J Physiology*, Lou JY, Laezza F, Gerber BR, Xiao M, Yamada KA, Hartmann H, Craig AM, Nerbonne JM, Ornitz DM. Fibroblast growth factor 14 is an intracellular modulator of voltage-gated sodium channels, 569:179-193, copyright 2005.

Our lab has seen similar discrepancies between heterologous expression systems and cardiomyocytes. As shown in Figure 9, Section 1.3.3, knockdown of FGF13 in ventricular cardiomyocytes reduces Naᵥ1.5 current density. However, when one co-expresses various FGF13 splice variants with Naᵥ1.5 in a HEK293 cell, the current density is also reduced (data by Chuan Wang, 2009, see Chapter 4). This again demonstrates that studying these modulatory proteins in heterologous expression systems does not accurately describe their roles in native cells.
Therefore, to elucidate the true physiologic effects of FHFs on cardiac function, I came up with a strategy to study FHFs in their native environment. We used isolated adult ventricular cardiomyocytes and manipulated their genetic environment with adenoviral gene transfer of shRNA or FHF genes. To study the electrophysiologic effects of FHFs on channel function, we used whole-cell patch clamp. These methods are summarized in the following sections.

2.2 Isolation and culture of ventricular cardiomyocytes using a Langendorff apparatus

2.2.1 Introduction

To eliminate the confounding data from heterologous expression systems, for the following studies, I designed a cardiomyocyte culture system in which I could knockdown endogenous FGF13 (as shown in Wang et. al., 2011), and rescue it with various FGF13 splice variants and other FHF isoforms. To be able to successfully infect cardiomyocytes with multiple viruses, I needed to come up with a way to not only isolate cardiomyocytes with minimal ischemic time, but also be able to keep them in culture such that the viruses could exert their genetic effects on the cell.

2.2.2 Materials and Methods

C57/Bl6 mice or Sprague-Dawley rats, age 6 to 8 weeks old, are initially anesthetized with 250 mg/kg tribromoethanol (Sigma) and anti-coagulated with 8,000 units/kg Heparin (Sigma) intraperitoneally (IP). Following induction of anesthesia (usually 2 to 5 minutes), another 8,000 units/kg Heparin is given IP to ensure full anti-coagulation of the coronary vessels. After another three minutes, the body is wiped with 70% ethanol and a bilateral thoracotomy performed. The heart is removed, taking care to leave the aorta and at least the first branch (the brachiocephalic artery) intact for
proper cannulation and placed in a cold bath of Ca\(^{2+}\)-free basal isolation solution (BIS, see Table 1 for recipe). This provides cardioplegia (paralysis of the heart) through a decrease in temperature and a lack of extracellular Ca\(^{2+}\). The heart is then moved to a second, cold bath of BIS and placed under the dissecting microscope for cannulation of the aorta. The aorta is tied onto the cannulus using 5-0 suture. When performing isolations of rat ventricular cardiomyocytes, if the aorta was too large for the cannula, the distal aorta would be tied off and the brachiocephalic artery cannulated. To ensure both coronary arteries are being perfused, the cannula is attached to a syringe containing BIS and the BIS is slowly pushed into the heart using the plunger. Washout of the blood from the coronary vessels ensures 1) that the proper vessel (the aorta) was cannulated and 2) the cannula is not blocking either the left or right coronary artery coming off the aorta.

Following confirmation of proper cannulation, the heart is hung on a Langendorff apparatus (Figure 13). A Langendorff apparatus is a glass tube encased in a water jacket. It is attached to a peristaltic pump that allows the constant flow of solution. Cannulation occurs in the aorta for retrograde perfusion of the ascending aorta. This forces the aortic valve closed and shunts the blood flow via the coronary ostia into to the coronary arteries. Thus, the solution flowing through the Langendorff apparatus provides the nutrition and oxygen such that the ventricular myocardium is still functional. For the first 3 to 5 minutes, the heart is perfused with BIS to wash out most of the blood. The solution is then switched to enzyme solution to dissociate the cells (BIS plus 150 u/ml collagenase type II (Worthington)). The perfusion with collagenase is continued until there is no blood left in the heart and it becomes white and boggy (typically 13 or 20 minutes for mouse or rat respectively). The heart is then removed from the Langendorff
apparatus and placed in a dish containing enzyme solution. The rest of the steps are performed in a sterile hood. The heart is minced into small chunks and trititerated using a sterile disposable pipette. The solution of cardiac tissue is placed in a conical tube and allowed to sit at 37 °C for two minutes. The solution is then filtered through 190 μm nylon mesh (Amazon.com) to remove any undigested tissue and centrifuged at 300 G for 2 minutes to pellet the ventricular cardiomyocytes. The cells are then washed sequentially with increasing amounts of Ca²⁺ (50 μM, 250 μM, 500 μM, and 1 mM) in BIS plus 500 mg/ml bovine serum albumin (BSA solution) to quench the excess enzyme and perform Ca²⁺ tolerance. Between each Ca²⁺ step, the cells are allowed to pellet by gravity and the supernatant switched to remove dead cells and any bacteria that might have come from the initial isolation. Following the final step of Ca²⁺ tolerance, the cells are resuspended in plating medium (Table 2) and plated onto sterile glass coverslips previously coated with 20 μg/ml laminin (Sigma) in Dulbecco’s Modified Eagles Medium in 24 well cell culture plates. The cells are then allowed to adhere for one hour. Following adherence, the cells are washed once with culture medium (Table 3), and culture medium plus or minus virus is added to the wells for 48 to 60 hour culture.

The culture medium has some components that are worth describing further. Most importantly, it contains two contractile inhibitors: 2,3-butanedione monoxime (BDM) and blebbistatin. BDM (Sigma) is a reversible myosin ATPase inhibitor that also has phosphatase-like properties (part of the oxime family). For that reason, high concentrations were originally used to rescue cholinesterase activity lost due to organophosphate insecticide poisoning. It has been shown to be cardioprotective during ischemia and perfusion. We use it during isolation and in the culture medium to prevent the cells from contracting down and to reduce myocardial perfusion damage.
While the potential effects on ion channel activity due to its phosphatase properties are less clear, it is known that lower concentrations of BDM (5 to 10 mM) do not have very high phosphatase activity and that BDM washes out within 5 minutes.\textsuperscript{124} Therefore during our electrophysiologic recordings, we do not include it in the bath solution, but ensure that the coverslip perfuses for 5 minutes with BDM-free solution prior to beginning recording.

Blebbistatin (Toronto Research Chemicals) is a cell motility inhibitor that preferentially binds the ADP-P\textsubscript{i} complex of myosin II, inhibiting myosin cycling.\textsuperscript{125} It has been shown to extend the life of cultured adult ventricular cardiomyocytes and improve adenoviral gene transfer. Additionally, it has been used in a whole heart Langendorff set-up to remove motion artifact such that imaging can be performed.\textsuperscript{126, 127} I also use blebbistatin at 7.5 $\mu$M in my recording solution for performing whole-cell patch clamp.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure13.png}
\caption{A, Langendorff apparatus schematic depicting the solution being sent through the peristaltic pump and into the water jacket for perfusion of the heart. B, comparison of freshly isolated cardiomyocytes and cultured cardiomyocytes. DIV, days in vitro. Scale bar 20 $\mu$m.}
\end{figure}
Table 1: Basal Isolation Solution for Cardiomyocyte preparation

<table>
<thead>
<tr>
<th>Component</th>
<th>FW</th>
<th>Concentration (mM)</th>
<th>Mass (g) for 1 L</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>58.44</td>
<td>112</td>
<td>6.55</td>
</tr>
<tr>
<td>KCl</td>
<td>74.56</td>
<td>5.4</td>
<td>0.40</td>
</tr>
<tr>
<td>NaH₂PO₄·H₂O</td>
<td>137.99</td>
<td>1.7</td>
<td>0.23</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>203.30</td>
<td>1.63</td>
<td>0.33</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>84.01</td>
<td>4.2</td>
<td>0.35</td>
</tr>
<tr>
<td>HEPES</td>
<td>238.30</td>
<td>20.04</td>
<td>4.78</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>180.16</td>
<td>5.4</td>
<td>0.97</td>
</tr>
<tr>
<td>Taurine</td>
<td>125.15</td>
<td>10</td>
<td>1.25</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>146.15</td>
<td>4.1</td>
<td>0.60</td>
</tr>
<tr>
<td>L-carnitine HCl</td>
<td>197.66</td>
<td>2</td>
<td>0.40</td>
</tr>
<tr>
<td>Creatine, anhydrous</td>
<td>131.13</td>
<td>2.3</td>
<td>0.30</td>
</tr>
<tr>
<td>2’3-butanedione monoxime</td>
<td>101.10</td>
<td>10</td>
<td>1.01</td>
</tr>
</tbody>
</table>

*pH to 7.2 with NaOH
*check osmolarity (~ 300 mOsm/L)
*filter sterilize
*store at 4 °C

Table 2: Plating Medium

<table>
<thead>
<tr>
<th>Component</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>MEM with Earle's salts and L-glutamine (Mediatech)</td>
<td>100 u/ml</td>
</tr>
<tr>
<td>Penicillin-Streptomycin</td>
<td>100 u/ml</td>
</tr>
<tr>
<td>Fetal bovine serum</td>
<td>5%</td>
</tr>
<tr>
<td>2,3-butanedione monoxime</td>
<td>10 mM</td>
</tr>
<tr>
<td>Table 3: Culture Medium</td>
<td></td>
</tr>
<tr>
<td>-------------------------</td>
<td></td>
</tr>
<tr>
<td>MEM with Earle's salts and L-glutamine (Mediatech)</td>
<td></td>
</tr>
<tr>
<td>Penicillin-Streptomycin</td>
<td>100 u/ml</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>0.1 mg/ml</td>
</tr>
<tr>
<td>2,3-butanedione monoxime</td>
<td>10 mM</td>
</tr>
<tr>
<td>Insulin</td>
<td>10 μg/ml</td>
</tr>
<tr>
<td>Transferrin</td>
<td>5.5 μg/ml</td>
</tr>
<tr>
<td>Selenium</td>
<td>6.7 ng/ml</td>
</tr>
<tr>
<td>Creatine, anhydrous</td>
<td>5 mM</td>
</tr>
<tr>
<td>Taurine</td>
<td>5 mM</td>
</tr>
<tr>
<td>L-carnitine HCl</td>
<td>2 mM</td>
</tr>
<tr>
<td>Blebbistatin</td>
<td>25 μM</td>
</tr>
</tbody>
</table>

2.3 Adenoviral transduction

2.3.1 Introduction

The major impediment to performing physiologic studies in adult ventricular cardiomyocytes is that they cannot be efficiently transfected by methods typically used with immortalized cell lines. Adult ventricular cardiomyocytes are terminally differentiated cells and do not divide, so liposomal or chemical strategies for transfection are ineffective.

Most investigators have therefore used viral vector-mediated gene transfer. Adenovirus was used first in the 1990s and proved to be very efficient in rat, rabbit,128

1 We were invited by Paul Insel, Editor-in-Chief for American Journal of Physiology-Cell Physiology to write an Editorial Focus to review methods of gene transfer in adult ventricular cardiomyocytes. These paragraphs are largely based on this review article.

Amenta JJ, Pitt GS. The glitter of gold: Biolistic transfection of fresh adult cardiac myocytes. Focus on "normal targeting of a tagged kv1.5 channel acutely transfected into fresh adult cardiac myocytes by a biolistic method". Am J Physiol Cell Physiol. 2010;298:C1305-1307

The American Journal of Physiology-Cell Physiology, does not require permission to reprint in a dissertation.
and mouse\textsuperscript{129} adult ventricular myocytes. Although there is question as to whether adenoviral-mediated gene transfer disrupts cellular physiology by promoting dedifferentiation,\textsuperscript{130} this technique has enjoyed substantial success. For example, Zhou et al.\textsuperscript{129} were able to express either β1- or β2-adrenergic receptors and rescue contractile and calcium current responses to β-adrenergic receptor stimulation in β1/β2-receptor double-knockouts, providing a means to study the effects of β-adrenergic signaling on Ca\textsuperscript{2+} currents in the ventricular cardiomyocyte. Lentivirus has also been used effectively and offers the advantage of having about two-fold greater transgene capacity relative to that of adenoviruses.\textsuperscript{131} A shortcoming, however, is very low transduction efficiency.

2.3.1.1 Adenoviral gene transfer versus other modes in terminally differentiated cells

Other methods of gene transfer in adult ventricular cardiomyocytes have been used successfully. For example, Dou et al.\textsuperscript{132} adapted the gene gun approach. This biolistic method, which uses high-speed propulsions of subcellular particles (typically gold) coated with DNA, has been successfully employed in neuronal tissue sections and primary neuronal culture systems, both of which share with ventricular myocytes the difficulty of transfection into terminally differentiated cells.\textsuperscript{133} Previous attempts to use this technique for ventricular myocytes failed to produce surviving, transfected myocytes—an experience that Dou et al. initially replicated. Systematic alterations to the standard protocol (e.g., omitting polyvinylpyrrolidone during preparation of the cartridges, and optimization of both gold particle size and bombardment pressure), however, yielded a transfection efficiency of almost 30% of surviving myocytes in less than 24 hours, and the transfected cells remained viable and retained their morphological
characteristics. In cells transfected with labeled Kv1.5, Dou et al. confirmed the localization of these channels at intercalated disks and demonstrated an increased, sustained K⁺ current component sensitive to 100 μM 4-aminopyridine, a Kv1.5 blocker. While these results establish that Kv1.5 channels are, indeed, localized to intercalated disks and, through use of expressed, truncated channels, identify channel determinants for proper trafficking to the plasma membrane, the most interesting result may be the demonstration of an effective and simple way to transfect genes into cardiac ventricular myocytes.

Although transfection by gene gun may not be suitable for all applications, it offers significant advantages over viral transduction for certain experimental questions. For example, the opportunity to use plasmid DNA obviates the need for generating viruses, which should save considerable time and effort that could then be applied to experimental studies, such as more thorough and creative testing of multiple constructs and mutants. Moreover, the maintenance of the normal rod-shaped myocyte morphology after transfection suggests that information gained from gene gun transfections will provide valuable insights into the function of cardiac myocytes.

Despite these bright spots, there are some areas in which this technique might lack luster. As reported by Dou et al., the gene gun bombardment method kills 90% of the viable myocytes, and of the 10% surviving, only ~30% are transfected. Thus, this technique may be especially suited for single-cell experiments, e.g., imaging and electrophysiology as performed in this study, in which the ability to perform analysis in transfected myocytes transcends the need for highly efficient transfection; in contrast, the relatively low level of transfection will render biochemical analyses challenging.
I attempted the gene gun approach with a similar protocol as described by Dou et. al. however was unsuccessful. Therefore, I utilized the adenovirus system, which I will describe in more detail in the following section. While it took more time and preparation to make the adenovirus, it ultimately provided the capability to perform both biochemical and single cell experiments in the same culture, with greater cell viability and reproducibility than reported by Dou et. al. with the biolistic approach.

2.3.2 Materials and Methods

We wanted to study the effects of knockdown of endogenous FGF13 on voltage-gated Na\(^+\) channel function and targeting to the cell surface. A shRNA to FGF13 and scrambled shRNA was designed by Chuan Wang (see Table 4 for sequence) with the RNA polymerase III H1 promoter.\(^{134}\) The FGF13 shRNA and scrambled shRNA was cloned into the pAdTrack-GFP plasmid obtained from ATCC using Sal I and Xho I restriction sites. The rest of the procedure was performed as stated in Luo et. al.\(^{135}\)

<table>
<thead>
<tr>
<th>FGF13 shRNA</th>
<th>Scrambled shRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’-GCACTTACACTCTGTTTAATTCAAGAGATTAACAGAGGTGTAAGTGC-3’</td>
<td>5’-GACCCTTAGTTTATACCTATTCAAGAGATAGGTATAAAACTAAGGGTC-3’</td>
</tr>
</tbody>
</table>

To test the efficiency of knockdown, three strategies were employed. The first was immunocytochemistry. Because the virus expressed a GFP simultaneously with the shRNA, we could identify infected cells and stain them using an antibody specific to FGF13 (Figure 13A).\(^{78}\) We observed a large decrease in the immunoreactivity of the FGF13 antibody with FGF13 KD (Figure 14A).
Figure 14: Specific knockdown of FGF13 by shRNA decreases Na⁺ channel current in adult mouse ventricular myocytes. (A) Confocal images of immunocytochemistry of FGF13 (red) after infection with adenovirus expressing FGF13 shRNA (bottom panels) or a Scrambled control (top panels) in ventricular myocytes. Infected myocytes were identified by GFP expression. Nuclei are stained with DAPI. Scale bar, 25 μm. (B) Quantitative analysis of FGF13 (using primers in the FGF-like core designed to amplify all FGF13 isoforms; see Online Table I) and FGF12B mRNA levels by qPCR in uninfected cultured myocytes (Control) or those infected with scrambled shRNA (Scrambled) or FGF13 shRNA (shRNA). All data were corrected with GAPDH and normalized to Control. **p<0.01, compare to control; n=6 for FGF13 and n=3 for FGF12-B. (C) Representative immunoblot of FGF13 protein expression in isolated control myocytes, or myocytes infected with Scrambled or shRNA. Result is representative of three independent experiments. (D) Histograms showing normalized amounts of FGF13 protein from Control, Scrambled, or shRNA treated myocytes. **p<0.01, compared to control; n=3. GAPDH was used as loading control. Reprinted with permission from Wolters Kluwer, Circ Res, Wang C, Hennessey JA, Kirkton RD, Wang C, Graham V, Puranam RS, Rosenberg PB, Bursac N, Pitt GS. Fibroblast growth factor homologous factor 13 regulates Na⁺ channels and conduction velocity in murine hearts. Sep 16;109(7):775-82, copyright 2011.
Then, because of the high efficiency of adenoviral infection, we could perform more quantitative biochemical studies on the efficiency of our shRNA. In Figure 14B, summarized data from quantitative reverse-transcriptase polymerase chain reaction shows about 90% knockdown of the total FGF13 transcripts. Additionally, western blot using the previously described antibody to FGF13 showed similar results (Figure 14C-D).  

Additionally, I devised strategies to rescue the loss of FGF13 with a second adenovirus encoding a shRNA-resistant human FGF13 splice variant. This is described in Chapter 3.

Adenovirus was made using the commercially available AdEasy System (Agilent Technologies) designed by Bert Vogelstein’s lab. The shuttle vector pAdTrack-GFP was used to insert the shRNA and pAdRFP for the overexpression constructs described in Chapter 3.

The adenovirus was purified using the VivaPure Adenopack 20 purification kit with some modifications to the protocol. After at least two rounds of initial amplification in HEK293 cells, adenovirus was amplified in low-passage HEK293 cells in three to four T-75 flasks (about 40 mls). Once almost 100% of cells were fluorescing (ideally 5 days, but could be longer), cells were detached with a scraper (no trypsin) and put in conical tube. Cells were centrifuge at 3500 g for 15 minutes to pellet cell debris and all but 5 ml of supernatant is removed. Cells were put through at least three freeze-thaw cycles (vortex, freeze in liquid nitrogen, thaw in 37 °C water bath) to lyse the cells and dissociate the majority of the virus which is stuck to the outside of the cells. The solutions from the three to four flasks are then combined and filtered through 0.45 μm cellulose acetate vacuum filters (Corning). Benzonase (from the kit) is then added at
1:1000 dilution and solution incubated for 30 minutes at 37 °C in order to digest cellular nucleic acids. The AdenoPACK 20 Maxi (clear top) is then equilibrated with 5 ml Wash Buffer and spun for 5 minutes at 500 g. Use a swing out rotor centrifuge for the rest of the steps. Digested supernatant is then loaded onto the Vivaclear Maxi (yellow top) and spun for 5 minutes at 500 g at 4°C or until whole volume passes through. Flow through is collected and 10x loading buffer is added while vortexing the solution at low speed. Sample is then loaded onto the AdenoPack Maxi (clear top) and spun for 5 minutes at 500 g or until whole volume has passed through the membrane. The column is then washed twice with 18 ml washing buffer and virus eluted with 1 ml of elution buffer. Elution buffer is pipetted onto column membrane and incubated for 20 minutes at 4 °C. The column is then spun for 5 minutes at 500 g to elute. This is repeated once to result in a total of 2 ml of eluate. Eluate is transferred to a Vivaspin 20 centrifugal concentrator and virus storage buffer (20 mM Tris HCl pH 8.0, 25 mM NaCl, 2.5% Glycerol in ddH$_2$O, filtered) is added to bring the volume to 10 ml. The concentrator is then spun for 30 minutes at 800 g or until the volume has gone below 1 ml. This is repeated again. Typically a volume of 700 μl produces $10^9$ to $10^{11}$ optical density units/ml using the A260/280 nm measurement on the Nanodrop (Thermo). Virus is then aliquoted and stored at 20 μl aliquots with 5 μl of virus added per well of a 24-well plate for 70% to 100% infection efficiency.

2.4 Electrophysiology

2.4.1 Introduction

With a method to isolate adult ventricular cardiomyocytes and effectively manipulate the levels of FGF13 in the cell, the next step is to test the effects of FGF13 on Ca$^{2+}$ and Na$^+$ currents. I performed whole-cell patch clamp and using a combination
of voltage changes and pharmacology, effectively isolated the individual Ca\textsuperscript{2+} and Na\textsuperscript{+} currents (I_{Ca} or I_{Na}, measured in amperes, A). I went to Cold Spring Harbor Laboratory in 2011 for the “Ion Channel Physiology” course. It was there that I learned the true intricacies of patch clamping. In this section, I will summarize the method of patch clamp, and the pharmacology and voltage-parameters used in the experiments that follow.

Before the specifics, it is important to understand what parameters are being controlled and which recorded. There are two modes of whole-cell patch clamp: voltage clamp and current clamp. Voltage-clamp is a true “clamp,” during which you hold (clamp) the voltage at a specific level and record ionic currents activated at that specific voltage. In contrast, current clamp does not actually clamp anything. During current clamp, the cell is at its own resting membrane potential, typically -85 mV for ventricular cardiomyocytes. The experimenter then injects depolarizing current beyond action potential threshold of the cell. This induces the cell, through activation of voltage-gated Na\textsuperscript{+} channels, to fire an action potential which is recorded as the changing voltage. In summary, current-clamp measures voltage changes and voltage-clamp is used to record ionic currents in response to a voltage step. Typically, voltage clamp is used to isolate a single type of current (Ca\textsuperscript{2+} or Na\textsuperscript{+} for example) and record its biophysical properties.

There are essentially four methods of recording ionic currents. They all begin with the fabrication of a pipette with a microscopic tip, filling the tip with solution, of which we will discuss the components later in this section, attaching the pipette to a head stage attached to a micromanipulator, and immersing the tip in the bath solution. The pipette is then pressed onto the cell to form a gigaseal (a high resistance, GΩ, seal). The voltage in the pipette is then hyperpolarized and the capacitance derived from the pipette is cancelled. It is after this step that the methods diverge (see Figure 15).
2.4.2 Types of electrophysiologic recordings

In cell-attached recording (Figure 15A) the pipette solution acts as the extracellular solution and the cytoplasm maintains its native state. It is useful when unknown cytosolic factors are required for gating, or to see if its activity is changed in response to a second messenger. One can add a ligand to the bath solution that the ion channel in question will be isolated from due to the pipette. Therefore, if a change in activity is seen, it is likely due to a second messenger. The disadvantages of this technique are the lack of ability to measure the resting membrane potential and no control over the intracellular cytosolic composition.

From the cell-attached configuration, one can pull the pipette away from the cell and form an inside-out configuration (Figure 15B). Because the inside of the cell is now immersed in the bath, this configuration allows one to change out the cytosolic solution to study cytosolic signals or enzymatic activities. However, one disadvantage is the complete loss of the endogenous cytosolic factors that control channel behavior.

From the cell-attached configuration as well, applying negative pressure leads to rupture of the membrane and movement into whole-cell configuration (Figure 15C). In whole-cell configuration, one records an average of all of the ion channels within the cell. Here, the experimenter controls the extracellular (bath) and the intracellular (pipette) solution, and thus can optimize conditions for the current of interest. However, there is loss of endogenous cytosolic factors because the pipette solution dilutes all of them out and it is impractical to change the pipette solution during a given recording.

Finally, in the whole-cell configuration, one can pull the pipette away to form an outside-out patch (Figure 15D). This allows rapid exchange of solutions on the
extracellular side and useful for recording ligand-gated ion channels. Again, the major disadvantage is the loss of the cytosolic environment.

In cell-attached, inside-out, and outside-out recording methods, one records single channel activity. This provides data on biophysical characteristics of the channel such as conductance, voltage-dependence, selectivity, and open probability. Whole-cell recording reveals the role of these channels in the context of whole-cell physiology. For my studies, I used whole-cell patch clamp on ventricular cardiomyocytes (Figure 15D) to explore the role of Ca$^{2+}$ and Na$^+$ channels in cardiomycyte physiology.
Figure 15: Different modes of patch clamping A, cell-attached patch. B, inside-out patch. C, whole-cell patch. D, outside-out patch. E, brightfield image of a ventricular cardiomyocyte with a patch pipette sealed, just prior to going into whole-cell configuration. Scale bar 20 um.
2.4.3 A closer look into whole-cell patch clamp

2.4.3.1 The cell as a simple circuit

The simplest way to understand the measurements taken in whole-cell patch clamp is to envision the cell as a circuit (Figure 16A).

![Diagram of a simple circuit with labels: RM, RS, VP, V, ΔV, in, SS, C.]

**Figure 16**: The cell as a simple circuit. A, The cell has membrane resistance (RM), a voltage gradient (ER), and capacitance, C. The pipette gives a test potential, VP, which drops over the series resistance of the pipette, RS, such that the cell sees test potential, V. B, example of a test potential given through the pipette and the response of the cell, C. I_in, instantaneous current; I_SS, steady-state current. Modified from Sakman and Neher, 2009. Permission not required from Springer Science and Business media if it is a “small excerpt for...scholarly analysis.”

The plasma membrane is a capacitor, defined as a narrow gap between two conductors. This creates a separation of charge in which the cell is more negative on the inside compared to the outside. Capacitance (C) is defined by how much charge (Q) needs to be transferred between the two conductors to set up a potential (V) difference between them.
Equation 2: Definition of capacitance

\[ C = \frac{Q}{V} \]

Capacitance is measure in Farads (F). Cell membranes act as capacitors with a specific capacitance of 1.0 μF/cm². Capacitance is directly proportional to the area of the cell and dielectric constant, and inversely proportional to the distance between the conductors. As the membrane bilayer is quite thin only about 2.3 nm, and the dielectric constant of hydrocarbons is 2.1, cells have a high electrical capacitance due to their molecular dimensions. This results in a small amount of charge having a large effect on membrane potential.⁴

As an example, we have gone into whole-cell configuration on a cell that is relatively small (~50 pF), and that is healthy and therefore has a high membrane resistance \( R_M \) of greater than 1 GΩ. The patch pipette has a series resistance \( R_s \). Note that it is named series resistance because in this circuit, there are two resistors in series, first the pipette, then the ion channel. In voltage clamp (Figure 16B), the cell is held at a hyperpolarized potential compared to the bath (ground electrode) and given a square pulse of amplitude ΔV, the current (Figure 16C) is measured as a single exponential, where \( V_0 \) is the original voltage, time is \( t \) as shown in Equation 3,

Equation 3: Discharge of an RC circuit

\[ V = V_0 e^{-t/RC} \]

It is assumed that \( R_M \gg R_s \) (typically 1 GΩ and 1-10 MΩ, respectively), three equations are obtained:
Equation 4: Instantaneous current

\[ I_{in} = \Delta V / R_S \]

Equation 5: Steady-state current

\[ I_{ss} = \Delta V / R_M \]

Equation 6: Tau

\[ \tau = R_S C \]

\( I_{in} \) is the first jump of current just after the change in voltage and \( I_{ss} \) is the steady state current. \( \tau \) is the time constant of the relaxation of the current, measured by fitting a single exponential equation to decay the decay from \( I_{in} \) to \( I_{ss} \).

Equation 7: Single exponential function to calculate \( \tau \)

\[ I_{ss} = I_{in} e^{-t/\tau} \]

Then, using Equations 4, 5 and 6, \( R_M \), \( C \), and \( R_S \) can be calculated. \( C \) and \( R_S \) are particularly important measurements. The specific capacitance of a cell is 1.0 \( \mu \)F/cm\(^2\). Thus the capacitance of the cell is directly proportional to the size of the membrane and therefore the size of the cell. We use this measurement to normalize the amount of current recorded such that we can compare among cells of different sizes, resulting in a measurement of current density (pA/pF). \( C \) is calculated from Equation 6. \( R_S \) is inversely proportional to how well the membrane has been ruptured. A full rupture of the membrane within the pipette will give a low \( R_S \) of about one to three times the pipette capacitance, thus giving a larger (and more accurate) calculation of cell capacitance. If \( R_S \) is high due to incomplete rupture or debris in the pipette, \( C \) can be artificially calculated as smaller than what would be measured, thus artificially increasing the calculation of current density.
Additionally, recall that $R_s$ is series resistance because there are two resistors in series with each other (Figure 16A), therefore the two resistances summate, leading to two voltage drops. This leads to a discrepancy between the pipette potential, $V_p$, and the cell potential, $V$. In the case of voltage-clamp, we want to measure the current in response to a specific voltage, and therefore this discrepancy would lead to the cell responding to a smaller voltage. Additionally, the high series resistance can lead to the cell firing an action potential upon a voltage step because it takes too long for the voltage to reach its max. This means the cell is no longer clamped at the $V_p$, leading to a complete loss of voltage control. Capacitance is also compensated such that no net current is recorded by the amplifier due to charging of the capacitor. This does not change the time course of $V$.

**2.4.3.2 Voltage-clamp protocols**

*2.4.3.2.1 Introduction*

Once the cell has been ruptured and proper compensation complete, it is time to switch to a recording solution specific to the channel of interest and run the protocols that elicit those currents.

*2.4.3.2.2 Solutions*

The proper composition of solutions is critical for ensuring the current of interest is being isolated. In this section, I will focus on the solutions used to record $Ca^{2+}$ currents as an example but these same principles apply for most currents of interest. It is useful to first look at the ionic content of a typical cell (Table 5), then decide which components need to be changed. The final solutions used in the experiments can be found in Section 3.3.3.
Table 5: Free ion concentrations and equilibrium potentials

<table>
<thead>
<tr>
<th>Ion</th>
<th>Extracellular concentration (mM)</th>
<th>Intracellular concentration (mM)</th>
<th>$\frac{[Ion]_o}{[Ion]_i}$</th>
<th>Equilibrium potential (mV)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>145</td>
<td>12</td>
<td>12</td>
<td>+67</td>
</tr>
<tr>
<td>K⁺</td>
<td>4</td>
<td>155</td>
<td>0.026</td>
<td>-98</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>1.5</td>
<td>100 nM</td>
<td>15,000</td>
<td>+129</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>123</td>
<td>4.2</td>
<td>29</td>
<td>-90</td>
</tr>
</tbody>
</table>

From Hille, 2001.⁴

*Calculated from Equation 1.

We will start with the intracellular solution. K⁺ is the most abundant ion inside the cell at 155 mM. K⁺ has a reversal potential of -98, thus any depolarizing steps will lead to an outward current. This outward current will contaminate (and potentially mask entirely) our inward Ca²⁺ current, therefore we need to replace K⁺ with another monovalent cation. We choose Cs⁺ for this purpose because it blocks K⁺ channels while giving us equal osmolality to K⁺. Most solutions contain CsCl, Cesium aspartate, and/or CsF. The salt provides balanced charge. Additionally, for cardiomyocyte recordings, we remove the Ca²⁺ from the solution completely because if not buffered down to the typical intracellular levels it will cause cell contraction and a loss of the seal. Mg²⁺ replaces Ca²⁺ and anecdotally makes it easier to seal on to the cell. Na⁺ is important for the Na⁺/K⁺ ATPase and NCX function therefore we will keep a similar amount of Na⁺ to the typical cell. Finally, Cl⁻ is the anion to counter all the cations we’ve added and stays about the same. The next component, HEPES is a buffer that keeps the solution at the physiologic pH of 7.2 to 7.3. HEPES is a “Good’s Buffer,” which maintains pH despite changes in carbon dioxide concentration produced by cell respiration better than
bicarbonate buffers. Ethylene glycol tetraacetic acid (EGTA) is a divalent cation chelator that has a much higher affinity for $\text{Ca}^{2+}$ than $\text{Mg}^{2+}$. This is required to maintain low free $\text{Ca}^{2+}$ even when we're recording $\text{Ca}^{2+}$ currents to keep the $\text{Ca}^{2+}$ from binding to the sarcomeres and leading to contraction. Finally, the cell needs energy to allow the channels to open and close in response to voltage. Energy is provided as GTP, ATP and phosphocreatine. Once a solution is made and brought to the goal pH with the proper acid or base (in this case CsOH), the osmolality should be calculated and also checked using a vapor pressure osmometer. While we are changing the components, we don't want to change the overall osmolality of the cell which could lead to changes in osmotic pressure.

Turning to the external solution, we need to think of which ions we want to allow through the channels when we depolarize the cell and which we want to block. We are recording $\text{Ca}^{2+}$, an inward current. Therefore, we do not want any $\text{Na}^+$ currents to contaminate our results. To do this, we get rid of all $\text{Na}^+$ in the extracellular solution. We replace the $\text{Na}^+$ with two components. The first is $\text{Cs}^+$, which was discussed previously. It is a monovalent cation that blocks $\text{K}^+$ channels. Additionally, N-methyl-D-glucamine (NMDG) is a membrane impermeable, monovalent cation that also blocks $\text{K}^+$ channels (again so that the outward $\text{K}^+$ current does not mask our inward $\text{Ca}^{2+}$ current). NMDG and $\text{Cs}^+$ are quite good at blocking the slow $\text{K}^+$ channels, but do a poor job blocking the fast, A-type current carried by the $\text{K}_{\text{V}}$4 channels and responsible for $\text{I}_{\text{to}}$ in the ventricular cardiomyocyte. To account for these currents, 4-aminopyridine is added to the extracellular solution, which acts as a specific blocker of A-type $\text{K}^+$ currents. Again, HEPES is present as a pH buffer and glucose provides energy for the cell. $\text{Ca}^{2+}$ is
necessary to record Ca\(^{2+}\) currents, although Ba\(^{2+}\) can also be used as a charge carrier. Mg\(^{2+}\) balances the osmolality and aids with sealing onto the cell with the patch pipette.

2.4.3.2.3 Voltage protocols

Now that the cell is sealed, compensation is complete and we have detailed the components of the solutions required to record currents, the final step is to give a voltage-protocol and record the currents elicited. Again, I will use Ca\(^{2+}\) currents as an example. The protocols for Na\(^{+}\) currents are generally the same with the exception of timing (because Na\(^{+}\) channels have a much faster activation and inactivation time). The analysis is the same for both. In whole-cell recordings, there are three basic measurements that are taken: Current density, voltage-dependence of activation, and steady-state inactivation. To get these three measurements, two protocols need to be run.

The first is a basic voltage step protocol. In this case, the cell is being held at a hyperpolarized potential (typically -80 mV for cardiomyocytes) and stepped to depolarizing voltages (Figure 17A) and an inward current is recorded (Figure 17B). The peak current for each voltage-step is recorded and plotted as the current (I, y-axis) versus voltage (V, x-axis) to give a typical I-V plot (Figure 17C). As stated earlier, this current is normalized to the cell size, measured as capacitance to give a current density measurement (pA/pF), so comparisons can be made between cells, although it is just shown as current in Figure 17C. Additionally, the voltage-dependence of activation, a measure of channel availability is calculated from a voltage-step protocol. While the I-V plot peaks at -10 mV in the example in Figure 17C and then starts decreasing, the channel is remaining open. However, the driving force for Ca\(^{2+}\) is beginning to reverse. Such a depolarized voltage results in a smaller driving force for Ca\(^{2+}\), a cation to go through the channel. Therefore, although it appears that some channels have started to
close, the channels are still open, but there is less driving force for Ca\(^{2+}\) to move inside the cell. To analyze the availability of the channel as a result of voltage, we express the current in terms of conductance (g) according to Equation 8.

**Equation 8: Conductance equation**

\[
I_{Ca} = g_{Ca} (V - V_{Ca})
\]

Where \(V_{Ca}\) is the reversal potential of for \(I_{Ca}\) and \(V\) is the potential of the voltage pulse. \(V - V_{Ca}\) is the net driving force on the Ca\(^{2+}\) ions. We can calculate the reversal potential from our solutions using Equation 1, the Nernst equation, we record the \(I_{Ca}\), and therefore the variable left is \(g\). We take this data and normalize it to its maximum \((G/G_{Max})\) to show what fraction of the total conductance is available at a given voltage. This data is then fit with a Boltzmann function,

**Equation 9: Boltzmann**

\[
\frac{G}{G_{Max}} = \frac{1}{1 + \exp \left( \frac{V_{1/2} - V_{M}}{k} \right)}
\]

where \(V_{M}\) is the membrane potential, and the \(V_{1/2}\), the voltage in which half the channels are activated, and the slope, \(k\), of the function are calculated (Figure 17D).
Figure 17: Typical recording and analysis of a voltage-step protocol. A, example of the voltage-steps given. Each step has six seconds in between to allow full recovery of the channel. B, recording of Ca\(^{2+}\) current in response to the voltage protocol in A. C, the Ca\(^{2+}\) current data directly derived from the protocol in A is the current-voltage (I-V) relationship. Using Equation 8, the I-V curve is converted into a sigmoidal activation plot from which we can derive the \(V_{1/2}\) and \(k\).

The second protocol provides data on steady-state inactivation, or the voltage-dependence of inactivation. In this protocol, the cell is held at a given test potential for 1 s (P1), and then given a pulse that activates its peak current (P2, for Ca\(^{2+}\) the step is to -10 mV or 0 mV). This is illustrated in Figure 18A. The basic idea is that a certain percentage of the channels will become inactivated during P1, then the rest that are still available will be elucidated during P2. The first potentials of P1 are at more hyperpolarized potentials compared to the activation of the channel and thus during P2, maximum current is obtained (Figure 18B). Take for example, at P1 = -50 mV (magenta
in Figure 18). During P1, there is no current recorded, and during P2, there is the maximal current. Then look at -10 mV for P1 (maroon). Maximal current is obtained during P1 and therefore little to no current is obtained at P2. The P2 current (I) normalized to the maximum P2 current (I_{Max}) is plotted against the test potential in P1 to create a steady-state inactivation curve (Figure 18C). This is fitted to a Boltzmann equation,

Equation 10: Steady-state inactivation

\[ \frac{I}{I_{Max}} = \frac{1}{1 + \exp\left(\frac{V_{1/2} - V}{k}\right)} \]

such that the \( V_{1/2} \) and \( k \) can be obtained (Figure 18C).
Figure 18: Typical two-step protocol with recording and analysis of channel steady-state inactivation. A, two-pulse protocol, and recording, B. Pulse 1 (P1) is stepped to different voltages and the relative current available during pulse 2 (P2) is plotted against the P1 voltage, C. This is fit with a sigmoidal function to derive $V_{1/2}$ and $k$.

When plotted together, the voltage-dependence of activation and the steady-state inactivation graphs demonstrate the window of voltage in which the channel is activated, but not inactivated yet (Figure 19), a measure of channel availability. Any shift in the activation or inactivation curves can change the size of that window, increasing or decreasing the availability of the channel at a given voltage.
Figure 19: The voltage-dependence of activation and steady-state inactivation curves plotted on the same graph to show the availability window of the channel, so called the window current (red striped region). This occurs at voltages which the channel is activated, but not inactivated.

2.4.4 Conclusion

While complex, electrophysiologic recordings provide a live, in vivo picture of the activity of ion channels within the cell. Using these methods in the following chapters, I will elucidate the roles of a modulator protein family, fibroblast growth factor homologous factors, in regulating voltage-gated Na⁺ and Ca²⁺ currents.
3. Fibroblast growth factor homologous factors modulate cardiac calcium channels

This chapter is based on a manuscript accepted to Circulation Research.

3.1 Summary

Fibroblast growth factor (FGF) homologous factors (FHF1, FGF11-14) are intracellular modulators of voltage-gated Na\(^+\) channels, but their cellular distribution in cardiomyocytes indicated that they performed other functions. Therefore, we aimed to uncover novel roles for FHF1s in cardiomyocytes starting with a proteomic approach to identify novel interacting proteins. Affinity purification of FGF13 from rodent ventricular lysates followed by mass spectroscopy revealed an interaction with Junctophilin-2, a protein that organizes the close apposition of the L-type Ca\(^{2+}\) channel, Ca\(_V\)1.2, and the ryanodine receptor, RyR2, in the dyad. Immunocytochemical analysis revealed overall T-tubule structure and localization RyR2 were unaffected by FGF13 knockdown in adult ventricular cardiomyocytes, but localization of Ca\(_V\)1.2 was affected. FGF13 knockdown decreased Ca\(_V\)1.2 current density, and reduced the amount of Ca\(_V\)1.2 at the surface due to aberrant localization of the channels. Ca\(_V\)1.2 current density and channel localization were rescued by expression of a shRNA-insensitive FGF13, indicating a specific role for FGF13. Consistent with these newly discovered effects on Ca\(_V\)1.2, we demonstrated that FGF13 also regulated Ca\(^{2+}\)-induced Ca\(^{2+}\) release, indicated by a smaller Ca\(^{2+}\) transient after FGF13 knockdown. Further, FGF13 knockdown caused a profound decrease in the cardiac action potential half width. This study demonstrates that FHF1s are not only potent modulators voltage-gated Na\(^+\) channels, but also affect Ca\(^{2+}\) channels and their function. We predict that FHF loss-of-function mutations would adversely affect currents through both Na\(^+\) and Ca\(^{2+}\) channels, suggesting that FHF1s may be arrhythmogenic loci, leading to arrhythmias through a novel, dual-ion channel mechanism.
3.2 Introduction

Despite an ever-growing understanding of ion channel structure, function, and regulation, many components of the macromolecular complexes anchored by ion channels are not yet known or well characterized. Identification of these channel interacting proteins and discovery of their functions within the channelsome provides important insight into physiologic and pathologic function. Mutations in newly defined channel interacting proteins often explain genetic causes of arrhythmias in cases where mutations in known arrhythmia loci are not found. Fibroblast growth factor (FGF) homologous factors (FHFs), a subfamily of FGF proteins (FGF11-FGF14) expressed predominantly in excitable cells, are prime examples of channel interacting proteins for which cardiac functions are not well understood. Although part of the FGF superfamily, FHFs do not function as growth factors and are incapable of activating FGF receptors. Rather, FHFs remain intracellular and have been shown to bind and modulate voltage-gated Na\(^+\) channels. Their roles as Na\(^+\) channel regulators have been studied most extensively in the brain, driven in large part by the identification of FGF14 as the locus for spinocerebellar ataxia and by observations that Fgf14\(^{-/-}\) mice display an ataxia phenotype that correlates with decreased Na\(^+\) channel function and diminished neuronal excitability.

FHFs are also expressed in cardiomyocytes, but their roles in regulating cardiac function have heretofore received less attention. We showed that FGF13, the most highly expressed FHF in murine heart, directly binds Na\(_\text{v}1.5\), the predominant cardiac Na\(^+\) channel, and participates in trafficking Na\(_\text{v}1.5\) to the sarcolemmal membrane and modulating Na\(^+\) channel kinetics. Consistent with these regulatory roles for Na\(^+\) channels, knockdown of FGF13 led to a reduction in conduction velocity and maximum
capture rate (the ability of the cells to recover from a stimulus of a specific speed) in a neonatal rat ventricular cardiomyocyte monolayer.\textsuperscript{78}

Several lines of evidence suggest that the effects of FHFs in excitable cells extend beyond Na\textsuperscript{+} channel modulation. For example, the complex changes in synaptic physiology in Fgf14\textsuperscript{-/-} mice are not consistent with a defect limited to Na\textsuperscript{+} channel dysfunction.\textsuperscript{142} Indeed, we recently found that FGF14 knockdown in cerebellar granule cells reduced presynaptic Ca\textsuperscript{2+} currents and synaptic transmission at the granule cell to Purkinje cell synapse.\textsuperscript{143} Moreover, as we reported,\textsuperscript{78} the cellular distribution of FHFs in ventricular cardiomyocytes extended beyond the distribution of Na\textsubscript{v}1.5 channels. We therefore aimed to determine novel roles for FHFs in cardiomyocytes by looking for new FHF interactors. Here we report the discovery that junctophilin-2 (JPH2) interacts with FGF13 in rodent ventricular myocytes. JPH2 is a protein responsible for coordinating the interaction of the sarcolemma and sarcoplasmic reticulum in the dyad. We therefore investigated the role for FGF13 in regulating ionic currents in the dyad and showed that FGF13 has essential roles in regulating the L-type, voltage-gated Ca\textsuperscript{2+} channel (Ca\textsubscript{v}1.2) currents in adult ventricular myocytes. These results provide to the best of our knowledge the first evidence that FHFs modulate ion channels other than voltage-gated Na\textsuperscript{+} channels in cardiomyocytes and highlight previously unknown modulatory roles for FHFs in cardiac physiology, such as regulation of Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) and the integrated electrical activity of the ventricular action potential. Our results lead to the hypothesis that loss-of-function mutations in FHFs could underlie inherited cardiac arrhythmias.
3.3 Materials and Methods

3.3.1 Adenovirus

The adenoviruses expressing FGF13 shRNA or scrambled shRNA with GFP has been previously described.78 FGF13 rescue viruses and the shRNA virus with the GFP removed were generated similarly using the AdEasy System (Agilent). For rescue, human FGF13VY was mutated at the site of shRNA recognition to replace each third nucleotide (see Table _ for primers), changing the DNA sequence but not the ultimate protein product. This construct was subcloned into pAdRFP (Addgene). The adenoviral plasmid was packaged in HEK293 cells. The recombinant virus was isolated by multiple freeze/thaw cycles, further amplified and then purified and concentrated using Vivapure Adenopack 20 (Sartorius Stedim Biotech). The viral titer was determined using optical density. All constructs were confirmed by sequencing.

Table 6: Mutagenesis primers

| Forward | 5’ CACCAAAGATGAGGACAGTACCTATACCCTATTCAATCTCATCCCTGTGGTCTG 3’ |
| Reverse | 5’ CAGACCCACAGGGATGAGATTGAATAGGGTATAGGTACTGTCCTCATCTTGGTG 3’ |

3.3.2 Cardiomyocyte isolation

Animals were handled according to National Institutes of Health Guide for the Care and Use of Laboratory Animals. The study was approved by Duke University Animal Care and Welfare Committee. Cardiomyocytes were isolated from 6-8 week old C57/B6 mice or Sprague Dawley rats and cultured as previously described.78 Animals were anesthetized with Avertin and anti-coagulated with heparin. Hearts were removed and the aorta was cannulated to perfuse the heart using a Langendorff apparatus. The hearts were first perfused with basal solution containing (in mM, from Sigma unless
otherwise specified): NaCl 112, KCl 5.4, NaH₂PO₄•H₂O 1.7, NaHCO₃ 4.2, MgCl•6H₂O 1.63, HEPES 20, glucose 5.4, taurine 30, L-carnitine 2, creatine 2.3, 2,3-butanedione monoxime (BDM) 10. After five minutes, the solution was switched to basal solution plus 150 u/ml Collagenase Type II (Worthington) and the heart was perfused until it was soft and boggy. The heart was then taken down from the Langendorff, minced, and trituated in enzyme solution until all cell clumps were broken. Calcium tolerance was performed in basal solution plus 5 mg/ml bovine serum albumin to quench the enzyme. For culture, cells were plated on laminin coated coverslips or glass bottom plates (MatTek) in plating medium of Minimal Essential Medium (MEM) with Earle’s Salts and L-glutamine (Mediatech), 10 mM BDM, 5% fetal bovine serum (Life Technologies) and 1% penicillin/streptomycin (Sigma). After cells had adhered to the plates, the cells were washed once and the medium changed to culture medium into which the proper adenovirus had been added. Culture medium contained MEM with Earle’s Salts and L-glutamine, bovine serum albumin 0.5 mg/ml, BDM 10 mM, 1X insulin-selenium-transferrin supplement (Life Technologies), creatine 5 mM, taurine 5 mM, L-carnitine 2 mM, and blebbisatin 25 µM (Toronto Research Chemicals). All solutions were oxygenated in 95% O₂/5% CO₂ for at least 30 minutes. Cells were then analyzed for electrophysiology, immunocytochemistry and Ca²⁺ transient recording 36-48 hours later.

### 3.3.3 Electrophysiology

Ca²⁺ currents (I_{Ca}) were recorded using the whole-cell patch-clamp technique as previously described.¹⁴⁴ Voltage-clamp experiments were performed at room temperature (22-24 °C), 36-48 hours after infection of adult cardiomyocytes with adenovirus. Bath (Tyrode) solution (Table 7) contained (in mM, from Sigma): NaCl 140, KCl 5.4, CaCl₂ 1, MgCl₂ 1, HEPES 5, glucose 10, pH 7.3 adjusted with NaOH. Once the
cell was ruptured, solution was quickly changed to recording solution (Table 8) containing (in mM, from Sigma): N-Methyl-D-glucamine 150, HEPES 10, CsCl 2, CaCl$_2$ 2, MgCl$_2$ 1.2, 4-aminopyridine 2, D-glucose 5.5, pH 7.3 adjusted with CsOH. Internal solution (Table 9) contained (in mM, from Sigma): CsOH•H$_2$O 70, aspartic acid 80, CsCl 40, NaCl 10, HEPES 10, EGTA 10, MgATP 5, Na$_2$GTP 0.2, Na$_2$-phosphocreatine 5, pH 7.3 adjusted with CsOH. Osmolarity was adjusted to ~300 mOsm with sucrose for all solutions. Recordings were filtered at 5 kHz and digitally sampled at 25 kHz. Amplitude was normalized to cell capacitance (pA/pF). Data analysis was performed using Clampfit 10.2 software (Axon Instruments) and Origin 8 (Originlab Corporation). For current clamp, perforated patch with 400 nM amphotericin (Sigma) was performed using the following internal solution (Table 10)$^{145}$ (in mM, from Sigma) KCl 110, NaCl 5, MgATP 5, Na$_2$-phosphocreatine 5, Na$_2$GTP 1, HEPES 10, pH 7.3 and Tyrode extracellular solution. Cells were stimulated with current injections at 1 Hz at 1.5x threshold to induce action potentials recorded with 25 kHz sampling frequency. Input resistance was not different between the groups and junction potential was calculated to be 5.6 mV and not corrected.

### 3.3.4 Sarcoplasmic reticulum load measurements

Cardiomyocytes were plated on glass bottom plates, cultured and infected with virus. After two days in culture, cells were washed twice with Tyrode solution and loaded with 0.25 µM Fura-2 AM for 15 minutes. Cells were then washed three times with Tyrode solution and allowed to de-esterify for 30 minutes. Cells were field stimulated at 1 Hz with a 50 V unipolar pulse for at least one minute prior to recording to allow them to reach steady state. Calcium transients were measured by excitation of Fura-2 with alternating 340 nm and 380 nm wavelengths of light (cycle time 4 ms) once
the cells had reached steady state. After a 10 s pause, 10 mM caffeine was rapidly applied. Following recording, cells were moved out of the field of view and background fluorescence was measured for subtraction. Background subtracted SR load peak height was measured using IonWizard software (IonOptix).

3.3.5 Immunocytochemistry and T-tubule staining

Immunocytochemistry methods have been previously described. Imager/analyze was blinded to the manipulation and all cells imaged were used for analysis. Cardiomyocytes on glass coverslips were washed in PBS and fixed for 15 minutes in 2% paraformaldehyde in PBS. Fixation was quenched with 10 mM glycine in PBS and cells were permeabilized with 0.2% triton X-100 in PBS for 8 minutes. Non-specific binding was blocked with 10% goat serum for one hour at room temperature. Cells were then incubated in primary antibody dissolved in antibody dilution solution containing 3% goat serum, 1% bovine serum albumin and 0.1% triton X-100 in PBS overnight at 4 °C. Primary antibody concentrations were anti-FGF13 1:400, anti-α1C 1:1000 (Alomone), anti-RyR 1:1000 (Sigma), anti-NCX 1:1000 (generously provided by G. Vann Bennett, Duke University). Cells were washed three times with PBS then incubated in secondary antibody in antibody dilution solution for 45 minutes at room temperature. Secondary antibodies were conjugated to Alexa-fluor 488, 633 (Life Technologies) or Cy3 (Jackson Immunoresearch). Following three more washes, coverslips were mounted in Vectashield (Vector Labs). For T-tubule staining, cells were plated onto glass bottom plates (MatTek Corp.) and cultured as above. Cells were then washed twice with Tyrode solution and incubated in 0.5 µM di-8-ANEPPS (Life Technologies) in Tyrode solution plus 25 µm blebbistatin to inhibit contraction. Cells were imaged live. For all image analysis, control plates were imaged first. To ensure
there were no effects of culture on cells, only those cultures in which greater than 95% of the controls had proper α_{1C} localization were used for analysis. All images were collected on a Zeiss 510 inverted confocal microscope with a Zeiss 63x oil (NA, 1.4) or a Zeiss 40x oil (NA, 1.3) lens for immunocytochemistry or live imaging, respectively at room temperature. The pinhole was set to 1.0 (Airy Disc) using Carl Zeiss Imaging software (version 4.0, SP1). For Ca_{v}1.2, NCX and RyR localization, 0.5 µm stacks were taken at 512 x 512 resolution with 3x digital zoom. For T-tubule staining, a single slice was imaged through the center of the cell. All cells were prepared identically, and imaged by using identical parameters (e.g. gain, offset, magnification, brightness, contrast, pinhole, scan time, resolution, etc.).

3.3.6 Image processing and Fast Fourier Transform

The experimenter analyzing images was blinded to treatment. Stacks were deconvolved using Hyugens software (Scientific Volume Imaging) and exported as Tiff files. Voxel colocalization was performed on deconvolved images using Pearson correlation coefficient. For channel localization, images were imported into ImageJ (NIH), and 15 µm by 5 µm, non-nuclear, non-sarcolemmal sections were selected and line scanned to create an average plot profile as in Figure 3B. The data was then imported into OriginLab software and a fast Fourier transform was performed at a 0.1 µm^{-1} sampling frequency. Peaks analyzed ranged from 1.8 µm to 2.1 µm intervals. The amplitude at the peak was then compared between the groups.

3.3.7 Simultaneous patch clamping and Ca^{2+} transient recording

Cardiomyocytes were plated onto glass bottom plates (MatTek Corp.) and cultured as above. Viruses expressing GFP were not used as GFP interferes with the Fura-2 emission. Therefore the cells were co-infected with a virus expressing mRFP to
identify infected cells. Cells were washed twice with Tyrode solution. Whole cell patch clamping was performed as above with the following modifications. Internal solution contained (in mM, from Sigma): CsOH•H₂O 70, aspartic acid 80, CsCl 40, NaCl 10, HEPES 10, Fura-2 pentapotassium salt 0.150 (Life Technologies), MgATP 5, Na₂GTP 0.2, Na₂-phosphocreatine 5, pH 7.3 adjusted with CsOH. Bath solution was a normal Tyrode solution. Prior to rupture, emission was recorded to account for autofluorescence. Upon rupture, cells were recorded at rest until a steady basal [Ca²⁺], was recorded for at least 30 seconds. Cells were then given a series of 50 ms voltage steps to 0 mV from holding at -40 mV (to inactivate Na⁺ channels) at 0.5 hz to equalize SR contents and then one 500 ms pulse to 0 mV that was used for measurement. Following recording, the patch pipette was removed and the cell moved out of the field to account for background fluorescence. Current was normalized to cell capacitance (pA/pF). For Ca²⁺ transient peak measurement, background emissions were first subtracted and the peak was measured as the difference from the baseline using IonWizard software (IonOptix). EC-coupling gain was defined as the Ca²⁺ transient peak divided by the Ca²⁺ current peak.

3.3.8 Immunoprecipitation

Fresh adult mouse ventricular heart lysate was prepared by homogenizing tissue on ice in lysis buffer containing 150 mM NaCl, 50mM Tris, 1% Triton X, and protease inhibitor cocktail (Roche) as previously described. 10 µg of anti-JPH2 (Santa Cruz), anti-FGF13, or anti-α₁C antibody, or control IgG rabbit/goat (Santa Cruz) were used. Samples were subjected to SDS-PAGE and co-immunoprecipitation was verified by western blot.
3.3.9 Proteomics

To crosslink anti-FGF13 antibody to agarose beads, 20 μg of FGF13 antibody or control rabbit IgG was coupled to 40 μl of protein A/G agarose beads in 1 ml PBS. After rocking overnight at 4 °C, beads were washed 3 times with 1 ml of 0.2M sodium borate (pH 9). Dimethyl pimelimidate (DMP) crosslinking reagent (Thermo Scientific) was dissolved in 0.2 M sodium borate (pH 9) to make 20mM DMP solution, and added to the coupled beads. After rocking at room temperature for 40 minutes, the sample was spun down and supernatant removed. The crosslinking reaction was quenched with 0.2M ethanolamine (pH 8) and the antibody cross-linked beads were ready for use.

Ventricular tissue lysate (~23 mg total protein) was added to cross-linked beads, and rocked overnight at 4 °C. Beads were washed 3 times with lysis buffer and eluted in 400 μl of 0.2% Rapigest SF Surfactant (Waters) in 50 mM ammonium bicarbonate. Samples were heated at 70 °C for 10 min, centrifuged, and the supernatant was subjected to an in-solution tryptic digestion. Peptide identifications were determined by the Duke Proteomics Core Facility using liquid chromatography/tandem mass spectrometry; following data acquisition, all spectra were searched against the SwissProt database with the mouse taxonomy selected.

3.3.10 Biotinylation and western blotting

Surface biotinylation and western blotting were performed as previously described. Cardiomyocytes were plated on laminin coated 60 mm plates, infected with adenovirus and cultured. After two days, cells were washed twice with cold PBS and incubated with 1 mg/ml EZ-Link Sulfo NHS-SS Biotinylation (Pierce) in cold PBS for 30 minutes. Biotinylation was quenched with 100 mM glycine in PBS and cells were lysed. Biotinylated proteins were incubated with NeutrAvidin (Pierce) overnight, then washed
three times and eluted in 2x LDS Sample Buffer (Life Technologies) plus 10 mM dithiothreitol. The biotinylated proteins and the whole lysate were run on 8-16% tris-glycine SDS page gels, transferred to PVDF membrane and western blotted. Primary antibodies used were anti-α1C 1:1000 (Alomone), anti-transferrin receptor 1:1000 (Life Technologies), anti-β-actin 1:5000 (Sigma) and anti-FGF13 1:200. Blotting for β-actin demonstrated if intracellular proteins had been biotinylated and those replicates were not quantified if the biotinylated fraction was positive. Transferrin receptor was used as a surface loading control.

### 3.3.11 Statistical analyses

Results are presented as means ± standard error of the mean; statistical significance of differences between groups was assessed using one-way analysis of variance (ANOVA) with Fisher’s Least Significant Difference as a post-hoc test. The cut off for statistical significance was set at P < 0.05.

#### Table 7: Normal Tyrode Bath Solution

<table>
<thead>
<tr>
<th></th>
<th>FW</th>
<th>mM</th>
<th>g/1 L</th>
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<tr>
<td>NaCl</td>
<td>58.44</td>
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<td>8.18</td>
</tr>
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<td>KCl</td>
<td>74.56</td>
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<td>0.40</td>
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<tr>
<td>CaCl$_2$·2H$_2$O</td>
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<td>1</td>
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<td>MgCl$_2$·6H$_2$O</td>
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<tr>
<td>HEPES</td>
<td>238.30</td>
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</tr>
<tr>
<td>D-glucose</td>
<td>180.16</td>
<td>10</td>
<td>1.80</td>
</tr>
</tbody>
</table>

pH to 7.2 with 1 N NaOH
Table 8: External $I_{Ca}$ Recording Solution

<table>
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<tr>
<th></th>
<th>FW</th>
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<th>g/1L</th>
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<tr>
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<td>D-glucose</td>
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pH to 7.2 with 12 N HCl

Table 9: $I_{Ca}$ Pipette Solution

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<td>MgATP</td>
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<td>Na$_2$GTP</td>
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<td>Na$_2$phosphocreatine</td>
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pH to 7.2 with 1 N CsOH
Table 10: Current Clamp Pipette Solution

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<tr>
<td>HEPES</td>
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</table>

pH to 7.2 with 1 N KOH

3.4 Results

3.4.1 Junctophilin-2 is in complex with FGF13

Although we previously showed that FGF13 is an intracellular modulator of voltage-gated Na$^+$ channels, close analysis of FGF13 immunocytochemistry in adult rat cardiomyocytes revealed an overall distribution that extended beyond what has been reported for Na$_V$1.5 in heart. Specifically, we observed a striated pattern similar to a T-tubule distribution (see Figure 20A, inset), in addition to the sarcolemmal and nuclear distribution we previously reported. This led us to hypothesize that FGF13 may have roles in cardiomyocyte physiology beyond Na$_V$1.5 regulation. To identify other potential FGF13 interactors, we performed immunoprecipitation using a previously validated FGF13 antibody or an IgG (as a control) from adult ventricular tissue and liquid chromatography / mass spectrometry analysis of the immunoprecipitated FGF13 protein complex. One interesting candidate we identified was Junctophilin-2 (JPH2). As shown in Figure 20B, we identified 4 unique peptides spanning all soluble domains of JPH2. We found this candidate to be of interest because it is a protein responsible for properly juxtaposing Ca$_V$1.2 and the Ryanodine Receptor 2 (RyR2) in the T-tubule (Figure 20B), consistent with the t-tubular distribution of FGF13. The specificity of the mass
spectrometry approach was confirmed through the identification of three FGF13 peptides after FGF13 immunoprecipitation, but none with the control IgG; and eight peptides (but none in the control IgG sample) from the known FGF13 binding partner Na\textsubscript{v}1.5 (see Table 11 for all peptide data). We further validated that FGF13 was in complex with JPH2 through co-immunoprecipitation, detecting JPH2 in FGF13 immunoprecipitates but not in the IgG control (Figure 20C).
Figure 20: FGF13 associates with JPH2 as part of the dyad macromolecular complex. A, immunocytochemical analysis shows FGF13 (green) in the nucleus, the sarcolemma and T-tubules, enlarged in the inset. DAPI is in blue. Scale bar 50 µm. B, schematic of JPH2 indicating the putative protein motifs and the location of the unique peptides identified by mass spectroscopy in red. MORN motifs are indicated in yellow. JPH2 is found in the dyad in which CaV1.2 is juxtaposed to RyR2. MORN, membrane occupation and recognition nexus; α-helix, alpha helical domain; divergent, divergent region; TMD, transmembrane domain; SR, sarcoplasmic reticulum. C, representative co-immunoprecipitation and western blot to validate the interaction of JPH2 and FGF13, repeated three times.
Table 11: Summary of peptides from proteomic screen

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<th># of Peptides</th>
<th>FGF13 IgG</th>
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<th>Peptide Sequence</th>
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<td></td>
<td>NKPAAHFLPKPLK SVSGVLNGGK VVAIQGVQTK</td>
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<tr>
<td>FGF13_MOUSE</td>
<td></td>
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<td>0</td>
<td></td>
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<tr>
<td>alpha SCN5A_MOUSE</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Junctophilin-2</td>
<td>4</td>
<td>0</td>
<td></td>
<td>ELAPDFYQPGPEYQK LLQEILENSESLLLEPPER RSDSAPPSPVSATVPEEEPPAPR YEGEWLDNLIR</td>
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<tr>
<td>JPH2_MOUSE</td>
<td></td>
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<td></td>
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</tr>
</tbody>
</table>

* Protein accession numbers from SwissProt 2011
3.4.2 FGF13 knockdown perturbs dyad organization by affecting Ca\textsubscript{\text{V}}1.2 localization

JPH2 is a protein responsible for coordinating the interactions between the sarcolemma and the sarcoplasmic reticulum (SR). JPH2 has a C-terminal transmembrane domain in the SR and its N-terminus interacts with the inner leaflet of the sarcolemma, providing a means to juxtapose the sarcolemma and SR so as to promote efficient CICR by apposing Ca\textsubscript{\text{V}}1.2 and RyR2.\textsuperscript{147} Based on our discovery that FGF13 was a component of the JPH2 macromolecular complex, we tested whether FGF13 participated in dyad ion channel targeting. We performed immunocytochemistry on mouse ventricular myocytes that were uninfected (CON) or that had been infected with a FGF13 shRNA adenovirus targeting all FGF13 splice variants (FGF13 KD) or a scrambled shRNA adenovirus (SCR, Figure 21). As we previously showed (see also Figure 23), the KD adenovirus reduced FGF13 protein by ~ 90% and the SCR virus had no effect.\textsuperscript{78} In the CON and SCR, we observed \(\alpha_{\text{iC}}\), the Ca\textsubscript{\text{V}}1.2 pore-forming subunit, colocalized with RyR2 in a striated pattern. Little \(\alpha_{\text{iC}}\) or RyR2 was observed between the striations. After FGF13 KD, however, we saw a loss of colocalization and a large portion of \(\alpha_{\text{iC}}\) was now present between the RyR2 striations (Figure 21A, Pearson’s correlation co-efficient of 0.52 ± 0.03 versus 0.65 ± 0.03 and 0.62 ± 0.03 in CON and SCR cardiomyocytes, respectively; P < 0.05 for FGF13 KD versus CON or SCR cardiomyocytes, N=8 cells per group).

We then performed quantitative analysis on the pattern of Ca\textsubscript{\text{V}}1.2. In CON adult mouse cardiomyocytes (Figure 21A) or after infection with the SCR virus (Figure 21B), \(\alpha_{\text{iC}}\) displayed the expected striated pattern with a periodicity of ~ 2 \(\mu\text{m}\), consistent with a t-tubular distribution.\textsuperscript{148} In contrast, the pattern in FGF13 KD cells was discontinuous.
with multiple punctae found between the residual striations (Figure 21B). With the analyzer blinded to treatment status, we quantified the change in distribution with intensity profiles of $\alpha_{1C}$ in confocal z-stacks of non-nuclear areas, as shown in Figure 21B. In SCR cardiomyocytes, we detected a regular pattern of intense $\alpha_{1C}$ staining at $\sim$ 2 µm intervals (analyzed by fast Fourier transform, Figure 21D), with almost no signal in the intervening intervals. In contrast, after FGF13 KD, the peak amplitude of $\alpha_{1C}$ staining at the $\sim$ 2 µm interval was reduced by more than 50% (FFT amplitudes (in arbitrary units): CON 0.284 ± 0.017 (n=8), SCR 0.234 ± 0.009 (n=8), FGF13 KD 0.116 ± 0.020 (n=12); P < 0.0001 for FGF13 KD vs. SCR and CON), and significant $\alpha_{1C}$ signal was observed in between the peaks (Figure 21B, D).

We tested whether this altered $\alpha_{1C}$ distribution was secondary to a general defect in T-tubule architecture or was more specific to $\alpha_{1C}$ using two separate analyses. First, we performed immunocytochemistry for the sodium-calcium exchanger (NCX), another T-tubule-localized protein, and observed that FGF13 KD did not affect NCX distribution (Figure 21C, D). Second, the membrane-binding dye, di-8-ANEPPS, revealed that T-tubular distribution was grossly unaffected by FGF13 KD (Figure 21E).

The specific change in Ca$_V$1.2 localization after FGF13 KD led us to hypothesize that FGF13, as a member of the JPH2 macromolecular complex anchored by JPH2, affected Ca$_V$1.2 targeting to T-tubules. We therefore tested whether Ca$_V$1.2 was a component of the JPH2 complex. Indeed, by co-immunoprecipitation, we were able to pull down the $\alpha_{1C}$ pore-forming subunit of Ca$_V$1.2 with an antibody to JPH2 but not a control IgG (Figure 21F). These data are consistent with a previous observation that JPH2 interacts with Cav1.1 in skeletal muscle,$^{149}$ but are, to the best of knowledge, the first demonstration of an interaction between JPH2 and an L-type Ca$^{2+}$ channel in in
cardiac muscle. Together, these data indicated that FGF13 KD did not affect T-tubule or SR architecture, but FGF13 has a specific role in targeting Ca\textsubscript{v}1.2 to its proper T-tubular location.
Figure 21: FGF13 perturbs dyad organization and Ca\textsubscript{V}1.2 localization. A, RyR2 is unaffected by FGF13 knockdown and Ca\textsubscript{V}1.2 no longer colocalizes with RyR2 (p < 0.05, see text for numbers). B, intensity plots for α\textsubscript{1C} were drawn of a 15 μm x 5 μm area of the cell. Similar studies were done for sodium-calcium exchange (NCX). C, D, the interval distance was calculated using a FFT for α\textsubscript{1C} (left) and NCX (right). Amplitudes for α\textsubscript{1C} were 0.28 ± 0.02, 0.23 ± 0.01 and 0.12 ± 0.02 for control, scrambled and shRNA respectively. P < 0.001 for FGF13 KD vs. CON (not shown) or SCR. C (right), NCX is not affected by FGF13 knockdown, (p=0.40 for n=10 per group). Scale bar 5 μm for A, B, and C. E, Di-8-ANEPPS staining of T-tubules showed no change in morphology. Scale bar 20 μm, n=20, 10 and 14 for CON, SCR, and KD respectively. F, representative co-immunoprecipitation and western blot of the pore-forming α\textsubscript{1C} subunit of Ca\textsubscript{V}1.2 with JPH2 from mouse ventricular lysate from three independent experiments showing an interaction with an antibody specific to JPH2 but not the IgG control.
3.4.3 FGF13 modulates Ca\textsubscript{v}1.2 trafficking to the surface and current density

We hypothesized that this aberrant Ca\textsubscript{v}1.2 localization would lead to a decrease in Ca\textsubscript{v}1.2 at the surface. We therefore quantified the relative amount of Ca\textsubscript{v}1.2 at the sarcolemma after FGF13 KD by labeling surface proteins with biotin and capturing them with avidin beads after cell lysis. Both the surface fraction and the whole cell lysate of adult mouse ventricular myocytes were then probed with an antibody against the pore-forming α\textsubscript{1C} subunit of Ca\textsubscript{v}1.2. These biotinylation experiments revealed a decrease in amount of α\textsubscript{1C} at the surface after FGF13 KD (Figure 22A-B). The total amount of α\textsubscript{1C} (in the cellular lysate) was unchanged. To test whether this decreased Ca\textsubscript{v}1.2 at the surface, led to physiologic changes, we measured Ca\textsuperscript{2+} channel current density using whole-cell patch clamp. In FGF13 KD cardiomyocytes, but not the SCR cardiomyocytes, Ca\textsuperscript{2+} channel current density was reduced by 35% compared to CON cardiomyocytes (Figure 22C, D). Channel availability, voltage-dependence of activation and steady-state inactivation, were unaffected (Table 12). Together, these results suggest that FGF13 knockdown reduced Ca\textsuperscript{2+} current density by affecting the number of channels at the sarcolemma.
Figure 22: FGF13 affects Ca\textsubscript{v}1.2 surface expression and current density. Surface expression of Ca\textsubscript{v}1.2 is reduced with FGF13 KD while total protein remains unchanged in surface biotinylation and western blotting, A. Summarized data of four independent biotinylation experiments in B. C, representative IC\textsubscript{a} traces from voltage-clamp of mouse ventricular cardiomyocytes (voltage protocol shown below). D, summarized IV curve. * $P < 0.05$ for $n=11, 6$ and $14$ for CON, SCR, and KD respectively.
3.4.4 Human FGF13VY can rescue Ca\(_v\)1.2 current density and localization

To confirm a role for FGF13 in targeting Ca\(_v\)1.2 to the T-tubules and regulating L-type Ca\(^{2+}\) channel current density, we performed “rescue” experiments on adult rat ventricular cardiomyocytes treated with FGF13 shRNA by co-expressing human, shRNA-insensitive and Hisx6-tagged, FGF13-VY (hFGF13-VY). FGF13 is differentially spliced at its N-terminus\(^{76}\) and we chose this FGF13 splice variant for rescue because it is the most highly expressed isoform in mouse and rat cardiomyocytes.\(^ {78}\) The adenovirus expressing FGF13 shRNA also expressed GFP (via a CMV promoter) and the adenovirus expressing hFGF13-VY also expressed mRFP, allowing us to identify cells in which hFGF13-VY was expressed in the context of endogenous FGF13 knockdown (Figure 23A). Immunostaining for endogenous FGF13 (with an anti-FGF13 antibody) and hFGF13-VY (with an anti-Hisx6 antibody) demonstrated not only effective knockdown of endogenous FGF13 but also that the expressed hFGF13-VY recapitulated the overall cellular distribution of endogenous FGF13 (Figure 23A), although the striated pattern was even more obvious when compared to endogenous FGF13. We suspected that the subtle differences in pattern reflected the specific distribution of hFGF13-VY compared to the distribution of all endogenous splice variants recognized by the antibody in CON cells.

Having established the efficacy of the hFGF13-VY rescue, we examined Ca\(_v\)1.2 Ca\(^{2+}\) current density in adult rat ventricular myocytes using whole-cell patch clamp. As in mouse cardiomyocytes (Figure 22C), FGF13 knockdown reduced Ca\(_v\)1.2 Ca\(^{2+}\) channel current density (Figure 23B, Table 12). Expression of hFGF13-VY restored Ca\(^{2+}\) current density to wild-type levels (Figure 23B). We also noted a hyperpolarizing shift in steady-state inactivation compared to CON cells (Table 12). The reasons for this are unclear,
but may result from the overexpression of the specific FGF13-VY splice variant in the context of the knockdown of other FGF13 splice variants, thus altering any counterbalancing effects imparted by these absent variants. Additionally, the cellular distribution of α₁c was restored with FGF13-VY overexpression, as indicated by the overall pattern and by Fourier transform (Figure 23C, D). This rescue strategy therefore firmly established a role for FGF13 in targeting Caᵥ1.2 to T-tubules and provided a potent confirmation of the specificity of the FGF13 KD virus.
Figure 23: Human FGF13-VY rescues Ca\(_{\alpha1.2}\) current density and localization. A, immunocytochemistry showing endogenous FGF13 and the level of knockdown with adenovirus expressing GFP and shRNA to all FGF13 splice variants (top two panels). Overexpression of shRNA-resistant, hFGF13-VY (also expressing mRFP) in the bottom panel. Scale bar 40 µm. hFGF13-VY rescued decreased current density (B) and the change in α\(_{1C}\) localization (C, D) seen with FGF13 knockdown (KD). Scale bar 5 µm. Fast Fourier transform amplitudes were CON, 0.27 ± 0.01 (n=5), FGF13 KD, 0.15 ± 0.24 (n=6) and FGF13 KD + hFGF13-VY, 0.27 ± 0.04 (n=5), P < 0.001 for FGF13 KD compared to CON and FGF13 KD + hFGF13-VY. *P < 0.05 for FGF13 KD vs. control and FGF13 KD + hFGF13-VY.
3.4.5 FGF13 affects Ca\textsuperscript{2+} transients but preserves excitation-contraction coupling gain

The mislocalization of Ca\textsubscript{v}1.2 after FGF13 knockdown, the newly described interaction between FGF13 and JPH2, and previous studies that defined clear roles for JPH2 in Ca\textsuperscript{2+} induced Ca\textsuperscript{2+} release (CICR)\textsuperscript{72} prompted us to query whether FGF13 influenced excitation-contraction (EC) coupling gain. We therefore simultaneously recorded Ca\textsuperscript{2+} currents and Ca\textsuperscript{2+} transients in CON, SCR, and FGF13 KD rat ventricular myocytes (Figure 24). Following a previously described protocol to ensure steady-state Ca\textsubscript{v}1.2 function,\textsuperscript{72} we held the cells at -40 mV to inactivate Na\textsuperscript{+} channels and gave a 500 ms pulse to 0 mV to induce influx of Ca\textsuperscript{2+} through Ca\textsubscript{v}1.2 and recorded it via whole-cell patch clamp. We simultaneously recorded Ca\textsuperscript{2+} transients using Fura-2 in the pipette internal solution. Representative traces are shown in Figure 24A. Knockdown of FGF13 not only reduced Ca\textsuperscript{2+} current through Ca\textsubscript{v}1.2 (Figure 24B), as previously shown, but also led to decreased Ca\textsuperscript{2+} release from the sarcoplasmic reticulum (SR, Figure 24C). Interestingly, it appeared that those channels that were at the surface were coupling with RyR2 appropriately as EC-coupling gain was not different between the groups. These changes were not due to decreased SR load as measured by rapid application of caffeine in Fura-2 loaded cells (peak height 0.28 ± 0.05 Δ340/380 (n=5), 0.32 ± 0.03 Δ340/380 (n=12), and 0.29 ± 0.03 Δ340/380 (n=14) for CON, SCR, and KD respectively, p=0.66). These data indicated that decreased Ca\textsuperscript{2+} transients after FGF13 KD were at least partially due to decrease current through Ca\textsubscript{v}1.2 and are consistent with its discovered interaction with JPH2, a known regulator of this function.
Figure 24: FGF13 knockdown reduces Ca\textsuperscript{2+} transients but preserves EC-coupling gain. A, representative traces of simultaneous whole-cell voltage clamp of Ca\textsuperscript{2+} current and Ca\textsuperscript{2+} transient recordings. B-D, summarized data for the labeled measurements. * P < 0.05 for KD vs SCR (n=10 per group).
3.4.6 FGF13 affects multiple phases of the cardiac action potential

Having established that FGF13 modulates not only voltage-gated Na\(^+\) channels,\(^{78}\) but also Ca\(_{\text{V}1.2}\) Ca\(^{2+}\) channels and Ca\(^{2+}\) transients, we hypothesized that loss of FGF13 would have measurable effects on the cardiac action potential. Therefore, using current clamp, we recorded evoked action potentials in CON, SCR, or KD adult rat ventricular cardiomyocytes (Figure 25A-D). In FGF13 KD cells, the action potential peak amplitude decreased by about 20% (Figure 25A, B), consistent with the previously defined effects of FGF13 on the cardiac Na\(^+\) channel current.\(^{78}\) Additionally, we also observed a shortening of the action potential half-width (Figure 25A, C) (control 18.60 ± 3.34 ms (n=7), scrambled shRNA 17.41 ± 3.40 ms (n=5), FGF13 shRNA 9.85 ± 1.34 ms (n=7), p < 0.05 for FGF13 KD vs. SCR and CON). These data are consistent with changes in phase 2 of the cardiac action potential that is predominantly mediated by Ca\(_{\text{V}1.2}\) and implicate FGF13 as a potent regulator of Na\(^+\) and Ca\(^{2+}\) channels in cardiac myocytes, leading to changes in the cardiac action potential.
Figure 25: FGF13 affects the cardiac action potential. A, induced action potentials in rat ventricular cardiomyocytes in current clamp mode at 1 Hz. B, normalized induced action potentials to emphasize the change in half width with FGF13 shRNA. C, D, Summarized data of action potential amplitude and half width. The number of cells analyzed is indicated in parentheses. * P < 0.05, ** P < 0.01.
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<th>$V_{1/2}$ of activation (mV)</th>
<th>$k$ of activation (pA/mV)</th>
<th>$V_{1/2}$ of inactivation (mV)</th>
<th>$k$ of inactivation (pA/mV)</th>
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<td>5.39 ± 0.25 (13)</td>
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<tr>
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<td>4.48 ± 0.36 (12)</td>
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<tr>
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<td>-11.65 ± 1.81 (9)</td>
<td>4.42 ± 0.41 (9)</td>
<td>-35.98 ± 1.91 (7) *</td>
<td>4.88 ± 0.21 (7) *</td>
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$I_{\text{Ca}}$ indicates calcium current
The number of cells analyzed for each parameter is in parentheses
* $P < 0.05$ by ANOVA
3.5 Discussion

Since the initial identification of FHFs their complete physiological roles have remained shrouded. Originally hypothesized to act as extracellular growth factors similar to canonical FGFs, these non-secreted FHF proteins do not appear capable of activating FGF receptors. The subsequent discovery that FHFs are binding partners for the intracellular CTD of voltage-gated Na+ channels provided context to appreciate how knockout of FGF14 might reduce neuronal excitability and cause ataxia in mice and to hypothesize mechanisms for SCA27, for which FGF14 was identified as the genetic locus. Our recent identification of a role for FHFs in the regulation of cardiac Na+ channel function and conduction demonstrated cardiac specific roles. Still, the cellular distribution of FGF13 in cardiomyocytes hinted at additional functions distinct from Na+ channel regulation.

Here, we show for the first time that FHFs regulate cardiac ion channels other than voltage-gated Na+ channels. As such, these results fit well with our recent demonstration that FGF14 regulates presynaptic Ca2+ channels in cerebellar neurons. Specifically, we demonstrated here that FHFs are part of the dyad macromolecular complex with JPH2 and CaV1.2. Through electrophysiologic and molecular biological methods we demonstrated that FGF13 affects Ca2+ current density and targeting of CaV1.2 to T-tubules. This effect on targeting is specific to CaV1.2, and not a general effect on T-tubule structure, as NCX and RyR localization were not altered, nor was overall T-tubule structure perturbed by FGF13 knockdown. We hypothesize that the abnormal localization of CaV1.2 after FGF13 knockdown represents a defect in intracellular sorting of these proteins in cardiomyocytes. Missorting of CaV1.2 was rescued by overexpression of a human FGF13 splice variant, demonstrating the
specificity of the FGF13 knockdown shRNA. Not only did these data provide the first evidence that FGF13 is required for proper targeting of Cav1.2 to the T-tubule in cardiac muscle, but they also show that FGF13 knockdown had profound physiologic effects on Ca\(^{2+}\) current density and Ca\(^{2+}\) cycling, culminating in a decrease in cardiac action potential amplitude and duration and a reduced Ca\(^{2+}\) transient amplitude in parallel with its reduction in Cav1.2 Ca\(^{2+}\) channel current.

The identification of JPH2 as a FGF13 interacting protein provides mechanistic insight to this effect. JPH2 is a structural protein found in the cardiac dyad, where the T-tubule is juxtaposed to one terminal cisterna of the sarcoplasmic reticulum. The structure of JPH2 fixes the distance between the plasma membrane and SR for efficient CICR (Figure 20C). JPH2 contains a cytosolic alpha helical domain that is capped on either side by membrane interaction motifs. On the N-terminus are multiple ‘membrane occupation and recognition nexus’ (MORN) motifs that interact with the inner leaflet of the sarcolemma. At the C-terminus is a transmembrane domain that anchors JPH2 to the sarcoplasmic reticulum.\(^{147}\) Reminiscent of our results after FGF13 knockdown, JPH2 knockdown in cardiomyocytes affects CICR. This provides further support that the interaction between JPH2 and FGF13 that we observed is functionally relevant. Moreover, analogous to loss-of-function mutations in JPH2, we hypothesize that FGF13 mutations may lead to “orphaned ryanodine receptors” that are no longer apposed to Cav1.2, and thus may be associated with heart failure.\(^{151}\) Further studies in the appropriate model are necessary to test that hypothesis.

Nevertheless, the phenotypes after FGF13 and JPH2 knockdown are not identical. JPH2 knockdown did not decrease Cav1.2 Ca\(^{2+}\) current and has no reported effect on voltage-gated Na\(^{+}\) currents. Moreover, JPH2 is observed only in a striated
pattern in cardiomyocytes, while we observed additional FGF13 throughout the cytoplasm and in the nucleus. Thus, the fraction of FGF13 that interacts with JPH2 likely represents only one component of the overall FGF13 pool, and is likely also distinct from the fraction interacting with Na\textsubscript{v}1.5. Other potential FGF13 interactors have previously been reported, such as microtubules in neurons,\textsuperscript{152} likely indicating additional FGF13 pools. The tubulin interaction site on FGF13 maps to a region in close proximity to where Na\textsubscript{v}1.5 interacts in our recent crystal structure.\textsuperscript{43} Thus, we predict that any FGF13 interacting with microtubules would be unable to bind Na\textsubscript{v}1.5 simultaneously, further underlining the concept of distinct FGF13 pools. While we have so far been unable to observe high affinity binding between tubulin and FGF13, the possibility of such an interaction in cardiomyocytes is attractive, particularly in light of the demonstration that Ca\textsubscript{v}1.2 is trafficked along microtubules via BIN1 in ventricular myocytes.\textsuperscript{69}

In summary, this study identified FHFs as novel modulators of the cardiac L-type Ca\textsubscript{v}1.2 Ca\textsuperscript{2+} channel and thereby significantly expands our understanding of the roles of these proteins. Our data have important clinical implications. FGF12, the dominant FHF in human heart (data not shown), is greater than 60% homologous with mouse FGF13. Our data suggest that FGF12 loss-of-function mutations would decrease both Ca\textsuperscript{2+} and Na\textsuperscript{+} channel currents. Since loss-of-function mutations in Na\textsubscript{v}1.5 or Ca\textsubscript{v}1.2 have been reported in BrS,\textsuperscript{153, 154} we specifically hypothesize that FGF12 should be explored as a candidate locus for BrS. Only ~ 30% of BrS patients have an identified mutation,\textsuperscript{155} so loss-of-function mutations in FGF12 may underlie the mechanism in at least some of the remaining cases by nature of its ability to affect both Na\textsuperscript{+} and Ca\textsuperscript{2+} currents.
3.6 Future Directions

In this chapter, I described a novel role for FGF13 in modulating cardiac Ca\textsuperscript{2+} channels, however there are still many questions behind the mechanism of its modulation and whether/how FHFs differentially modulate Na\textsuperscript{+} and Ca\textsuperscript{2+} channels in a given cell.

3.6.1 Does FGF13 directly interact with JPH2?

While we have been able to co-immunoprecipitate FGF13 with JPH2 in ventricular cardiomyocytes and a heterologous expression system overexpressing both proteins, whether they associate through a direct interaction is unclear. If the interaction is direct, we can then use biochemical tools to decipher the domain of interaction on both molecules. This is clinically interesting question as mutations in JPH2 lead to hypertrophic cardiomyopathy.\textsuperscript{74} If these mutations lie in the interaction domain with FHFs and a change in their binding affinity, FHFs might contribute to the pathophysiology of hypertrophic cardiomyopathy.

Additionally, with the previously published crystal structures of FHFs alone,\textsuperscript{79,138} and in complex with Na\textsubscript{v}1.5 and calmodulin,\textsuperscript{43} knowledge of the interaction site can help us determine if JPH2 competes with the Na\textsubscript{v}1.5 CTD for binding or if it likely binds to a separate pool of FHF in the cell. Because JPH2 and Na\textsubscript{v}1.5 are in separate microdomains within the cardiomyocyte, I hypothesize the domain of interaction is different.

3.6.2 How does FGF13 modulate Ca\textsubscript{v}1.2 targeting to the T-tubule membrane?

It remains unclear whether FGF13 binding to JPH2 is actually responsible for its modulation of Ca\textsubscript{v}1.2. While there is overlap in phenotype with loss of JPH2 or FGF13
(mislocalization of Ca\textsubscript{V}1.2 compared to RyR with reduced CICR), van Oort et. al.\textsuperscript{72} did not see any reduction in Ca\textsubscript{V}1.2 current with JPH2 knockdown. I hypothesize that FGF13 might be the link between Ca\textsubscript{V}1.2 and JPH2 because in heterologous expression systems, co-expression of Ca\textsubscript{V}1.2 with various FGF13 splice variants effects current density and channel kinetics. Specifically, FGF13-S increases current density without affecting steady-state inactivation, whereas FGF13-VY does not affect current density but shifts steady-state inactivation in a depolarizing direction (data not shown).

In order to answer this question, and begin to separate the differences in Na\textsuperscript{+} and Ca\textsuperscript{2+} modulation, using the crystal structure of FGF13 and the Na\textsubscript{V}1.5 CTD in our lab, Chaojian Wang has designed an FGF13 that is incapable of binding to Na\textsubscript{V}1.5 as tested by isothermal titration calorimetry and co-expression/co-purification studies. Using the rescue system I established in this chapter, we can determine whether this FGF13 can rescue the electrophysiologic phenotypes of the Na\textsuperscript{+} channel described in the introduction (decreased current density, decreased availability, slower recovery from inactivation) and the Ca\textsuperscript{2+} defects described in this chapter (decreased Ca\textsuperscript{2+} current density and mislocalization of the channel). If for example, the current density and surface expression of the Na\textsuperscript{+} channel can be rescued, but the availability of the channel cannot, one can deduce that the binding is necessary for channel kinetics but not for surface expression and that a more general mechanism of trafficking might be at play for the two ion channels. If concomitant measurement of Ca\textsuperscript{2+} channel current density and localization is unchanged, that will provide further evidence that Na\textsuperscript{+} and Ca\textsuperscript{2+} channel trafficking occurs through a different mechanism than modulation of Na\textsuperscript{+} channel kinetics. If all of the Na\textsuperscript{+} channel phenotype cannot be rescued, but all of the Ca\textsuperscript{2+} channel can, then it is likely that these channels are being modulated by FHFs through
completely separate mechanisms. This, taken together with JPH2 binding data can help to demonstrate the role of FHFs in the dyad as compared to the intercalated disc and sarcolemma. I hypothesize that there is a general mechanism of trafficking that FHF utilizes for both channels. Na\textsubscript{V}1.5 has Ankyrin-G as its structural marker of where in the cell it should be. JPH2 is similar for Ca\textsubscript{V}1.2. Therefore, I hypothesize that FHFs are responsible for getting the channels to their structural partners, which then further delineate proper localization.

**3.6.3 FGF13 decreases Na\textsuperscript{+} and Ca\textsuperscript{2+} channel surface expression, but does not change protein levels; where are all the channels?**

In both studies of FGF13 modulation of Na\textsuperscript{+} and Ca\textsuperscript{2+} channels, we showed decreased surface expression, but no change in total protein levels. Additionally, using immunocytochemistry I showed that the Ca\textsuperscript{2+} channels were not in the typical striated pattern of the cardiac T-tubule, even though T-tubule structure was grossly intact. An important question is what part of trafficking do FHFs take part in? Figure 26 is a schematic of the anterograde ion channel trafficking pathway. FHFs can take part in anything from translation on the ribosome to endoplasmic reticulum and golgi export to transport of the vesicles along microtubules. Indeed, data from the interaction proteomics experiment on FGF13 in mouse ventricle revealed multiple ribosomal subunits, suggesting it might take part in translation of the protein or targeting that protein to the proper place once it has been translated. Additionally, Wu et. al.\textsuperscript{152} showed that FGF13 binds to and stabilizes microtubules. Evidence suggests that both Ca\textsuperscript{2+} and Na\textsuperscript{+} channels are trafficked along microtubules.\textsuperscript{69,156} While this idea is promising, I could not replicate the interaction of FGF13 and tubulin in a heterologous expression system or cardiomyocytes.
To define where it is the channels are being held with FGF13 KD, ultracentrifugation to separate the individual organelles with a sucrose gradient can be performed. In that way, we can isolate the nucleus, the endoplasmic reticulum, the golgi and the plasma membrane and look at expression of the FHFs in that subcellular compartment and the ion channels of interest. This will provide insight into where the FHFs are exerting their effects on trafficking and might even hint at how they traffic Na⁺ channels to the AIS in neurons as well.

Figure 26: The anterograde vesicular trafficking pathway. Schematic representation of the forward trafficking pathway that de novo ion channels (white cylinders) traverse to reach their specific destinations. Nascent ion channel polypeptides are translocated to the membrane of the rough endoplasmic reticulum (ER), after which they are modified and move through the ER–Golgi intermediate compartment (ERGIC), Golgi apparatus, and trans-Golgi network (TGN). From the TGN, vesicles containing ion channels are loaded onto microtubules and are transported to their destinations. Microtubule plus-end binding proteins interact with scaffolding proteins specific to particular membrane subdomains to achieve targeted delivery of vesicular cargo. Reprinted from Heart Rhythm, Vol 7, Smyth JW, Shaw RM, Forward trafficking of ion channels: What the clinician needs to know, 8;1135-1140, copyright 2010 with permission from Elsevier.
Retrograde trafficking and endocytosis also must be considered. It has been shown that the Ca\(^{2+}\) channel β subunits drives dynamin-dependent endocytosis of the channel,\(^{157}\) as have islet-brain 1 in neurons. Islet-brain 2 is a member of the islet-brain protein family. It has been shown to bind to FHF\(^{158, 159}\) and modulate vesicle trafficking in neurons.\(^{160}\)

### 3.6.4 Do FHFs play a role in cardiac hypertrophy or heart failure?

With loss of FGF13, I observed a mislocalization of the Ca\(_v\)1.2 Ca\(^{2+}\) channel compared to RyR, decreased Ca\(^{2+}\) current density and decreased EC coupling. Mislocalization of Ca\(^{2+}\) channels relative to RyR and reduced CICR have been reported in multiple models of heart failure.\(^{73, 151, 161}\) To test whether FGF13 plays a role in heart failure, I was provided ventricular tissue lysate of mice that had been subjected to transverse aortic constriction (TAC) or sham operated by Dennis Abraham, MD from the laboratory of Howard Rockman, MD. TAC mice were shown to have decreases in ejection fraction by at least 30% indicating that they were in heart failure. I took the lysates and western blotted them to compare levels of FGF13 in sham or TAC mice. As shown in Figure 27, levels of FGF13 are doubled in mice in heart failure due to TAC.
Figure 27: FGF13 levels are increased in heart failure. Example of one of three western blots for FGF13 from sham versus TAC mice. Five TAC mice and four sham mice were quantified to show a doubling of FGF13 protein levels in heart failure. GAPDH as loading control.

This suggests that FHFs are important in heart failure, through what I hypothesize to be a compensatory mechanism as heart failure typically leads to reduction in channel protein function. In order to study this further, the proper model system is necessary in which FGF13 is knocked out and we can test the response to TAC-induced heart failure on whole-animal physiology compared to wild-type controls. Unfortunately, FGF13−/− mice are embryonic lethal at day E10.5 and exhibit large pericardial effusions. Because FGF13 is found on the X-chromosome (in mice and humans), the FGF13X− females are actually mosaics due to random X-inactivation. While they do display a post-partum cardiomyopathy, they are not a proper system to study FHFs in heart failure. Eric Q. Wei, a MD-PhD student in the Pitt lab, is designing a mouse with the FGF13 locus floxed, mouse such that these mice can be crossed with a cardiac-specific Cre-recombinase mouse to create organ-specific knockout. This will provide the model necessary to study FHFs in heart failure in greater detail.

3.6.5 Do FHFs play a role in inherited cardiac arrhythmias?

I showed that loss of FGF13 in ventricular cardiomyocytes has profound effects on action potential peak height and half width, correlating with the decrease in Na+ and
Ca\textsuperscript{2+} current density demonstrated. As described in section 1.5.3, the effects of FHF's on Na\textsuperscript{+} channel currents and availability would predict loss of function mutations would lead to a BrS phenotype due to a loss of the action potential dome and increased transmural dispersion due to loss of Ca\textsuperscript{2+} and Na\textsuperscript{+} channel function.\textsuperscript{163} In the next chapter, I will demonstrate the effects of a FHF mutation leading to BrS on Na\textsuperscript{+} and Ca\textsuperscript{2+} currents and action potential morphology.
4. FGF12 is a novel Brugada syndrome locus

For this study, we collaborated with Michael J. Ackerman at the Mayo Clinic in Minnesota to identify patients with mutations in FHF5 that lead to BrS. The following is a physiologic study of a single mutant in FGF12 in a patient with BrS. It is currently in preparation for submission to Circulation.

4.1 Summary

**Background:** Less than 30% of Brugada syndrome (BrS) cases have an identified genetic cause. Of the known BrS-susceptibility genes, loss-of-function mutations in SCN5A or CACNA1C and their auxiliary subunits are most common. Based on the recent demonstration that fibroblast growth factor homologous factors (FHFs; FGF11-FGF14) regulate cardiac Na\(^+\) and Ca\(^{2+}\) channel currents, we hypothesized that FHFs are candidate BrS loci.

**Methods and Results:** We identified FGF12 as the major FHF expressed in human ventricle and used a candidate gene approach to query a phenotype-positive and genotype-negative BrS biorepository. We identified a single missense mutation in FGF12-B (Q7R-FGF12), among 102 subjects. Biochemical assays demonstrated this mutant reduced binding to the Na\(\text{v}1.5\) C-terminus, but not to Junctophilin-2, which mediates Ca\(^{2+}\) channel regulation. We then developed a novel adult rat ventricular cardiomyocyte system to query the effects of the mutation on multiple ionic currents in their native milieu and on the cardiac action potential. We replaced the endogenous FHF with human wild-type- or Q7R-FGF12 and using whole-cell patch clamp, demonstrated that the mutant reduced Na\(^+\) channel current density and availability without affecting Ca\(^{2+}\) channel function. Further, the mutant, but not wild-type FGF12, reduced action potential amplitude, consistent with the mutant-induced loss of Na\(^+\) channel function.

**Conclusions:** These multilevel investigations strongly suggest that Q7R-FGF12 is a disease-causing BrS mutation. Moreover, these data demonstrate for the first time that FHF effects on Na\(^+\) and Ca2\(^{+}\) channels are separable. Most significantly, this study establishes a new method to analyze effects of human arrhythmogenic mutations on cardiac ionic currents.
4.2 Introduction

Brugada syndrome (BrS) is a life-threatening inherited cardiac arrhythmia characterized by ST-segment elevation in the right precordial leads of the electrocardiogram (ECG).\textsuperscript{155} BrS is a channelopathy. The most commonly mutated locus is SCN5A, which encodes the pore-forming subunit of the major cardiac voltage-gated Na\textsuperscript{+} channel, Na\textsubscript{v}1.5, responsible for the phase 0 upstroke of the ventricular action potential. BrS mutations in SCN5A lead to a loss of function in Na\textsubscript{v}1.5, through a decrease in channel availability or surface expression.\textsuperscript{164} Mutations have also been found in the CACNA1C-encoded pore-forming subunit of the cardiac Ca\textsubscript{v}1.2, L-type Ca\textsuperscript{2+} channel; these mutations are also loss-of-function.\textsuperscript{98,153} In addition to loci encoding ion channel pore-forming subunits, genes associated with BrS include those encoding channel modulatory proteins such as β subunits of both the Na\textsuperscript{+} and Ca\textsuperscript{2+} channels, as well as proteins responsible for their trafficking and targeting to the proper location within the cardiomyocyte. For most BrS patients, however, genetic analysis does not identify a cause, suggesting the existence of additional, yet unidentified BrS loci.

Among candidate ion channel modulatory subunit genes are those encoding fibroblast growth factor (FGF) homologous factors (FHF; FGF11-14), which can modulate both voltage-gated Na\textsuperscript{+} and Ca\textsuperscript{2+} channels.\textsuperscript{78,165,166} FHFs are part of the FGF superfamily, but are not secreted, cannot bind or activate FGF receptors, and do not function as growth factors.\textsuperscript{138} Instead, FHFs remain intracellular where they perform multiple tasks. Their best characterized intracellular binding partner is the C-terminal domain (CTD) of voltage-gated Na\textsuperscript{+} channels.\textsuperscript{80} Knockdown of the predominant FHF expressed in murine ventricular myocytes, FGF13, leads to decreased Na\textsuperscript{+} channel current density and channel availability in isolated ventricular cardiomyocytes, and a
reduction in conduction velocity in a myocyte monolayer. Additionally, FGF13 knockdown reduces Ca\textsubscript{v}1.2 current density and prevents proper targeting of Ca\textsubscript{v}1.2 channels to the T-tubule.

Because FHFs modulate two cardiac ionic currents that, when perturbed, lead to BrS, we hypothesized that loss-of-function mutations in FHFs would be associated with BrS. To test this hypothesis, we identified the major FHF expressed in human ventricle, the “B” splice variant of FGF12, and then applied a candidate gene approach to patients with phenotype-positive but heretofore genotype-negative BrS. Among 102 unrelated patients with BrS, we found a single, rare missense mutation in FGF12-B (Q7R-FGF12). To test the physiological effects of Q7R-FGF12, we developed a system to query the effects of the Q7R or WT FGF12 in an adult cardiomyocyte by replacing the endogenous FGF13 with the human variants. With this novel approach, we showed that the Q7R-FGF12 mutation leads to a Na\textsuperscript{+} channel loss-of-function phenotype consistent with BrS, thereby identifying FGF12 as a new BrS locus.
4.3 Materials and Methods

4.3.1 Study Population

The study population consisted of 102 unrelated patients with BrS who were referred to either the Molecular Cardiology Laboratory, Fondazione IRCCS Policlinico San Matteo, Pavia Italy or to the Windland Smith Rice Sudden Death Genomics Laboratory at Mayo Clinic, Rochester, Minnesota for laboratory-based genetic testing. All BrS patients included in this study remained genotype negative after comprehensive genotyping for mutations in the fourteen known BrS-susceptibility genes listed here: SCN5A, GPD1L, CACNA1C, CACNB2B, SCN1B [including the alternatively spliced exon 3A; SCN1Bb], SCN3B, KCNE3, KCNJ8, KCND3, CACNA2D1, MOG1 and HCN4, KCNE1L and SLMAP. This study was approved by both the Mayo Foundation Institutional Review Board and the Medical Ethical Committee of Fondazione IRCCS Policlinico San Matteo. Informed consent was obtained for all patients.

4.3.2 Mutational Analysis and Control Population

Comprehensive open reading frame/splice site mutational analysis of all amino acid coding exons and intron borders of FGF12 was performed using polymerase chain reaction (PCR), denaturing high performance liquid chromatography (DHPLC), and DNA sequencing as previously described.\textsuperscript{167} PCR primer sequences and PCR / DHPLC conditions are available upon request.

In order to be considered as a putative pathogenic mutation, any FGF12 variant had to be i) non-synonymous and ii) absent among at least 1000 ethnically-matched controls obtained from the European Collection of Cell Cultures (HPA Culture Collections, UK), the Human Genetic Cell Repository sponsored by the National Institute of General Medical Sciences and the Coriell Institute for Medical Research (Camden,
New Jersey), and from the Blood Transfusional Centre in IRCCS Policlinico San Matteo of Pavia (Italy) and all available online databases, including the 1000 Human Genome Project database\textsuperscript{168,169} (www.1000genomes.org/ensembl-browser, n=1094 individuals; 381 Caucasian; 246 African-American, 286 Asian, and 181 Hispanic), the NHLBI GO Exome Sequencing Project\textsuperscript{170} (http://evs.gs.washington.edu/EVS/, n=5379 individuals; 3510 Caucasian and 1869 African-American), and the Exome Chip Design\textsuperscript{171} (http://genome.sph.umich.edu/wiki/Exome_Chip_Design, n=12000 individuals). Mutations were annotated using the single letter nomenclature whereby F45M for example denotes a non-synonymous variant producing a missense mutation involving a substitution of Phenylalanine (F) by a methionine (M) at amino acid position 45.

4.3.3 Subcloning and adenovirus production

Human FGF12-B (accession no. NM_004113.5) in pIRES2-AcGFP\textsuperscript{81} was mutated using Quickchange II Site-directed Mutagenesis (Agilent Technologies) to form Q7R-FGF12 and then both were subcloned into the pAdRFP adenovirus shuttle vector. The adenoviruses expressing FGF13 shRNA with GFP has been previously described.\textsuperscript{78} WT-FGF12 and Q7R viruses were generated similarly using the AdEasy System (Agilent). The adenoviral plasmid was packaged in HEK293 cells. The recombinant virus was isolated by multiple freeze/thaw cycles, further amplified and then purified and concentrated using Vivapure Adenopack 20 (Sartorius Stedim Biotech). The viral titer was determined using optical density. All constructs were confirmed by sequencing.

4.3.4 HEK293T cell transfection, electrophysiology and co-immunoprecipitation

Transfection, \( \text{Na}_v 1.5 \) \( \text{Na}^+ \) current recording with FGF12-B and immunoprecipitation techniques have been previously described in HEK293T cells.\textsuperscript{81}
The construct encoding wild-type human JPH2 was generously provided by Xander Wehrens (Baylor College of Medicine, Houston, TX).

4.3.5 Cardiomyocyte isolation

Animals were handled according to National Institutes of Health Guide for the Care and Use of Laboratory Animals. The study was approved by Duke University Animal Care and Welfare Committee. Cardiomyocytes were isolated from 6-8 week old Sprague Dawley rats and cultured as previously described.78

4.3.6 Cardiomyocyte electrophysiology

Ca$^{2+}$ currents ($I_{Ca}$) and Na$^{+}$ currents ($I_{Na}$) were recorded using the whole-cell voltage-clamp technique in cardiomyocytes after 36 to 48 hours as previously described.78,172 Cardiac action potentials were recorded in current clamp as previously described.172 Input resistance was not different between the groups and junction potential was calculated to be 5.6 mV and not corrected.

4.3.7 Surface biotinylation and western blotting

Surface biotinylation and western blotting were performed as previously described.78

4.3.8 Immunocytochemistry

Immunocytochemistry was performed as previously described.172

4.3.9 Statistical analysis

Results are presented as means ± standard error of the mean; statistical significance of differences between groups was assessed using one-way analysis of variance (ANOVA) and was set at P < 0.05.
4.4 Results

4.4.1 FGF12, the most highly expressed FHF in human ventricle, is a BrS candidate locus

As summarized in Figure 28, we have shown that, in adult ventricular cardiomyocytes, FHFs regulate Na\textsubscript{v}1.5 trafficking and function; and Ca\textsubscript{v}1.2 targeting to T-tubules, likely through a junctophilin-2 (JPH2)-mediated process.\textsuperscript{78,165} This prompted us to hypothesize that loss-of-function mutations in FHFs could perturb Na\textsubscript{v}1.5 and/or Ca\textsubscript{v}1.2 function and lead to BrS. To determine the complement of FHFs expressed in human ventricle, we extracted mRNA from non-failing human ventricular tissue and performed quantitative reverse-transcriptase polymerase chain reaction (qPCR) for all FHF splice variants (see Table 13 for primer sequences). Each of the four FHFs undergoes alternative splicing of their first exon, which confers unique Na\textsuperscript{+} channel modulatory properties.\textsuperscript{82} Summarized in Figure 28B, qPCR demonstrated that FGF12-B is the most highly expressed FHF splice variant. There was a negligible amount of FGF12-A. FGF13-Y and/or FGF13-VY (the qPCR strategy cannot distinguish between these two variants) mRNAs were also detected, at ~ 40% of the amount of FGF12-B.

We therefore screened 102 unrelated BrS patients in whom genetic testing had not detected a known BrS locus for FGF12 mutations. Clinical demographics of the BrS genotype negative study cohort are shown in Table 14. Overall, the majority of our cohort were male (82%), of Caucasian descent (100%) with a mean age at diagnosis of 45 ± 13 years and a mean QTc of 407 ± 27 ms. Forty-six patients (45%) had a spontaneous type 1 Brugada ECG pattern at baseline, 50 patients (49%) had a type 1 Brugada ECG pattern induced after intravenous injection of a sodium channel blocker, and 6 patients had a type 1 Brugada ECG pattern with fever (6%). Of the 102 patients in
the study cohort, 12 patients (12%) had a personal history and 27 patients (26%) had a family history of arrhythmic syncope, sudden cardiac arrest, or sudden death.

Following comprehensive mutational analysis of *FGF12*, one novel putative pathogenic missense mutation, resulting in a substitution of glutamine (Gln, Q) at amino acid residue 7 by arginine (Arg, R), Q7R-FGF12, was identified in our cohort of genotype negative/phenotype positive patients with BrS. This mutation involved a residue that is highly conserved across species and was absent in over 1000 ethnically matched, internal control individuals and all publically available whole exome databases (> 12,000 individuals).

The Q7R-FGF12 mutation was identified in a 61-year-old male who exhibited an abnormal ECG pattern suggestive of BrS during flecainide therapy for atrial fibrillation (AF). He subsequently underwent a flecainide challenge that induced a diagnostic type 1 Brugada ECG during his electrophysiology study and AF ablation. His personal history revealed an episode of supraventricular tachycardia during surgery for a hiatal hernia; however, he had a negative family history of SCD.

This mutant was of particular interest because it directly abuts the Na\(_V\)1.5 interaction surface and leads to a change in charge (neutral to positive) near an important conserved electrostatic interaction site, K9 (Fig. 28A, inset), which we had previously showed forms a salt bridge with E1890 in Na\(_V\)1.5. Disruption of this salt bridge markedly reduced the affinity of FGF12 for Na\(_V\)1.5 and affected the regulation of Na\(_V\)1.5 by FGF12.\(^{43}\) We therefore suspected that Q7R might adversely affect interaction with Na\(_V\)1.5 and consequent regulation of Na\(_V\)1.5 function. Because we recently discovered that FHFs also affect Ca\(_V\)1.2, we considered that the Q7R mutant might also perturb Ca\(_V\)1.2 regulation.
Figure 28: FHFs modulate cardiac Ca$^{2+}$ and Na$^+$ channels and FGF12 is a locus for BrS. A, FHFs modulate Ca$_{v}$.1.2 trafficking to the T-tubule in the cardiac ventricular myocyte through interactions with JPH2. FHFs also bind to the Na$_{v}$.1.5 CTD, modulating trafficking to the sarcolemma and channel kinetics. Right, a surface rendering of the interface between the Na$_{v}$.1.5 CTD (blue) and FHF core domain (red). Q7 (aqua) is in the same plane as the salt bridge between the Na$_{v}$.1.5 CTD (E1890, yellow) and FHF core (K9, yellow). B, FGF12-B is the most highly expressed FHF in human ventricle by qPCR. C, denaturing high performance liquid chromatography (DHPLC) profiles (wild-type, brown trace, and Q7R, green trace). D, DNA sequence chromatograms showing the nucleotide change at position resulting in a Glutamine (Q) to Arginine (R) substitution at position 7 (Q7R) versus normal. E, sequence conservation across species for Q7R in FGF12B. F, Leads V1 and V2 of an electrocardiogram from the proband during a diagnostic flecainide challenge.
**Table 13: qPCR primer pairs used for detecting FHF mRNA levels in adult human ventricle**

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<tr>
<th>FGF isoforms</th>
<th>Forward primer (5’ – 3’)</th>
<th>Reverse primer (5’ – 3’)</th>
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<tr>
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<td>GTAGATTAGTGACTGTACAT</td>
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<td>GAPDH</td>
<td>CATCAGCAATGCCTCCTGCA</td>
<td>CCGTTAGCTCGGGAGTAC</td>
<td>220</td>
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*BP denotes length in base pairs*
Table 14: Demographics of genotype-negative BrS patient cohort

<table>
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<tr>
<th>Patient Demographic</th>
<th>Cohort</th>
</tr>
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<tbody>
<tr>
<td>No. of Probands</td>
<td>102</td>
</tr>
<tr>
<td>Age at Diagnosis (years)</td>
<td>45 ±13</td>
</tr>
<tr>
<td>Range (yrs)</td>
<td>9-81</td>
</tr>
<tr>
<td>Males</td>
<td>84 (82%)</td>
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<tr>
<td>Females</td>
<td>18 (18%)</td>
</tr>
<tr>
<td>Average QTc (ms)</td>
<td>407 ± 27</td>
</tr>
<tr>
<td>Average PQ Interval (ms)</td>
<td>169 ± 27</td>
</tr>
<tr>
<td>Symptomatic Patients (%)</td>
<td>12 (12%)</td>
</tr>
<tr>
<td>Family History of Cardiac Events / Unexplained Sudden Death (%)</td>
<td>27 (26%)</td>
</tr>
<tr>
<td>Type 1 ST-segment Elevation at Baseline (%)</td>
<td>46 (45%)</td>
</tr>
<tr>
<td>Type 1 ST-segment Elevation with Sodium Blockade (%)</td>
<td>50 (49%)</td>
</tr>
<tr>
<td>Type 1 ST-segment Elevation with Fever (%)</td>
<td>6 (6%)</td>
</tr>
</tbody>
</table>
4.4.2 The Q7R mutant in FGF12-B decreases binding of FGF12-B to Na\textsubscript{v}1.5 CTD

To test whether Q7R produces Na\textsubscript{v}1.5 or Ca\textsubscript{v}1.2 dysregulation, we first overexpressed Na\textsubscript{v}1.5 in HEK293T cells with His6-tagged FGF12-B wild type (WT-FGF12) or Q7R-FGF12 and performed co-immunoprecipitations to examine whether the mutant affected the interaction with the Na\textsubscript{v}1.5 CTD or JPH2. There was no difference in co-immunoprecipitation of WT-FGF12 or Q7R-FGF12 with JPH2, as shown in Figure 29A. In contrast, markedly less (33 ± 7 %, n= 3, p < 0.05 ) Na\textsubscript{v}1.5 co-immunoprecipitated with Q7R-FGF12 than with WT-FGF12 (Figure 29B-C), consistent with the mutant’s location at the FGF12-Na\textsubscript{v}1.5 interface (see Fig. 28A). Additionally, isothermal titration calorimetry with purified recombinant WT-FGF12 or Q7R-FGF12 with the Na\textsubscript{v}1.5 CTD showed a two-fold reduction in binding affinity (Figure 29D) for the mutant. These data show that Q7R-FGF12 specifically affects interaction with Na\textsubscript{v}1.5 and suggest that functional perturbation of cardiac ion currents would likely be limited to Na\textsubscript{v}1.5 rather than Ca\textsubscript{v}1.2.
Figure 29: Q7R-FGF12 decreases affinity for the Na\textsubscript{V}1.5 CTD. A, co-immunoprecipitation and western blot of FGF12-B WT or Q7R with JPH2 from at least three independent experiments. B, co-immunoprecipitation and western blot of FGF12-B WT or Q7R with Na\textsubscript{V}1.5 shows reduced Na\textsubscript{V}1.5 pulled down with Q7RF-FGF12 compared to WT; * p < 0.05. Quantified in C from at least three independent experiments. D, ITC data shows a two-fold decrease (p < 0.05) in binding affinity for Q7R-FGF12 versus WT from three independent experiments.
4.4.3 Q7R-FGF12 affects Na\textsubscript{v}1.5 current density and kinetics in HEK293T cells

To test the functional effects of Q7R-FGF12 on Na\textsubscript{v}1.5, we co-expressed Na\textsubscript{v}1.5 alone or together with WT-FGF12 or Q7R and recorded Na\textsuperscript{+} currents using whole-cell patch clamp. When co-expressed with Na\textsubscript{v}1.5, WT-FGF12 decreased current density compared to Na\textsubscript{v}1.5 alone (Figure 30A, Table 15). This result was unexpected since we had previously shown that knockdown of FGF13 in adult ventricular cardiomyocytes reduced current density and trafficking of Na\textsubscript{v}1.5 to the plasma membrane. We considered the possibility that FGF12 and FGF13 exert distinct effects on Na\textsubscript{v}1.5, consistent with the isoform-specific differences in interaction between FHFs and Na\textsubscript{v} CTDs that we previously documented,\textsuperscript{81} and isoform-specific effects on voltage-gated Na\textsuperscript{+} currents previously reported,\textsuperscript{82} but observed that co-expression of FGF13 also reduced Na\textsubscript{v}1.5 current density (Figure 31). Nevertheless, we were readily able to distinguish between WT-FGF12 and Q7R-FGF12, since the degree of modulation of current density by Q7R-FGF12 was reduced compared to WT-FGF12 (Figure 30A). We also tested the effect of FGF12-WT and FGF12-Q7R on steady-state inactivation. We had observed that knockdown of FGF13 in adult rat ventricular cardiomyocytes induced a hyperpolarizing shift in the \textit{V}_{1/2}. As with measurement of current density, we could distinguish between WT-FGF12 and Q7R-FGF12, as WT-FGF12 induced a +4 mV shift in the \textit{V}_{1/2} of steady-state inactivation, while co-expression of Q7R-FGF12 produced no change (Figure 30B, Table 15). Although the differences between WT-FGF12 and Q7R-FGF12 on Na\textsubscript{v}1.5 modulation were consistent with the mutant’s reduced affinity for Na\textsubscript{v}1.5, as observed in Figure 29, these data suggest that HEK293T cells cannot recapitulate all key features of Na\textsubscript{v}1.5 modulation by FHFs previously observed in murine adult cardiomyocytes.
Figure 30: Q7R-FGF12 affects Na\textsubscript{v}1.5 current density and inactivation differently than WT in a heterologous expression system. A, WT-FGF12 significantly reduces Na\textsubscript{v}1.5 current density, whereas Q7R-FGF12 does not; * p < 0.05 versus Na\textsubscript{v}1.5 only. B, WT-FGF12 significantly depolarizes the steady-state inactivation curve by 4 mV (p< 0.05 versus Na\textsubscript{v}1.5 only). Q7R-FGF12 is unable to shift the curve from control levels.

Figure 31: Overexpression of FGF13 reduces Na\textsubscript{v}1.5 current density in a heterologous expression system. A, representative current traces of Na\textsubscript{v}1.5 only, or Na\textsubscript{v}1.5 plus FGF13-VY (the most commonly expressed splice variant in ventricular cardiomyocytes) or FGF13-U. B, summarized data for the peak current density at -30 mV (280.01 ± 32.18 pA/pF, n=11 for Na\textsubscript{v}1.5 only; 119.15 ± 17.89 pA/pF, n=12, for Na\textsubscript{v}1.5 + FGF13-VY; 121.24 ± 22.70 pA/pF, n=10, for Na\textsubscript{v}1.5 + FGF13-U. * p < 0.01.
Table 15: Summary of electrophysiology data from recordings in HEK293T cells

<table>
<thead>
<tr>
<th></th>
<th>$I_{Na}$ peak at -55 mV (pA/pF)</th>
<th>$V_{1/2}$ of activation (mV)</th>
<th>$k$ of activation (pA/mV)</th>
<th>$V_{1/2}$ of inactivation (mV)</th>
<th>$k$ of inactivation (pA/mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaV1.5 only</td>
<td>-481.7 ± 77.1 (6)</td>
<td>-50.6 ± 2.2 (6)</td>
<td>3.1 ± 0.7 (6)</td>
<td>-87.6 ± 1.0 (9)</td>
<td>5.0 ± 0.1 (9)</td>
</tr>
<tr>
<td>NaV1.5 + WT-FGF12</td>
<td>-213.1 ± 44.9 (11) *</td>
<td>-45.0 ± 1.9 (11)</td>
<td>4.1 ± 0.5 (11)</td>
<td>-83.8 ± 0.6 (12) *</td>
<td>5.1 ± 0.1 (12)</td>
</tr>
<tr>
<td>NaV1.5 + Q7R-FGF12</td>
<td>-309.4 ± 101.4 (5)</td>
<td>-51.0 ± 1.4 (5)</td>
<td>3.0 ± 0.4 (5)</td>
<td>-86.4 ± 0.8 (9)</td>
<td>5.0 ± 0.3 (9)</td>
</tr>
</tbody>
</table>

$I_{Na}$ denotes Na$^+$ current

* p < 0.05

Numbers recorded are in parentheses
4.4.4 A system to study FHF modulation of ion channels in their native milieu

We therefore designed a system to explore the physiologic effects of FHFs in adult rat ventricular cardiomyocytes. Our aim was to “replace” the endogenous FGF13 with human WT-FGF12 or Q7R-FGF12 and query the resulting effect on Na⁺ currents. We began by using our established adenovirally-mediated shRNA knockdown of FGF13 in cultured adult rat ventricular cardiomyocytes (marked by GFP), in which we achieve a >50% reduction of FGF13 protein within two days in culture, and co-infected with a separate adenovirus expressing either WT-FGF12 or Q7R-FGF12 (marked by mRFP). We confirmed effective knockdown of the endogenous rat FGF13 and proper subcellular localization of expressed FGF12 by immunocytochemistry with antibodies to FGF13 or to the Hisx6 tag on the expressed FGF12 C-termini (Figure 32).
Figure 32: A system to study the effects of FGF12 on ventricular cardiomyocyte physiology. Control cell stained for FGF13 in Cyan shows endogenous FGF13 distribution in the cardiomyocyte at the intercalated disc, T-tubules and nucleus; magnified inset (2X) emphasizes distribution at the intercalated disc and in a striated pattern. Knockdown with FGF13 using a virus that expresses GFP and shRNA reduces the reactivity of the FGF13 antibody, even with overexpression of WT-FGF12, indicating no cross-reactivity. Using a virus expressing FGF13 shRNA without GFP and overexpressing WT-FGF12 (indicated by RFP), there is a decreased immunoreactivity for FGF13 (green) and a signal using the His6 antibody that correlates to endogenous FGF13 expression. A similar pattern of His6 immunoreactivity is observed for Q7R-FGF12. Insets are magnified 2X to emphasize pattern of distribution. Scale bar 50 μm for large images and 12.5 μm for inset.
4.4.5 Q7R-FGF12 differentially affects Na\textsuperscript{+} and Ca\textsuperscript{2+} currents in a ventricular cardiomyocyte

Using whole-cell patch clamp, we recorded voltage-gated Na\textsuperscript{+} currents in uninfected Control (CON) cardiomyocytes, those with FGF13 knocked down (FGF13 KD), and cells with FGF13 KD and concomitant expression of WT-FGF12 or Q7R-FGF12 (Figure 33, Table 16). Consistent with our previous results, FGF13 KD reduced Na\textsuperscript{+} current density without affecting voltage-dependence of activation (Figure 33A-D, -69.47 ± 9.03 pA/pF, n=14, for Control versus -34.45 ± 5.06 pA/pF, n=9, for FGF13 KD, p < 0.05) and decreased channel availability through a -4 mV shift in the $V_{1/2}$ of steady-state inactivation (Figure 33E). Co-expression of WT-FGF12 compensated for the loss of FGF13, significantly increasing Na\textsuperscript{+} channel current density compared to FGF13 KD or Control (-100.28 ± 18.69 pA/pF, n=9, p < 0.05 versus Control and FGF13 KD) and inducing a +6 mV shift in the $V_{1/2}$ of steady-state inactivation compared to FGF13 KD (Table 16, Figure 33E). In contrast, co-expression of Q7R-FGF12 failed to rescue current density or the hyperpolarizing shift in steady-state inactivation (Table 16, Figure 33E).

While the diminished ability of FGF12-Q7R to rescue FGF13 KD compared to FGF12-WT is consistent with the mutant’s decreased binding affinity for the Na\textsubscript{v}1.5 CTD, the augmentation of Na\textsuperscript{+} channel current density above Control cells was unexpected, and suggested three possibilities: FGF12 is an even more potent FHF for Na\textsuperscript{+} currents than FGF13; FGF12 has additional effects on Na\textsuperscript{+} currents compared to FGF13; or Na\textsubscript{v}1.5 is not saturated by endogenous FGF13 so that FGF12 over-expression modulates additional Na\textsubscript{v}1.5 channels that were not regulated by a limiting pool of endogenous FGF13. To distinguish among these possibilities, we overexpressed WT-FGF12 without knocking down FGF13 and measured the effects upon Na\textsuperscript{+} current...
density. Overexpression of WT-FGF12 did not significantly increase Na\(^+\) channel current density in these conditions (Figure 34), indicating that FGF12 does not exert additional effects on Na\(_{\text{v}}\)1.5 beyond FGF13 and that the effects of FGF13 on Na\(_{\text{v}}\)1.5 are saturated in Control cells. Thus, we conclude that FGF12 is more potent than FGF13 for increasing Na\(_{\text{v}}\)1.5 at the sarcolemma. The lack of an effect of FGF12 in the presence of FGF13 is consistent with our previous data showing that FGF13 has a higher affinity for the Na\(_{\text{v}}\)1.5 CTD than FGF12;\(^{81}\) in the absence of the higher affinity FGF13 (after knockdown), expressed FGF12 is capable of exerting its increased potency. We also considered that the failure of Q7R-FGF12 to increase Na\(^+\) current density in the context of FGF13 knockdown could be due to a dominant negative effect, similar to effect of the F150S mutation in FGF14, which causes spinocerebellar ataxia 27.\(^{84}\) We therefore expressed Q7R-FGF12 without knocking down FGF13. Under these conditions, the effects of Q7R-FGF12 were negligible (Figure 34). These results not only rule out a dominant negative effect, but also support the hypothesis that endogenous FGF13 effects are saturating in Control cells.

As shown in Fig. 29, the Q7R mutation did not appear to affect interaction with JPH2, leading us to expect that the mutant would have minimal effects upon \(I_{\text{Ca}}\). We tested this hypothesis with the same replacement strategy. Consistent with our previous data, FGF13 KD in adult ventricular cardiomyocytes reduced Ca\(^{2+}\) current density (Figure 35A-B). Adenoviral expression of WT-FGF12 rescued the effect of FGF13 KD. Expression of Q7R-FGF12 potentiated Ca\(^{2+}\) currents, increasing current density to a level 30% higher than Control or rescue with WT-FGF12, although this difference was not significantly different from WT-FGF12 (Figure 35, \(p=0.09\)). Additionally, both WT and Q7R FGF12 were able to rescue Ca\(_{\text{v}}\)1.2 mislocalization observed after FGF13
knockdown (Figure 35C). These results indicated that FHFs modulate Na\(^+\) and Ca\(^{2+}\) currents via separable mechanisms.

Figure 33: Q7R-FGF12 cannot rescue reduced Na\(_V\)1.5 current density and availability from FGF13 KD while WT can. A, representative Na\(^+\) current traces for the four groups tested. B-C, I-V curve and summarized peak current data. D, activation curve and E, steady-state inactivation curve; see Table 3 for quantification. * p < 0.05 vs. Control, ** p < 0.05 versus WT-FGF12B, † p < 0.01 versus WT-FGF12B.
Figure 34: WT and Q7R FGF12 do not affect Na\(^+\) channel current density without FGF13 KD. I-V curve depicting the current density for CON and those cells with overexpression of WT or Q7R FGF12 (peak current densities at -45 mV were -18.05 ± 3.02 pA/pF (n=5), -20.49 ± 3.73 pA/pF (n=10), -15.53 ± 1.96 pA/pF (n=7) for Control, WT-FGF12, and Q7R-FGF12, respectively, p = 0.55.
Figure 35: Both WT and Q7R FGF12 rescue Ca\textsuperscript{2+} current density and localization from FGF13 KD. A, representative Ca\textsuperscript{2+} current traces from the four groups. B, I-V curve depicting the rescue of Ca\textsubscript{v}1.2 current density with WT and Q7R FGF12. Peak current densities at 0 mV are -13.01 ± 0.88 pA/pF (n=9) for Control, -8.09 ± 1.30 pA/pF (n=13) for FGF13 KD, -12.86 ± 1.50 pA/pF (n=13) for FGF13 KD + WT-FGF12, -17.05 ± 1.46 (n=13) for FGF13 KD + Q7R-FGF12. C, immunostaining for Ca\textsubscript{v}1.2, green and ryanodine receptor, red, showing that Ca\textsubscript{v}1.2 is mislocalized with FGF13 KD and the localization is rescued with WT and Q7R FGF12. * p < 0.05 versus control.
Table 16: Summary of electrophysiology data

<table>
<thead>
<tr>
<th>$I_{Ca}$</th>
<th>$I_{Ca}$ peak at 0 mV (pA/pF)</th>
<th>$V_{1/2}$ of activation (mV)</th>
<th>k of activation (pA/mV)</th>
<th>$V_{1/2}$ of inactivation (mV)</th>
<th>k of inactivation (pA/mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
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<td>-13.01 ± 0.88 (9)</td>
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<td>4.91 ± 0.33 (9)</td>
<td>-32.41 ± 1.16 (10)</td>
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<tr>
<td></td>
<td>-8.09 ± 1.30 (13) *</td>
<td>-15.34 ± 0.3 (6)</td>
<td>6.17 ± 0.27 (6) *</td>
<td>-30.37 ± 0.82 (6)</td>
<td>3.90 ± 0.14 (6)</td>
</tr>
<tr>
<td><strong>FGF13 KD + FGF12-B WT</strong></td>
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<td></td>
<td>12.86 ± 1.50 (13)</td>
<td>-17.59 ± 0.84 (13)</td>
<td>5.90 ± 0.17 (13) *</td>
<td>-36.33 ± 2.19 (7) *</td>
<td>5.31 ± 0.29 (4)</td>
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<tr>
<td><strong>FGF13 KD + FGF12-B Q7R</strong></td>
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<tr>
<td></td>
<td>-17.05 ± 1.46 (13) *</td>
<td>-15.97 ± 0.60 (13)</td>
<td>6.04 ± 0.14 (13) *</td>
<td>-36.58 ± 1.50 (4) *</td>
<td>5.21 ± 0.31 (4)</td>
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<table>
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<th>$I_{Na}$</th>
<th>$I_{Na}$ peak at -55 mV (pA/pF)</th>
<th>$V_{1/2}$ of activation (mV)</th>
<th>k of activation (pA/mV)</th>
<th>$V_{1/2}$ of inactivation (mV)</th>
<th>k of inactivation (pA/mV)</th>
</tr>
</thead>
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<tr>
<td><strong>Control</strong></td>
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<td>-69.47 ± 9.03 (14)</td>
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<td>-99.71 ± 1.39 (15)</td>
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<td>-34.45 ± 5.06 (9) *</td>
<td>-57.07 ± 1.73 (9)</td>
<td>4.56 ± 0.53 (9)</td>
<td>-103.64 ± 1.27 (10) *</td>
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<td>-100.28 ± 18.69 (9) *</td>
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<td>-97.22 ± 1.93 (6)</td>
<td>5.82 ± 0.41 (6)</td>
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<td><strong>FGF13 KD + FGF12-B Q7R</strong></td>
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</tr>
<tr>
<td></td>
<td>-38.23 ± 4.28 (9) *</td>
<td>-61.17 ± 1.48 (8)</td>
<td>4.73 ± 0.27 (8)</td>
<td>-104.51 ± 1.52 (12) *</td>
<td>6.36 ± 0.26 (12)</td>
</tr>
</tbody>
</table>

* p < 0.05 by ANOVA and compared to Control

$I_{Ca}$ denotes Ca$^{2+}$ current

$I_{Na}$ denotes Na$^{+}$ current
4.4.6 Q7R-FGF12 leads to changes in action potential morphology consistent with BrS

Finally, we assessed the effects of WT-FGF12 and Q7R-FGF12 on the ventricular action potential. As we previously reported,165 FGF13 KD decreased the action potential peak amplitude and half-width, consistent with observed reductions in Na\(^+\) and Ca\(^{2+}\) channels. Rescue with WT-FGF12 restored both peak amplitude and half-width to control levels. In contrast, rescue with Q7R-FGF12 failed to restore the peak amplitude, consistent with its lack of efficacy in modulating Na\(_V\)1.5 currents, and increased the half-width (Figure 36A-C), which we hypothesize results from potentiating effect of Q7R-FGF12 on Ca\(_V\)1.2 Ca\(^{2+}\) current density (see Discussion).
Figure 36: Q7R-FGF12 recapitulates the BrS action potential. A, representative evoked action potentials from cardiomyocytes of the four groups. B-C, summarized data for action potential peak amplitude and half width, respectively. * p < 0.05 versus control, ** p < 0.01 versus control, † p < 0.01 versus FGF13 KD + WT-FGF12.
4.5 Discussion

Inherited cardiac arrhythmias such as BrS can result from mutations in ion channel pore-forming subunits or their modulator proteins, providing consequent functional changes in ionic currents. Although still only accounting for less than 25% of BrS, the most commonly affected current in BrS is the voltage-gated Na\(^+\) channel, and most identified mutations are loss-of-function in SCN5A or in genes that encode regulators of Na\(_v\)1.5 currents, leading to a reduction in Na\(^+\) current density.\(^{165}\) Less commonly, loss-of-function mutations in Ca\(_v\)1.2 and its beta subunit have been linked to BrS. In this context, we suspected that loss-of-function mutations in FHFs, which we showed can modulate the cardiac voltage-gated Na\(^+\) and Ca\(^{2+}\) channels,\(^{78,165}\) were likely BrS candidate loci. After identifying the FGF12-B splice variant as the most abundantly expressed FHF in human ventricle, we queried a BrS repository and discovered a Q7R mutation in FGF12-B that leads to a Na\(^+\) channel loss-of-function phenotype consistent with the BrS diagnosis, using a novel, adult ventricular cardiomyocyte system.

By providing extensive evidence that this rare FGF12 variant is the disease-causing mutation, we demonstrate that FGF12 is a new BrS locus. We showed that this mutation decreased the affinity of FGF12 for the Na\(_v\)1.5 CTD; that functional regulation of Na\(_v\)1.5 by FGF12 is adversely affected; and that ventricular action potentials are perturbed. We also demonstrated that Q7R-FGF12 is fully capable of modulating Ca\(_v\)1.2 Ca\(^{2+}\) currents. Thus, this mutant appears causal for BrS in this patient due to loss-of-function effects on Na\(^+\) channel currents. Further, these results allow for the first time a separation of FHF-dependent effects on Na\(^+\) channels from Ca\(^{2+}\) channels. Because the Q7R mutation is on the Na\(_v\)1.5 CTD interface and affects the affinity of FGF12 for the CTD, these data suggest that the modulatory actions of FHFs on Ca\(_v\)1.2 channels result
from a different domain of the FHF. Although the interaction site between FHFs and JPH2, which is associated with the FHF-dependent effects on Ca\(^{2+}\) channels, has not yet been mapped, we hypothesize that the JPH2 interaction site is distinct from the Na\(_v\)1.5 CTD interaction site. As such, this BrS mutation will be a useful tool for further differentiating among distinct FHF pools within a cardiomyocyte.

The increase in action potential half width in the context of the Q7R-FGF12 was unexpected but possibly reflects the potentiating effect of the mutant FGF12 on the cardiac Ca\(^{2+}\) current density (Figure 35). Use of exome sequencing combined with systems biology recently identified a gain-of-function mutant in CACNA1C (increase in Ca\(^{2+}\) channel current) associated with Long QT Syndrome,\(^{173}\) demonstrating that increased Ca\(^{2+}\) current density can produce a prolonged QT interval that would manifest as a longer ventricular action potential duration. Alternatively, the Q7R-FGF12 might have multiple effects upon Na\(_v\)1.5, leading to a prolonged action potential or a decrease in peak amplitude as has been observed in specific individuals with “overlap syndromes” that result from mutations in SCN5A.\(^{174}\) Within these families, the same mutation (e.g., 1795insD) can have disparate clinical and electrophysiological phenotypes.\(^{175}\) Similar to results obtained with 1795insD, we observed that overexpression of WT-FGF12 in HEK293T cells (Fig. 30) decreases Na\(_v\)1.5 current density (loss-of-function), but depolarizes the V\(_{1/2}\) of steady-state inactivation (gain-of-function). Additionally, although not significant (p = 0.09), there is a slight increase in Ca\(_v\)1.2 current density in cardiomyocytes expressing Q7R-FGF12 in the context of FGF13 KD from control or those overexpressing WT-FGF12 (Figure 35). Together, these data provide evidence that FHFs may be involved in “overlap syndromes,” resulting in opposing effects on multiple ion channels depending on environmental and other genetic factors. Finally, as
our recent data demonstrates that FHFs are modulators of multiple ion channels,\textsuperscript{6,10} it is possible that FGF12 could affect additional ion channels or two properties of one ion channel, not necessarily in the same direction.

Critical to our ability to define the pathogenesis of this FGF12 mutant was the development of an adult cardiomyocyte-based system for investigation. Initial studies in a heterologous expression system, although demonstrating differences between the WT- and Q7R-FGF12, did not yield mechanistic insight consistent with the disease phenotype. While heterologous expression systems can show changes in intrinsic channel properties and its regulators isolated from the complex environment of a cardiomyocyte, these systems cannot accurately recapitulate the ion “channelsomes” and are not suited to query combinations of unanticipated cardiomyocyte-specific factors or the particular anatomy of a cardiomyocyte (e.g., T-tubules). Indeed, differences in results from a heterologous expression system and from native cell types have been previously reported for FHF regulation of Na\textsuperscript{+} channel currents\textsuperscript{82,84} and likely derive from a requirement for aspects of the native channel environment for complete expression of the FHF-regulated phenotype. While induced pluripotent stem cells (iPSCs) offer a similar strategy for testing endogenous effects of arrhythmogenic mutations,\textsuperscript{176} the resulting cardiomyocytes are often immature, precluding definitive assessment of the adult phenotype, and iPSC technology does not currently offer a high throughput method for analysis of multiple mutants.

In summary, using a native cardiomyocyte system, we demonstrated that Q7R-FGF12, is a new BrS locus and that the mutant affects Na\textsuperscript{+} channel trafficking and kinetics with minimal effects on Ca\textsuperscript{2+} channel function. These results also demonstrate a clear separation of the effects of FHFs on Na\textsuperscript{+} channels from Ca\textsuperscript{2+} channels. Since BrS
and other inherited arrhythmias can result from changes in Na⁺ channel or Ca²⁺ channel function, we hypothesize that other mutations in FGF12 may underlie genotype-negative cases of inherited arrhythmias.
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Biography

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