EVALUATING NON-CANONICAL ROLES OF KCHIP2 IN THE HEART

by

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DEDICATION

Without a doubt, this body of work is dedicated to my family. Their constant consideration and help in accommodating the inconsistent working hours of a Ph.D. has meant so much. My wife, Michelle, whose understanding of the work and particularly of me, has provided a limitless amount of support and been a source of constant endurance, and my son Liam, who has already been forced to get his feet wet in the lab.

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LIST OF ABBREVIATIONS

HF Heart Failure
KChIP Potassium Channel Interacting Protein
SCD Sudden Cardiac Death
MI Myocardial Infarction
AP Action potential
SR Sarcoplasmic Reticulum
RyR Ryanodine Receptor
NCX Sodium-Calcium Exchanger
CICR Calcium-Induced Calcium Release
APD Action Potential Duration
VT Ventricular Tachycardia
EAD Early Afterdepolarization
DAD Delayed Afterdepolarization
SERCA2a Sarcoplasmic Reticulum Ca\(^{2+}\) ATPase
IP3R 1,4,5-Trisphosphate Receptor
NMDA N-methyl-D-aspartate
DRE Downstream Regulatory Element
Evaluating Non-Canonical Roles Of Kchip2 In The Heart

Abstract

By

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Cardiac arrhythmias are a leading cause of morbidity and mortality within developed nations, resulting in more than 300,000 deaths per year in the U.S. alone. These sudden arrhythmias are frequently associated with acquired heart diseases, notably cardiac hypertrophy and heart failure (HF), where the dysregulation of numerous ion channels and transporters are observed. This provides a challenge in identifying which alterations are essential in driving disease pathogenesis and conferring susceptibility to lethal cardiac arrhythmias. Notably, one of the most consistent changes and most frequently associated with compromised repolarization, is selective reduction in the transient outward potassium current, $I_{to}$. $I_{to}$ is generated primarily by the voltage-gated potassium (Kv) channel, Kv4 and its interacting auxiliary subunit known as the Potassium Channel Interacting Protein 2 (KChIP2). Under hypertrophy and HF there is rapid and consistent loss of KChIP2, thought to cause the destabilization of Kv4 channels and subsequent $I_{to}$ depletion. While it is well understood that KChIP2 allows for the enhanced expression, trafficking, and modulation of Kv4 channels, emerging evidence suggests KChIP2 may not be limited to cell surface channel regulation of Kv4.

Supporting this notion is the conserved expression of KChIP2 in the myocardium of the guinea pig, where $I_{to}$ and the underlying subunit Kv4 are absent, reinforcing the concept of additional capacities for KChIP2. Notably, other members of the KChIP family not expressed in the myocardium have been shown to express multimodal functions outside
of Kv4 modulation. Therefore, the focus of this dissertation sought to identify what expanded functions KChIP2 might perform and whether those functions are relevant in myocardial reprogramming in response to disease signaling.

In my first project, we used the guinea pig as a platform for identifying novel KChIP2 functions pertaining to electrical reprogramming, motivated by the absence of endogenous Kv4 and therefore $I_{to}$. We isolated primary guinea pig ventricular myocytes and treated with an adenovirus encoding a KChIP2 antisense sequence to silence KChIP2 expression, which led to a significant prolongation of the cardiac action potential. This was attributed to increases in the depolarizing current $I_{Ca,L}$ in response to increased Cav1.2 expression, the primary alpha subunit encoding $I_{Ca,L}$ expression. We also observed significantly decreased $I_{Na}$ density coinciding with reductions in Nav1.5, the subunit encoding $I_{Na}$. The second project sought to observe the functional performance changes in these guinea pig myocytes, given that Ca$^{2+}$ alterations can have a significant impact on myocyte contractility. Unexpectedly, despite the enhanced delivery of Ca$^{2+}$, Ca$^{2+}$ transient amplitudes and correspondingly sarcomeric shortening were significantly attenuated following KChIP2 loss. While expression of the most significant Ca$^{2+}$ handling proteins was preserved, we instead found relocalization of a recently implicated ryanodine receptor modifier, presenilin 1. This corresponded to decreases in ryanodine receptor open probability and translated to attenuated Ca$^{2+}$ release.

The third project sought more specifically to identify a potential transcriptionally capacity for KChIP2, driven by the observation that in neonatal rat ventricular myocytes $SCN5A$/Nav1.5, $SCN1B$/Navβ1, and $KCND3$/Kv4.3 were found to experience transcriptional changes following KChIP2 silencing. Indeed, we observed the potential
for KChIP2 to bind DNA and repress promoter activity for two miRNAs, miR-34b and miR-34c, which subsequently targeted these three ion channel genes and suppressed corresponding current densities. Notably, the therapeutic manipulation of these pathways following cardiac stress successfully preserved current densities, leading to the complete attenuation of arrhythmia susceptibility. Collectively, the outcome of these investigations clearly identify that KChIP2 actions are dramatically more expansive than modulation of Kv4 channels alone, and that these mechanisms are potent contributors to adverse remodeling events characterized in the diseased heart.
Chapter 1

Introduction and Background
1.1 Motivation

Over the last several decades, the incidence of death from cardiac hypertrophy and heart failure (HF) has been steadily rising with the aging population. Currently, the annual death toll exceeds 250,000 in the US alone\(^1\). Even in spite of significant improvements in medical therapy, the outlook of heart failure remains poor, with nearly 20% of patients dying within the first year of HF prognosis. As a reflection of these discouraging numbers, up to half of those deaths are the consequence of sudden cardiac death (SCD). SCD is the result of profound electrical deterioration on the heart, most commonly observed in the form of pathologic ventricular arrhythmias, which dramatically compromises the heart's normal pumping action. This condition arises from complex structural and electrical remodeling events following prolonged stress on the heart and is driven by virtually all forms of structural heart disease, including myocardial infarction (MI), pressure overload (aortic stenosis and hypertension), myocarditis, idiopathic dilated cardiomyopathy, and volume overload. Paradoxically, while early cardiac remodeling is initially an adaptive response in an attempt to sustain cardiac performance, over time those changes eventually become maladaptive, driving the formation of the pathologic state. Despite this predictable prognosis, there exists a fundamental lack in understanding of the core mechanisms responsible for establishing the remodeled state. Therefore, while current therapies are able to slow disease progression, they ultimately prove insufficient in treating and preventing the adverse developments that lead to SCD. This is the fundamental purpose driving investigations aimed at the identification and intervention of novel pathways in HF remodeling. As a result of these efforts, a cascade of physiologic, neurohumoral, and biochemical
alternations have been linked to cardiac remodeling, however, the lack of effective therapeutics suggests that challenges remain in identifying the distinctions between simple markers of disease progression and true mechanisms that sustain decompensation. Therefore, the goal of revealing the underlying source(s) of cardiac remodeling continue to be explored. The focus of this dissertation investigates one such novel signaling cascade dependent on previously uncharacterized activity of the potassium channel interacting protein 2 (KChIP2) and its contribution to ion channel remodeling and cardiac disease.

1.2 Normal Cardiac Electrophysiology

One of the most pronounced and early readouts used to evaluate cardiac disease progression are the effects of electrical remodeling on the cardiac action potential (AP). The AP represents the cardiac transmembrane potential as a function of time. It is an intrinsic property of cardiac myocytes and is essential for the normal pumping action of the heart. A panel of various depolarizing (inward) and repolarizing (outward) currents establish the coordinated passage of sodium, potassium, and calcium ions across cellular membranes, generating an action potential waveform (Figure 1.1). This allows for the highly consistent and coordinated propagation of electrical signal throughout the heart, and the translation of electrical energy into mechanical to achieve pumping of blood throughout the body\(^2\). Notably, in acquired cardiac disease the proper balance of these underlying currents becomes disrupted and affects the mechanical forces generated. Importantly, this disruption begins at the cellular level. Through understanding of normal
presentation of these ionic currents, we can begin to understand how their alteration during disease contributes to pathogenesis and arrhythmia susceptibility.

The cellular resting potential of a cardiac ventricular cell is set by a large resting K⁺ conductance established by the inward rectifying current, $I_{K1}$, encoded by the Kir2.x channels. The high conductance of this channel at rest puts the resting membrane potential near the K⁺ equilibrium potential of -80 to -90 mV. Upon activation, cells are rapidly depolarized through quick entry of Na⁺ ions through voltage-gated Na⁺ channels, generating the very large inward current, $I_{Na}$, encoded by Nav1.5 ($SCN5A$), defining phase 0 of the action potential. The maximum rate of depolarization, known as the upstroke velocity, is heavily determined by the size of this depolarizing current, and plays a critical role in determining impulse propagation and conduction velocity across the

Figure 1.1: Panel of various depolarizing and repolarizing currents in the human ventricular action potential. Major sarcolemmal ionic currents; inward currents elicited by sodium current ($I_{Na}$) and L-type calcium current ($I_{Ca,L}$) and the outward currents which consist of multiple distinct K⁺ currents such as the inward rectifying current ($I_{K1}$), the transient outward current ($I_{to}$), the rapid and slow activating delayed rectifier current ($I_{Kr}$ and $I_{Ks}$, respectively). Phase 0, rapid depolarization; phase 1, rapid early repolarization phase; phase 2, slow repolarization phase (or plateau); phase 3, rapid late repolarization phase; phase 4, resting membrane potential.
myocardium. Within 10 ms this current inactivates and begins to give way to the rapidly activating and inactivating transient outward K\(^+\) current, \(I_{to}\), defined by the channels Kv1.4/Kv4.x. This current is largely responsible for establishing the traditional “spike and dome” morphology, or phase 1, of the action potential. In smaller mammals this current is very pronounced, yielding action potentials with a triangular morphology and therefore shorter APD. In humans, however, a smaller current density results in incomplete repolarization and entry into the plateau phase (phase 2) of the AP, marked by a balance of depolarizing (Ca\(^2\)+ entry through the L-type Ca\(^2\)+ current, \(I_{Ca,L}\)) and repolarizing (additional outward K\(^+\)) currents. \(I_{Ca,L}\) is encoded by the channel Cav1.2. The inflow of Ca\(^2\)+ through these channels triggers a much more substantial release of Ca\(^2\)+ from the intracellular storehouse known as the sarcoplasmic reticulum (SR) through activation of the ryanodine receptor (RyR) in a process referred to as Calcium-Induced Calcium Release (CICR). The activity of L-type Ca\(^2\)+ channel is critical in determining the amount of Ca\(^2\)+ released, directly affecting the degree of contractility of the myocyte, as well as maintaining the amount of Ca\(^2\)+ available to the SR. Eventually, this channel inactivates, giving way to the still active repolarizing K\(^+\) currents (phase 3 of the AP). These outward K\(^+\) currents are labeled delayed-rectifiers for their delayed activation kinetics. There is a rapid (\(I_{Kr}\)) and a slower (\(I_{Ks}\)) component that together lead to complete repolarization of the action potential. Overall, \(I_{Kr}\) is more active in determining terminal repolarization, while \(I_{Ks}\) becomes more pronounced during more rapid heart rates and adrenergic tone, providing a repolarization reserve to sustain faster action potential firing. Additional reactivation of \(I_{K1}\) during this phase assists in repolarization and stabilizes the potential back to the resting membrane potential (phase 4).
The description of these currents and the AP waveform they represent characterize the ventricular action potential. In addition to these currents, the atrial myocardium expresses several additional underlying $K^+$ currents including the ultra-rapid delayed rectifier current, $I_{Kur}$ encoded by Kv1.5, the acetylcholine-dependent inward-rectifier $K^+$ current, $I_{K,Acch}$ encoded by Kir3.1/3.4, and the small-conductance Ca$^{2+}$-activated K$^+$ (SK) current, $I_{K,Ca}$ encoded by the channels SK1/2/3. The contribution of these additional repolarizing $K^+$ currents leads to an overall shorter AP through an attenuated plateau phase. While the derangement of ionic currents are prolific in pathologic remodeling of the atria, this review and the investigations that follow focus on remodeling events and the underlying currents of the ventricle which contribute to the prominence of ventricular tachycardia and fibrillation that underlie the morbidity and eventual SCD observed in HF.

1.3 Remodeling of the AP in the stressed heart

As one might expect given the multitude of channels that comprise the cardiac AP, the waveform can be highly regulated to satisfy the acute demands of the body. Adjustments to the rate of repolarization or the amount of Ca$^{2+}$ entry and release are necessary for the fine turning and continued orchestration of currents in altering parameters such as heart rate or enhanced contractility without disrupting proper cardiac performance beat-to-beat. However, the electrical reprogramming that leads to early performance compensation can also be what drives disease remodeling. Invariably the cells from a failing heart, independent of the disease etiology, will display a prolonged action potential duration (APD). Increases in inward (depolarizing) or decreases in
outward (repolarizing)\textsuperscript{7,8} current can achieve a prolonged AP, both of which are well characterized in the failing heart (Figure 1.2). Ultimately, the prolonged AP is used to establish elevated levels of $[\text{Ca}^{2+}]_i$ to enhance cardiac contractility\textsuperscript{9}. However, alteration of numerous Ca\textsuperscript{2+} sensitive signaling pathways in the presence of sustained, heightened levels of Ca\textsuperscript{2+} is a significant factor in what drives maladaptive processes, leading to declining function, cardiac decompensation, and arrhythmia formation. A variety of mechanisms, including modulation of gene transcription, mRNA translation, protein processing, subunit assembly, membrane transport, protein complex assembly, and post-translational regulation have all been implicated in the pathologic modification of the cardiac AP. The details of these regulatory changes, and how they impact the electrophysiology of the heart will be discussed next.

\textbf{Figure 1.2: Ventricular remodeling of cardiac ion channels in heart failure.} Shown are the representative changes to the underlying currents comprising the cardiac action potential. Changes in the expression of the primary alpha subunit and auxiliary subunits primarily contribute to reductions in repolarizing potassium currents, leading to a loss in the phase 1 notch and APD.
1.4 Remodeling of K+ Channels and their influence in cardiac arrhythmias

As mentioned, one of the most consistent features of action potential modification in ventricles from patients with HF and in animal models investigating HF is APD prolongation\textsuperscript{10-13}. Reduced inward $I_{K_1}$ density in heart failure is believed to contribute in part to this, while also providing a mechanism for enhanced susceptibility to spontaneous membrane depolarizations, critical in triggered arrhythmias\textsuperscript{12-15}. However, the observations of reduced $I_{K_1}$ remain controversial, owing in part to inconsistent observations within different regions of the heart, differences between species, and even differences in disease models and formation, therefore resulting in settings where no reduction in $I_{K_1}$ is also observed\textsuperscript{16}. When reductions in $I_{K_1}$ are observed, it has been seen to be the result of reduced protein expression of the underlying channel Kir2.1\textsuperscript{3}. Notably this reduction occurs in the absence of reduced mRNA, suggesting a post-transcriptional mode of regulation. The consequence of this loss leads to membrane depolarization during the diastolic interval which contributes to the potential arrhythmic mechanism known as a delayed after-depolarization (DAD) resulting in enhanced triggered activity\textsuperscript{17}. Specifically, elevated cytosolic Ca\textsuperscript{2+} levels can lead to depolarizing currents translated from forward mode sodium-calcium exchanger (NCX). Normally this depolarizing current is buffered, however, with attenuated $I_{K_1}$ this can lead to NCX triggering an action potential, contributing to ectopic activity and arrhythmogenic potential\textsuperscript{15}.

At the same time, loss of $I_{K_1}$ can directly slow the rate of phase 2 and 3 repolarization. Additional contributors at this stage of the AP are $I_{Kr}$ and $I_{Ks}$, which have both also been shown to experience loss in HF remodeling\textsuperscript{18}. Importantly, small reductions in either of these currents lead to significant AP prolongation\textsuperscript{19}. Much like
$I_{K1}$, however, these reductions are not always consistent. Numerous studies have revealed that there is also no change in $I_{Kr}$\textsuperscript{19-21} as well as $I_{Ks}$\textsuperscript{12}. At the same time, where loss in current can be observed, there is also not a clear decrease of the underlying subunits encoding the channel, suggesting complex regulation of the channel and its subunits yet to be elucidated.

Notably, however, a reduction in the outward $K^+$ current over phase 2 of the AP predisposes the heart to the development of another condition of arrhythmia known as early after-depolarizations (EADs) (Figure 1.3) as well as a loss in repolarization reserve leading to increased transmural dispersion of repolarization promoting transmural conduction block\textsuperscript{20, 22, 23}. Together, these conditions act as substrates that promote the initiating events in arrhythmogenesis that lead to sustained electrical re-entry.

1.5 Remodeling of $I_{Na}$ and its influence in cardiac arrhythmias

While it appears that repolarizing currents are the primary target of reprogramming, depolarizing currents are equally capable of being targeted. $I_{Na}$ has been shown to be the target of significant downregulation of current density, acceleration of its inactivation, and slowed recovery from inactivation\textsuperscript{12}. Numerous signaling mechanisms collectively contribute to sodium channel dysfunction. Isoform switching\textsuperscript{24} has been shown to lead to formation of a truncated Nav1.5, leading to nonfunctional channels, in addition to overall decline in protein expression. Post-translational modifications are also relevant in altering channel activity\textsuperscript{25}, leading to alterations in the kinetics of $I_{Na}$ which restrict the magnitude and open probability of the channel. Given the importance of this current in sustaining normal wavefront propagation, this can have profound implications
on the electrical dysfunction of the heart. In addition to these loss of function
characteristics, however, disease signaling has also revealed gain of function alterations
to $I_{\text{Na}}$. It is frequently observed in the tissue of failing hearts that there is incomplete
inactivation of $I_{\text{Na}}$, leading to increases in “late” current, markedly prolonging APD and
disrupting intracellular sodium and calcium homoeostasis. Therefore, we see that
changes to $I_{\text{Na}}$ density and kinetics can predispose the heart to arrhythmias by disrupting
conduction and/or prolonging repolarization.

1.6 Calcium remodeling and its influence in cardiac arrhythmias

Excitation-contraction coupling is the fundamental principle by which a
myocyte’s ionic properties are coordinated to its mechanical function. Defective Ca$^{2+}$
handling has profound effect on electrophysiology because it controls not just the state of
the intracellular Ca$^{2+}$ transient mediating contractile strength and duration but also the AP
duration, contributing as well to arrhythmogenic substrates.

As mentioned previously, Ca$^{2+}$ entry as a result of $I_{\text{Ca,L}}$ triggers the release of Ca$^{2+}$
from the SR. This amount of Ca$^{2+}$ released is directly correlated to the amount of
“trigger” Ca$^{2+}$ provided. In the early stages of cardiac remodeling when cardiac
compensation is preserved, $I_{\text{Ca,L}}$ is enhanced, providing enhanced contractility in response
to cardiac stressors. However, in the later stages of HF, when decompensation occurs
and ejection fractions are lost, $I_{\text{Ca,L}}$ is decreased or in some cases unchanged. Overall, it appears that the expression of Cav1.2, the primary alpha subunit encoding $I_{\text{Ca,L}}$
is reduced in HF. However, this is compensated by an enhancement of channel
open probability as a consequence of saturating levels of channel phosphorylation,
leading to the passage of more current for each channel\textsuperscript{38}. Additionally, there is a desensitization in the normal increase in $I_{Ca,L}$ normally observed in response to $\beta$-adrenergic stimulation\textsuperscript{30}. Lastly, there is significant slowing of $I_{Ca,L}$ inactivation in HF that alters Ca\textsuperscript{2+} handling and will dramatically prolong the AP by sustaining the duration of the plateau phase\textsuperscript{39}.

While the contributions of $I_{Ca,L}$ can indirectly influence the Ca\textsuperscript{2+} transient of a given AP, the overall amplitude and duration of the transient are associated with alterations to the SR associated Ca\textsuperscript{2+} handling proteins. Importantly, the amplitude and rate of decay of the Ca\textsuperscript{2+} transient are consistently attenuated in the myocardium from failing hearts, which has been observed to be the aggregate outcome of multiple facets of remodeling. The loss of transient amplitude is in large part associated with defective sequestration of Ca\textsuperscript{2+} within the SR, largely caused by a decrease in expression and function of the sarcoplasmic reticulum Ca\textsuperscript{2+} ATPase (SERCA2a). This SR membrane associated protein is responsible for the reuptake of Ca\textsuperscript{2+} from the cytosol on a beat-to-beat basis. With its compromised activity, this contributes not only to the loss of SR stores minimizing the driving force of Ca\textsuperscript{2+} release, but also leads to the elevation of cytosolic Ca\textsuperscript{2+} levels, driving alternative regulatory cell signaling cascades activated by increased Ca\textsuperscript{2+} levels which contribute to multiple transcriptional pathways which potentiate adverse remodeling. Moreover, decreased phosphorylation status of the SERCA2a inhibitor phospholamban have been observed in the failing heart. This protein in the non-phosphorylated state inhibits SERCA2a activity, therefore, potentiating the loss in SERCA2a function (Figure 1.3). The recognition of the highly consistent nature of this remodeling event has led to investigations therapeutically restoring ventricular
SERCA2a levels through gene therapy, leading to restoration of contractility in failing hearts.

While Ca\textsuperscript{2+} reuptake is clearly compromised in the failing heart, there is also a regulated state of Ca\textsuperscript{2+} release through the RyR described previously through the process of CICR. Similar to the loss of SERCA2a, reduced expression of RyR at both the mRNA and protein levels are seen, leading to further loss of the Ca\textsuperscript{2+} transient amplitude and contributing to the declining state of contractility. Moreover, elevated states of phosphorylation attributed with tonic adrenergic tone result in a hyperactive RyR, contributing to a state of diastolic Ca\textsuperscript{2+} leak. This, combined with the compromised SERCA2a function, have additive effects on the pathologic contributions of Ca\textsuperscript{2+} deregulation described above (Figure 1.3).

Lastly, another major contributor to Ca\textsuperscript{2+} handling in a given cardiac cycle is NCX. This protein is expressed in the sarcolemma space and is responsible for either Ca\textsuperscript{2+} extrusion fueled by an electrogenic exchange for extracellular Na\textsuperscript{+} to produce a depolarizing current, or alternatively Ca\textsuperscript{2+} delivery, becoming a source of ionotropic support. Notably in the failing ventricle, NCX expression is enhanced at both the mRNA and protein level. While this increase may be compensatory to the compromised SERCA2a function by assisting in the removal of elevated Ca\textsuperscript{2+} levels, this extrusion over time leads to a depletion of intracellular Ca\textsuperscript{2+} stores. At the same time, this activity can lead to significant depolarizing currents that may trigger abnormal AP firing, becoming a significant substrate for arrhythmia induction (Figure 1.3).
1.7 Remodeling of $I_{to}$ and its influence in cardiac arrhythmias

The alterations to electrophysiology discussed up to this point have shown variations in results between studies and between species. It is not clear what these inconsistencies are a reflection of: study design, species, unaccountable variances in data collection, or simply that these changes are not hardened outcomes to disease pathogenesis. Remarkably however, the most consistent and pronounced change to ionic currents across all these variables, is downregulation of the transient outward potassium current ($I_{to}$)\textsuperscript{40} (Table 1.1 and Table 1.2). The consequence of this loss is very clear when it comes to small mammals where $I_{to}$ is the primary repolarizing current, drastically
prolonging the AP and sustaining enhanced Ca$^{2+}$ entry during the plateau phase of the AP. In larger mammals, however, where $I_{to}$ density is less, its influence over APD remains much more controversial. In these settings, the smaller magnitude of $I_{to}$ is thought to establish the membrane potential leading into the phase 2 potential and $I_{Ca,L}$ activation, establishing the initial driving force for Ca$^{2+}$ entry. With the loss in $I_{to}$ in the stressed heart, the phase 2 potential becomes more depolarized, weakening the driving force for Ca$^{2+}$ entry. In theory, this would work to shorten the plateau phase and overall duration of the AP by limiting the degree of depolarizing Ca$^{2+}$ entry. In fact, computer simulations of the canine APD prove this phenomenon. Therefore, with the early, sustained, and consistent loss of $I_{to}$ throughout the progression of heart failure, there is considerable interest in elucidating its components and contribution in cardiac disease remodeling.

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<td>Mice overexpressing tumor necrosis factor-α</td>
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<td>Rose et al. (14)</td>
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<td>VTP-induced CHF, rabbit</td>
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<td>VTP-induced CHF, rabbit</td>
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Table 1.1. Changes in K$^+$ channel function in HF. Loss in $I_{to}$ is one of the most consistent remodeling events in cardiac disease, regardless of species or stress induction strategy. CHF, congestive HF; VTP, ventricular tachypaced
Components of Ito

Biophysical analysis reveals that $I_{\text{to}}$ can be divided into two separate components, a fast ($I_{\text{to},f}$) and a slow ($I_{\text{to},s}$), which are created by separate pore forming alpha subunits known as the Kv4 subfamily. Kv4.2 ($\text{KCND2}$) and Kv4.3 ($\text{KCND3}$) are responsible for $I_{\text{to},f}$ and Kv1.4 ($\text{KCNA4}$) constitutes $I_{\text{to},s}$. Electrophysiological and pharmacologic techniques can be used to reveal these separate current components. While $I_{\text{to},f}$ and $I_{\text{to},s}$ both have fast activation and inactivation, they are distinguished based on their rate of recovery from inactivation $^{51, 52}$. $I_{\text{to},f}$ is capable of recovering from inactivation within 60-100 ms, while $I_{\text{to},s}$ recovers significantly slower on the order of seconds. Alternatively, Heteropoda toxin derived from the crab spider, is selective against $I_{\text{to},f}$ in the nM range, but requires significantly higher dosage before it begins to affect $I_{\text{to},s}$ $^{53}$. The importance of these molecular distinctions are critical to identifying regional heterogeneities of $I_{\text{to}}$ distribution throughout the heart. Collectively, $I_{\text{to},f}$ densities are most prominent in the

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<th>Property</th>
<th>CHF</th>
<th>MI</th>
<th>AF</th>
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<tr>
<td>$I_{\text{to}}$</td>
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Table 1.2. A comparison of ion current and transporter remodeling in CHF, MI, and AF (atrial fibrillation). Notably, dramatic loss in $I_{\text{to}}$ underlies the remodeled state in three of the primary cardiac pathologies, suggesting its potential influence over driving disease progression.
epicardium and midmyocardium compared to the endocardium and septal regions\textsuperscript{54}. This effect is witnessed in the characteristic “spike and dome” morphology of epicardial action potentials, which is less pronounced in endocardial cells. Interestingly, this heterogeneity is believed to assist in myocardial electrical coupling by favoring the propagation of action potentials in the endocardial to epicardial orientation by providing more coupling current to depolarize adjacent cells\textsuperscript{55}. Indeed, this is the normal orientation for propagation of action potentials through the myocardium that allows for the most efficient pumping of blood. Interestingly, in the occurrence of heart failure this directional preference of conduction is blunted because of the associated loss of $I_{\text{to}}$ and therefore the transmural gradient, promoting abnormal impulse propagation. Indeed, epicardial-site pacing is associated with VT in patients with HF.

Functional Kv channels result from the assembly of four pore-forming Kv α-subunits\textsuperscript{56, 57}. Each subunit contains six transmembrane segments (S1 to S6), including a voltage sensor in the S4 domain, a single pore between the S5 and S6, and cytoplasmic N- and C-termini\textsuperscript{58}. In heterologous expression systems, Kv α-subunits can actually assemble as homo- or heterotetramers, containing different α subunits form the same subfamily. As mentioned previously, significant evidence suggests that both Kv4.2 and Kv4.3 underlie $I_{\text{to,f}}$. Multiple \textit{in vivo} studies which genetically deleted or expressed a dominant negative mutant of Kv4 channels in mice eliminated the detection of $I_{\text{to,f}}$, showing the critical role for Kv4 in the generation of $I_{\text{to}}$\textsuperscript{59}. \textit{In vitro} gene silencing has also been able to tell us that both Kv4.2 and Kv4.3 compose $I_{\text{to,f}}$ in rodent cells, while Kv4.3 is the dominant isoform in humans\textsuperscript{60}. Physiologically, gradients of Kv4 expression across
the myocardial layers correspond with higher current densities seen in the epicardium relative to the endocardium\textsuperscript{51, 61}

Consistent with the differences in their biophysical properties, investigations have shown that $I_{\text{to},s}$ is defined by a functionally distinct gene, Kv1.4\textsuperscript{51}. Heterologous expression of the channel reveals slowly recovering currents on the order of seconds, that is also 4-AP sensitive, resembling that of $I_{\text{to},s}$. Endogenously, higher expression of Kv1.4 is found in septal and endocardial cells, reflecting the gradient of $I_{\text{to},s}$ expression. Kv1.4 null animals were found to eliminate $I_{\text{to},s}$, reinforcing this relationship.

\section*{1.9 Role of accessory subunit, Potassium Channel Interacting Protein 2 (KChIP2)}

Interestingly, the heterologous studies that identified the relationship between $I_{\text{to}}$ and the underlying molecular correlate, were unable to completely reproduce the native currents observed in endogenous tissues. For example, expression of Kv4 channels produced currents that inactivated and recovered from inactivation much slower than what was observed for native $I_{\text{to},f}$, suggesting the involvement of additional subunits required for the construction of the mature current. Indeed, a number of molecularly diverse accessory subunits have been demonstrated to modulate both the biophysical properties of the cell surface expression of heterologously expressed Kv4 channels\textsuperscript{62, 63}. From these studies, the auxiliary subunit KChIP2 has been shown to be an essential component of myocardial $I_{\text{to},f}$\textsuperscript{64}.

KChIP2 belongs to a family of small neuronal calcium sensing proteins that consists of four different family members (KChIP1-4) ranging from 216-270 amino acids\textsuperscript{65}. They are very aptly named for their primary function, potassium channel
interacting proteins, which were first discovered in the brain using the Kv4 N-terminus as bait in a yeast-two-hybrid assay. The consequence of this interaction is three fold on the Kv4 channel. Co-expression leads to a dramatic increase in the cell surface expression of the Kv4 channel through occlusion of an endoplasmic reticulum retention motif, it slows the kinetics of current inactivation, and accelerates recovery from inactivation, together producing a more prominent $I_{to}$. These effects are lost with the deletion of the N-terminus of Kv4. Notably in the brain where KChIP and Kv4 expression persist, this current is referred to as $I_A$. The four KChIP proteins consist of a highly conserved C-terminus representing nearly 75% of the protein, and a highly variable N-terminus. Within the highly conserved region are four calcium binding EF-hand domains responsible for controlling its regulatory activity. The kinetic effects of KChIP2 on Kv4, for example, are dependent on the binding of calcium to the EF-hand domains, while the binding of KChIP2 to the N-terminus is independent of calcium. The remaining N-terminal variable region of the KChIP family contains motifs capable of post-translational modifications including myristoylation and palmitoylation which have been shown to enhance membrane localization through anchoring KChIP to the plasma membrane.

While all four KChIPs can be found in the brain, only KChIP2 is expressed in the heart. However, several different isoforms for KChIP2 have been identified in the mouse, and up to seven isoforms have been observed in human, producing variants within the N-terminal region, potentially altering motifs important in post-translational modifications and perhaps influencing KChIP2 localization. Reinforcing the critical role for KChIP2 in establishing $I_{to,t}$, the genetic deletion of KChIP2 in a mouse transgenic led
to the complete elimination of $I_{to,f}$ through a mechanism of Kv4 destabilization and enhanced degradation$^{70}$. Additionally, Kv4.2 and Kv4.3 mRNA levels were reduced, suggesting an influence of KChIP2 over gene level expression as well. Alternatively, Kv4.2$^{-/-}$ mice produced a loss in KChIP2 expression, showing an intimate reciprocated regulation of these proteins$^{71}$. Even more telling is that a gradient of KChIP2 expression across the ventricular wall parallels the gradient in $I_{to,f}$, reinforcing the functional effect of KChIP2 over $I_{to,f}$$^{72}$ (Figure 1.4).

![Figure 1.4. KChIP2 transmural gradient underlies heterogeneities in $I_{to}$ density.](image)

### 1.10 Expanded Role of KChIP2

Notably, in addition to the initial discovery of KChIP2 activity on Kv4, there have since been several other investigations revealing other roles for KChIP2 in the heart. KChIP2 was also found to be able to bind the N-terminus of Cav1.2, the $\alpha$-subunit for L-type Ca$^{2+}$ current$^{73}$. This interaction was initially prompted by the observation of reduced $I_{Ca,L}$ in myocytes from KChIP2$^{-/-}$ mice, while heterologous expression of KChIP2 with
Cav1.2 yielded increased current density. It was discovered that KChIP2 led to the masking of an autoinhibitory (NTI) domain on Cav1.2 responsible for channel inactivation. In the absence of KChIP2, this domain would occlude the channel pore, decreasing the open probability. This loss in current occurred despite an increase in Cav1.2 protein expression, showing the potency of this NTI domain. Interestingly, however, acute silencing of KChIP2 in neonatal rat cardiomyocytes did not affect Cav1.2 protein levels, but it was not determined if there was a change in $I_{\text{Ca,L}}$.

KChIP2 has also been shown to regulate potassium channels other than Kv4. Kv1.5 constitutes the current $I_{\text{K,slow}}$, also known as the atrial-specific $I_{\text{Kur}}$. This current, with the assistance of $I_{\text{to}}$, enhances the repolarization rate in atrial cells, leading to much abruptly ended APDs compared to the ventricle\textsuperscript{74}. Interestingly, however, KChIP2 has been reported to reduce Kv1.5 current by attenuating Kv1.5 trafficking, with no effect on channel kinetics. Supporting this inverse relationship, the KChIP2\textsuperscript{-/-} mouse shows elevated Kv1.5 mRNA.

KChIP2 has also been suggested to both functionally and structurally regulate $I_{\text{Na}}$. Following the acute silencing of KChIP2 in neonatal rat myocytes, there was a significant loss to Nav1.5 (SCN5A) and its accessory subunit (Navβ1) SCN1B, to where no $I_{\text{Na}}$ could be recorded\textsuperscript{75}. On the other hand, co-expression of KChIP2 with Nav1.5 was able to enhance $I_{\text{Na}}$ density. Intriguingly, co-immunoprecipitation revealed an association of Kv4 with Navβ1 as well, suggesting a macromolecular complex between the molecular components of $I_{\text{Na}}$ and $I_{\text{to}}$. The regulation on the transcriptional components of this pathway, however, begin to suggest a multi-faceted state of KChIP2 activity over cardiac ion channel expression. Yet, the KChIP2\textsuperscript{-/-} mouse was unable to reproduce these results\textsuperscript{76}.

20
While there was a mildly trended reduction in upstroke velocity, a surrogate for $I_{Na}$, mRNA and protein levels for Nav1.5 and Navβ1 were unchanged from control mice. It is unclear why these discrepancies exist, however, it is feasible to believe that the constitutive absence of KChIP2 in these mice can lead to compensatory regulation or mechanisms that adapt around the missing KChIP2, when the alternative is embryonic lethality.

Most interesting of all, however, are the discovered roles for the other KChIP family members that have been identified outside the established role of ion channel modulation. Specifically, KChIP3 was independently discovered on three occasions, resulting in the use of three separate names later identified to be the same protein. One of these discoveries made use of another yeast-two-hybrid screen using a peptide sequence from the protein presenilin, a protein involved in beta-amyloid formation and apoptosis relevant in molecular pathways of Alzheimer disease\textsuperscript{77}. They were able to pull down a neuronal protein which they named calsenilin for its association with presenilin and its discovery to bind calcium. Investigations into the sequence of this protein later revealed it to be the same as KChIP3. Since its discovery, experiments have identified this interaction to lead to the regulation of presenilin activity. Such activity includes generation of the neurotoxic amyloid-beta peptide\textsuperscript{78} as well as effects on cytosolic calcium concentrations related to the interaction presenilin maintains with the inositol 1,4,5-trisphosphate receptor (IP3R)\textsuperscript{79}, the N-methyl-D-aspartate (NMDA) receptor\textsuperscript{80}, and the ryanodine receptor (RyR)\textsuperscript{81-83}.

In addition to these identified roles, the promiscuity of KChIP3 function was expanded even further when it was discovered under the name of DREAM for
Downstream Regulatory Antagonistic Modulator. In this context it was discovered for its role as a transcriptional repressor, specifically binding to downstream regulatory elements (DRE) sequences in DNA made up of a core nucleotide binding sequence of ‘GTCA’ with an affinity modified by flanking residues\textsuperscript{84, 85}. Its capacity to bind DNA and hinder the transcription of downstream targets was found to be regulated by the level of nuclear Ca\textsuperscript{2+}, the interaction with other nuclear proteins such as cAMP response element binding protein CREB, and activation of the PI3K pathway. Notably, while binding of CREB to DREAM can prohibit its interaction with DNA, the effect can also be reciprocated. The obstruction of CREB binding, which performs as a transcriptional activator therefore offers an alternative mode by which DREAM can repress gene expression. Therefore, KChIP3 transcriptional activity is modulated by both mechanisms of Ca\textsuperscript{2+} signaling as well as cAMP, making it a highly adaptive and versatile signaling molecule.

Of considerable interest, however, is that these diverse functions for KChIP3 have not been investigated for KChIP2, let alone whether or not they are significant to molecular signaling and regulation in the heart. Considerably, all KChIP family members, including KChIP3/calsenilin/DREAM, are capable of comparable regulation on Kv4 trafficking and kinetic modulation. Moreover, other members of the KChIP family, most notably KChIP2, were previously shown in heterologous expression systems to also display the same ability to bind to the DRE motif recognized by KChIP3 and repress the expression of downstream targets.
Notably, these similarities are reflective of the high degree of protein sequence homology shared between these family members (Figure 1.5). More importantly, however, this begins to suggest that some of these diverse mechanisms may potentially be preserved for native activity of KChIP2 in the heart. Intriguingly, the loss in cardiac KChIP2 expression determined directly or indirectly through attenuated \( I_{\text{to}} \) density has been observed to be one of the earliest and most consistent remodeling events in the course of HF development. The commonality and early state of this remodeling begins to suggest KChIP2 loss might not just be one of the casualties in the course of disease progression, but may actually represent an initiating factor driving pathogenesis. Given the profound scope of pathologic remodeling in HF, a pathway that is implicated early and represents the potentially expansive and diverse mechanisms of the KChIP family reinforces the notion that KChIP2 may be a critical factor in the overall trajectory of HF. Moreover, very little is understood about the mechanisms that lead to compromised

![Figure 1.5. Splice variants for KChIP2 and KChIP3 show significant sequence homology. C-terminal residues marked by green (EF hands) and red regions are identical. However, the variable N-terminal regions are thought to provide localization dependent differences. Residues marked with (●) represent lipid post-translational modification sites, shown to confer greater membrane localization.](image)
electrical patterns in the heart during HF. While current therapeutic applications may stem or mask the underlying remodeling events, this limited understanding leaves little therapeutic opportunity to stall, let alone recover the heart from a worsening state. Importantly, in this dissertation we therefore look to not only reveal a much expanded significance of KChIP2 in the heart, but also to implicate these potential activities in mediating events that may drive the pathologic state.

1.11 Summary Statement of Thesis Project

To reiterate, the first part of this dissertation (Chapter 2) sought to identify novel contributions of KChIP2 in the guinea pig myocardium, given that Kv4 expression and a measureable $I_{to}$ are absent in this species. Acute silencing of KChIP2 in isolated ventricular cells resulted in a significant increase in Cav1.2 protein, providing augmented $I_{Ca,L}$ and APD prolongation. It also led to attenuated $I_{Na}$ through a loss in Nav1.5 protein. Chapter 3 sought to identify the functional changes in contractility in response to these ionic current changes. Against expectations, acute KChIP2 silencing led to significant reductions in Ca$^{2+}$ transient amplitudes and attenuated sarcomeric shortening, despite the enhanced delivery of Ca$^{2+}$ from $I_{Ca,L}$. These observations were reconciled by an observed loss in RyR open probability, which we attributed to the dispersion of presenilin, an established modulator of RyR, away from sacromeric regions. These observations were the first set to link non-canonical KChIP2 activity with actions previously established for KChIP3. Notably, Chapter 4 next sought to identify if another KChIP3 action was conserved for KChIP2 in the heart; its capacity for transcriptional repression. Indeed, we observed KChIP2 was capable of mediating a decline in $I_{to}$ and $I_{Na}$ following cardiac
stress through a transcriptionally repressive action on a miRNA which targeted the genes underlying these currents. Restoration of KChIP2 or repression of the miRNA target led to the normalizing of these currents and completely eliminated susceptibility to induced arrhythmias. Overall, these observations reveal a prolific multimodal capacity for KChIP2 but more importantly identify the significance of these actions in mediating established events in cardiac electrical remodeling relevant to HF morbidity and mortality.
Chapter 2

Myocardial KChIP2 Expression in Guinea Pig Resolves an Expanded Electrophysiologic Role


Conceived and designed the experiments: DMN XW ID. Performed the experiments: DMN XW HL. Analyzed the data: DMN XW. Contributed reagents/materials/analysis tools: XW HL ID. Wrote the paper: DMN XW ID.
Myocardial KChIP2 Expression in Guinea Pig Resolves an Expanded Electrophysiologic Role

Short title: KChIP2 regulation beyond $I_{to}$

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2.1 Abstract

Cardiac ion channels and their respective accessory subunits are critical in maintaining proper electrical activity of the heart. Studies have indicated that the K\(^+\) channel interacting protein 2 (KChIP2), originally identified as an auxiliary subunit for the channel Kv4, a component of the transient outward K\(^+\) channel (I\(_{to}\)), is a Ca\(^{2+}\) binding protein whose regulatory function does not appear restricted to Kv4 modulation. Indeed, the guinea pig myocardium does not express Kv4, yet we show that it still maintains expression of KChIP2, suggesting roles for KChIP2 beyond this canonical auxiliary interaction with Kv4 to modulate I\(_{to}\). In this study, we capitalize on the guinea pig as a system for investigating how KChIP2 influences the cardiac action potential, independent of effects otherwise attributed to I\(_{to}\), given the endogenous absence of the current in this species. By performing whole cell patch clamp recordings on isolated adult guinea pig myocytes, we observe that knock down of KChIP2 significantly prolongs the cardiac action potential. This prolongation was not attributed to compromised repolarizing currents, as I\(_{Kr}\) and I\(_{Ks}\) were unchanged, but was the result of enhanced L-type Ca\(^{2+}\) current due to an increase in Cav1.2 protein. In addition, cells with reduced KChIP2 also displayed lowered I\(_{Na}\) from reduced Nav1.5 protein. Historically, rodent models have been used to investigate the role of KChIP2, where dramatic changes to the primary repolarizing current I\(_{to}\) may mask more subtle effects of KChIP2. Evaluation in the guinea pig where I\(_{to}\) is absent, has unveiled additional functions for KChIP2 beyond its canonical regulation of I\(_{to}\), which defines KChIP2 as a master regulator of cardiac repolarization and depolarization.
**Key Words:** KChIP2, transient outward potassium current, sodium current, L-type Ca$^{2+}$ current

**List of Abbreviations**

K+ channel interacting protein (KChIP)

Action Potential Duration (APD)
2.2 Introduction

K+ channel interacting proteins (KChIPs) represent a class of highly diverse Ca\textsuperscript{2+}-
sensors originally discovered for their interaction with the cytoplasmic N-terminus of the
Kv4 family of potassium channels\textsuperscript{65}. The assembly of these two proteins creates the
native current known as A-type current (\textit{I}_A) in neuronal tissue and the fast-inactivating
transient-outward potassium current (\textit{I}_{to,f}) in the heart\textsuperscript{86-90}. While the expression of Kv4
alone is sufficient to observe this current, co-expression with KChIP results in currents
with slowed inactivation, faster recovery from inactivation, and increased current
densities, effectively creating native Kv4 current\textsuperscript{65,91}.

In total, there are four KChIP genes (KChIP1-4)\textsuperscript{65,92}. While all four KChIPs can
be observed in the brain, the heart is seemingly simplified by expressing only KChIP2.
Together, these proteins are characterized by a highly conserved C-terminal domain
containing 4 EF-hand motifs and a highly variable N-terminus which is thought to
provide both altered localization and activity\textsuperscript{67,93}. Indeed, the KChIP family has become
the most diverse of the Ca\textsuperscript{2+}-sensing proteins\textsuperscript{94,95} not just in numbers, but in their breadth
of function. In addition to serving as K\textsuperscript{+}-channel subunits, KChIP3 and 4 were discovered
in the brain to both regulate presenilin\textsuperscript{77,92}, affecting the processing of amyloid precursor
protein. Additionally, KChIP3 was discovered to act as a transcriptional repressor, an
activity later shown by all four KChIP isoforms\textsuperscript{84,96}. Even cardiac KChIP2 expression
shows behavior of interacting with more than Kv4, including Kv1.5, which has been
shown to display impaired trafficking by KChIP2\textsuperscript{74}. Additionally, there is evidence that
KChIP2 interacts with the N-terminus of Cav1.2, affecting channel open probability\textsuperscript{73,97},
while we have shown a regulation on $I_{Na}$ through a potential macromolecular interaction with $I_{to}$\textsuperscript{75}.

This concept of multimodal function is reinforced in the setting of the guinea pig where we show expression of myocardial KChIP2 is maintained despite having a complete absence of Kv4 expression\textsuperscript{98}, making it unclear what function KChIP2 satisfies. Frequently, mice have been used to investigate the impact of KChIP2 expression, where KChIP2 loss invariably diminishes $I_{to}$, the main repolarizing current in rodents, and therefore prolongs the cardiac action potential. However, the guinea pig offers a unique perspective to investigate what other influences emerge as a consequence of KChIP2 without the dominant influence of $I_{to}$ present. Here, we demonstrate using the guinea pig that KChIP2 possesses additional functions beyond its canonical regulation of $I_{to}$ that define KChIP2 as a master regulator of cardiac repolarization and depolarization.
2.3 Materials and Methods

Detection of relative kcnip2 (KChIP2) expression across species

Left ventricular tissue was excised from the hearts of rat, guinea pig, dog, and human samples. Both ventricles were taken from neonatal rat hearts. Tissue was immediately frozen in liquid nitrogen and pulverized on dry ice for better homogenization. Total RNA was isolated from the pulverized heart tissue using Trizol Reagent (Invitrogen) according to the manufacturer’s instructions. 20 ng/μl RNA per sample was used as a template for cDNA synthesis in reverse transcriptase reactions using the Multiscribe Reverse Transcriptase kit (Invitrogen). Real-time quantitative PCR reactions were performed with the ABI 7500 Real-Time PCR system using Power SYBR green PCR Master Mix (Invitrogen) technology. Relative quantification of kcnip2 across samples was normalized using gapdh and the 2^{ΔΔCt} method was implemented to determine relative quantification. A melting curve was performed to verify amplification of discrete products. All primer pairs used spanned at least a single intron to select against any contaminating genomic amplification. The following primers were used for detection: rat kcnip2.F: 5’-ACTTTGTGGCTGGTTTGTCG-3’, rat kcnip2.R: 5’-TGATACAGCCGTCCTTTGAG-3’; rat gapdh.F: 5’-AGTTCAACGCGCAGTGCAAG-3’, rat gapdh.R: 5’-ACTCCACGACATCTACGAC-3’; guinea pig kcnip2.F: 5’-AGAAACAAGGATGCGTGGT-3’, guinea pig kcnip2.R: 5’-CAAAGAGCTGCATGGATCGC-3’; guinea pig gapdh.F: 5’-GCGCCGAGTATGCTAGTGGAA-3’, guinea pig gapdh.R: 5’-
TGATTCACGCCCATCAGAA-3', canine knip2.F: 5'-
ACGTATCCTGCACCTCCAGA-3', canine knip2.R: 5'-
GCCATCCTTGTCTCTGTCCA-3', canine gapdh.F: 5'-
GGGCGTGAAACCAGAGAGAAG-3', canine gapdh.R: 5'-
CAGTGATGCCAATGGACGGT-3', human knip2.F: 5'-
TGTACCGGGGCTCAAGAAAC-3', human knip2.R: 5'-
GGCATGAAAGAGAAAGTGCA-3', human gapdh.F: 5'-
TCCTCTGACTACAGCAAGAAG-3', human gapdh.R: 5'-
GGGTCTTACTTGTGGAGGC-3'

Viral Constructs

cDNA of the identified guinea pig KChIP2 antisense sequence was created and cloned into an adenoviral vector under the regulation of a CMV promoter (KChIP2-KD). The vector coexpressed GFP through an internal ribosomal entry site. A control adenoviral vector was used that omitted the antisense sequence and expressed GFP alone.

Immunoblotting

Freshly isolated guinea pig ventricular myocytes were cultured for 24 h at 37 °C in M199 medium under control conditions or with KChIP2 antisense virus. Upon collection, cardiomyocytes were washed in ice-cold PBS and resuspended in a RIPA lysis buffer (150 mM sodium chloride, 1.0% NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulphate), 50 mM Tris, pH 8.0, plus Roche Inhibitor tablet) and then sonicated on ice to disrupt cell membranes. 30-40 μg of whole cell extract was
loaded into SDS-PAGE gels, transferred to nitrocellulose membrane, and western blotting performed using KChIP2 mouse monoclonal antibody (UC Davis NeuroMab 75-017) at a 1:500 dilution, beta-actin mouse monoclonal antibody (Sigma-Aldrich, A1978) at a 1:1000 dilution, Cav1.2 mouse monoclonal antibody (UC Davis NeuroMab 75-257) at a 1:500 dilution, Nav1.5 mouse monoclonal antibody (Sigma S8809) at a 1:800 dilution, and pan-cadherin rabbit monoclonal antibody (Cell Signaling 4068S) at a 1:1000 dilution. Protein concentrations were determined by the BCA method (Pierce).

Guinea Pig Ventricular Myocytes: Isolation and Short-term Culture

Single ventricular myocytes were isolated from adult guinea pigs as described previously. Briefly, guinea pigs were anesthetized by injection of fatal plus. Hearts were quickly removed and perfused via the aorta with a physiological salt solution (PSS) containing (in mmol/L) NaCl 140, KCl 5.4, MgCl₂ 2.5, CaCl₂ 1.5, glucose 11, and HEPES 5.5 (pH 7.4). After 5 minutes, perfusate was switched to a nominally calcium-free PSS with collagenase (Roche, 0.5 mg/mL) being added after an additional 5 minutes. After 20 to 35 minutes of digestion, hearts were perfused with a high K⁺ solution containing (in mmol/L) potassium glutamate 110, KH₂PO₄ 10, KCl 25, MgSO₄ 2, taurine 20, creatine 5, EGTA 0.5, glucose 20, and HEPES 5 (pH 7.4). Ventricles were minced in high K⁺ solution, and single myocytes were obtained by filtering through a 115-μm nylon mesh. Myocytes were left to settle for 2 hours at room temperature before being collected in a low-speed spin. Cell pellets were resuspended in M199 medium supplemented with antibiotic and plated on uncoated 10 cm dishes. Cultures were left untreated or they were treated with GFP adenovirus or adenovirus with a kcnip2 (KChIP2) mRNA antisense
coding sequence in IRES with GFP. Cultures were then incubated in 5%CO₂ at 37°C for 24 hrs. Untreated and GFP treated cells were found to have no significant differences across the studies conducted, and therefore the datasets between these two groups were combined to comprise our control group.

Cellular Electrophysiology

$I_{K1}, I_{Ks}, I_{Kr}$, and action potentials were recorded in isolated ventricular guinea pig myocytes cultured overnight in M199 medium using the following intracellular solution: 119 mM potassium gluconate, 15 mM KCl, 3.75 mM MgCl₂, 5 mM EGTA, 5 mM HEPES, 4 mM K-ATP, 14 mM phosphocreatine, 0.3 mM Tris-GTP, and 50 U/ml creatine phosphokinase, pH 7.2. The extracellular solution was 132 mM NaCl, 4.5 mM KCl, 1.2 mM MgCl₂, 1.8 mM CaCl₂, 10 mM Glucose, and 5 mM HEPES, pH 7.4. $I_{K1}$ currents were elicited from a holding potential of -40 mV with depolarizing voltage pulses from -100 mV to 40 mV. $I_{Ks}$ currents were elicited from a holding potential of -40 mV with depolarizing voltage pulses from -30 mV to 60 mV for 2.5 s and then return to -40 mV to generate outward tail currents in the presence of 5 uM E4031 to block $I_{Kr}$. $I_{Kr}$ currents were isolated as E4031 sensitive current. $I_{Ca,L}$ were recorded with an intracellular solution of 130 mM CsMES, 20 mM TEA Cl, 1 mM MgCl₂, 10 mM HEPES, 10 mM EGTA, 0.3 mM TRIS GTP, 14 mM Phosphocreatine, 4 mM Mg ATP, 2 mM Creatine phosphokinase and brought to a pH of 7.3. Myocytes were placed in the solution containing 137 mM NaCl, 5.4 mM CsCl, 1.8 mM MgCl₂, 2 mM CaCl₂, 10 mM glucose, 10 mM HEPES, pH 7.3. $I_{Ca,L}$ were elicited from a holding potential of -40 mV with depolarizing voltage pulses from -30 mV to 60 mV for 300 ms. $I_{Na}$ was recorded in the
solution containing 20 mM NaCl, 120 mM N-methyl D-glucamine, 5.4 mM CsCl, 1 mM MgCl₂, 10 mM glucose, 10 mM HEPES, pH 7.3. 1 uM of nisodipine was used to block L-type Ca currents. \( I_{Na} \) were elicited from a holding potential of -120 mV with depolarizing voltage pulses from -80 mV to 60 mV for 16 ms. Ionic current density (pA/pF) was calculated from the ratio of current amplitude to cell capacitance. pClamp software (Molecular Devices) was used for generation of voltage-clamp protocols and data acquisition. All experiments were performed at 35°C except \( I_{Na} \) (room temperature).

**Statistical Analysis:**

The experimental data were expressed as mean ± SEM. A Student’s \( t \)-test or a paired Student’s \( t \)-test (for western blot analysis) was performed. All tests were two-sided and a significance level of \( p < 0.05 \) was defined as statistically significant (SPSS 18.0 software, SPSS, Chicago, IL).

**Ethics Statement and Tissue Acquisition**

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol for tissue isolation from adult guinea pig (Protocol Number: 2012-0175), adult and neonatal rat (Protocol Number: 2013-0015), and canine (Protocol Number: 2014-0026) samples were approved by the Committee on the Ethics of Animal Experiments of Case Western Reserve University. 14 Hartley guinea pigs, 3 adult Sprague Dawley rats, and 3 neonatal hearts from 3 separate litters were provided from Charles River to conduct our studies. 3 separate hearts of canine left ventricular tissue were sourced from purpose
bred canines from Marshall Farms. Tissue from the left ventricular free wall of 3 non-failing, human samples were acquired from the Cleveland Clinic Foundation (CCF) tissue repository from unmatched organ donors. All protocols were approved by the CCF Institutional Review Board (IRB# 2378). Samples were received coded and no identifying metrics were documented for the study.

2.4 Results

We began our investigation by first identifying the maintained expression of KChIP2 in the guinea pig myocardium. Sequence alignment of the identified transcript shows significant sequence homology compared to other mammalian species which possess Kv4 encoded $I_{to}$ (Fig. 2.1A). Variability exists, however, in the relative levels of kcnip2 (KChIP2) mRNA expression. Left ventricular tissue isolated from guinea pig expresses approximately 35-fold less KChIP2 than the ventricular tissue from an adult rat (Fig. 2.1B), making the level of KChIP2 mRNA expression comparable to neonatal rat ventricular myocytes. Previous work has established the degree of KChIP2 expression directly correlates with the amount of $I_{to}$ density $^{72,100,101}$. Indeed, adult rat which has high levels of KChIP2 has the most prominent $I_{to}$ density of the animals shown, while neonatal rats which express much less KChIP2 has a smaller $I_{to}$ density which reflects the lower expression $^{102}$. An important distinction, however, is that neonatal rat myocytes display measurable $I_{to}$, while the guinea pig myocardium, while maintaining comparable
KChIP2 levels expresses no $I_{to}$, suggesting a functional consequence of KChIP2 presence beyond the modulation of $I_{to}$.

To begin to address if guinea pig cardiac KChIP2 contributes to cardiac excitability and modulation of other ionic currents, we evaluated the guinea pig action potential following acute changes to KChIP2 expression. Ventricular cells isolated from the adult guinea pig heart were left untreated or were treated for 24 hrs with an adenovirus expressing GFP or an mRNA antisense sequence for KChIP2 to acutely silence KChIP2. Evaluation by western blot shows we were able to significantly reduce KChIP2 protein in KChIP2 antisense treated cells compared to control cells (Fig. 2.2A). Critically, the consequence of this reduction was significant prolongation of the cardiac action potential, extending APD$_{90}$ from 211±10.8 ms in control cells to 262±14.6 ms in anti-sense treated cells (Fig. 2.2B). This prolongation was significant at both 90% and 50% of repolarization and occurred at multiple pacing frequencies (Fig. 2.2C, D). The overlap at phase 1 of the action potential traces between both groups is reflective of the absence of $I_{to}$ or any other comparable repolarizing current that KChIP2 has been shown to effect. This data implicates a clear influence of KChIP2 on factors beyond $I_{to}$. Notably, action potential amplitude, resting membrane potential, and $I_{K1}$ density were all unaltered by reductions in KChIP2 (Fig. 2.3A, B, C), showing that these factors were not responsible for changing action potential morphology or duration.

We next sought to identify the currents responsible for the prolonged repolarization observed following KChIP2 silencing. Given the observed prolongation of
the plateau phase of the guinea pig action potential, we focused on changes to $I_{Kr}$, $I_{Ks}$, and $I_{Ca,L}$. While there is no history for KChIP2 regulation on either of the delayed rectifier currents, we measured their activity to determine if these currents might be responsible for the delayed repolarization. However, assessment of the tail current densities for $I_{Kr}$ and $I_{Ks}$ displayed no significant change to either current (Fig. 2.4A, B). Overall, the loss of KChIP2 does not appear to affect outward repolarizing currents in the guinea pig myocardium. Therefore, we next looked at potential changes to $I_{Ca,L}$, to determine if enhancement of this depolarizing current might explain a longer APD. Indeed, when looking at the changes to L-type Ca$^{2+}$ current we see that reduction of KChIP2 produced an increase in current density across multiple potentials when compared to control cells (Fig. 2.5A, B). Peak current density at 10 mV was enhanced from $-5.43 \pm 0.73$ pA/pF in control cells to $-10.68 \pm 1.46$ pA/pF in KChIP2 anti-sense treated cells, nearly doubling the current density. Analysis of channel activation and voltage-dependent inactivation revealed that the kinetics of $I_{Ca,L}$ were unaltered by KChIP2 KD and could not account for the increase in current density. However, assessment of Cav1.2 protein expression showed that KChIP2 KD led to a significant increase in expression compared to control cells ($1.85 \pm 0.05$ fold change). Given that current kinetics were left unaltered, this suggests $I_{Ca,L}$ augmentation occurs through an increase in channel expression following the loss of KChIP2.

Lastly, to evaluate the full panel of currents relevant in the guinea pig cardiac action potential, changes to $I_{Na}$ was assessed. Evaluation of $I_{Na}$ density showed a decrease in the peak current from $-24.51 \pm 1.70$ pA/pF in control cells, to $-19.14 \pm 2.09$ pA/pF in
KChIP2 knock down treated cells (Fig. 2.6A, B). To understand this loss in current, Nav1.5 protein was assessed, which showed a mild but significant decrease following KChIP2 loss (0.71 ± 0.07 fold change), reflecting the change in current density. This suggests that KChIP2 not only has influence over the rate of repolarization, but may potentially have regulation over cardiac excitability as well.
2.5 Discussion

This study sought to characterize the influence of KChIP2 expression in the guinea pig myocardium. The species was specifically chosen for its unique, endogenous absence of $I_{to}$ \textsuperscript{98} despite our observation of maintained KChIP2 expression, suggesting an alternative significance for this protein. Additionally, this model provided a means of evaluating the electrophysiological consequences of KChIP2 loss without the influence of diminished $I_{to}$ density. Indeed, when we silenced KChIP2, we observed a significantly longer plateau phase of the action potential, resulting in a prolonged APD. This prolongation was due to enhanced L-type Ca\textsuperscript{2+} current density, with no change to the repolarizing currents $I_{Kr}$ and $I_{Ks}$. Additionally, we measured significant reduction to $I_{Na}$ density. Together, these data show that KChIP2 is indeed a multimodal effector of cardiac ionic currents.

In rodents, $I_{to}$ serves as the primary repolarizing current maintaining a strong influence over APD \textsuperscript{103}. In response to cardiac stressors like myocardial infarction and pressure overload, both KChIP2 and Kv4 experience reduced expression, significantly contributing to APD prolongation \textsuperscript{70, 104, 105}. It was found that maintaining KChIP2 expression even in the presence of cardiac stress could normalize the APD back towards baseline levels \textsuperscript{106}. This had the therapeutic effect of mitigating the hypertrophic response by minimizing increases in the Ca\textsuperscript{2+} transient and turning off Ca\textsuperscript{2+} activated hypertrophic signaling pathways. In larger mammals, however, this relationship is less straightforward as $I_{to}$ is comparatively smaller and therefore does not fully repolarize the membrane \textsuperscript{9}, which means decreased $I_{to}$ does not necessarily translate to prolonged APD. In fact, data suggests reductions in $I_{to}$ can actually lead to APD shortening by altering the driving
force for Ca\(^{2+}\) entry\(^9,107\). Yet, independent of species, cardiac pathologies almost invariably result in diminished KChIP2 expression and a prolonged APD\(^{43,108,109}\). Therefore, the observations in the guinea pig of KChIP2 loss directly augmenting \(I_{Ca,L}\) through increases in Cav1.2 protein (Fig 5) is highly significant in fully understanding its contributions to disease pathogenesis. It shows that independent of its effect on \(I_{to}\), KChIP2 depletion can still prolong the cardiac action potential (Fig 2) and possibly drive disease progression through Ca\(^{2+}\) dependent signaling cascades.

This is not the first instance showing that KChIP2 is capable of causing changes to L-type Ca\(^{2+}\) current. Thomsen \textit{et al} previously identified in a mouse KChIP2 knockout model a significantly reduced \(I_{Ca,L}\) in the absence of KChIP2\(^73\), the opposite regulation measured in the guinea pig here. This regulation was discovered to be the consequence of KChIP2 interaction with an N-terminal auto-inhibitory (NTI) domain on Cav1.2, which was interrupted when KChIP2 was bound, resulting in an increased open probability for the channel. However, when identifying this NTI domain in the guinea pig, alignment with the mouse shows an incomplete conservation of the amino acid residues, suggesting that the KChIP2 interaction site within the NTI domain may be absent in guinea pig. Without this mode of regulation present, this may explain why we do not observe a decrease in L-type Ca\(^{2+}\) current density, and instead may even be unmasking a secondary mode of regulation by KChIP2 on Cav1.2. Indeed, the KChIP2 null mice were observed to have increased Cav1.2 protein expression, despite the diminished current density\(^73\). This reinforces the observations here in the guinea pig where increased Cav1.2 protein expression results in a corresponding increase in \(I_{Ca,L}\). Notably, alternative splicing to the N-terminus of Cav1.2 can produce transcripts that omit this NTI domain\(^110\). While these
variants predominate in endothelial cells, it does suggest KChIP2 may in fact have differential regulation on Cav1.2, depending on which variant is being expressed. That we see increased current density in the guinea pig suggests KChIP2 regulation of $I_{Ca,L}$ has additional regulatory pathways yet to be determined.

The implications of cardiac KChIP2 expression grows even further when we consider its impact on $I_{Na}$. We have previously shown that silencing KChIP2 in rat myocytes resulted in loss of $I_{to}$ and $I_{Na}$\textsuperscript{75}. Immunoprecipitation studies suggested that the subunits comprising the currents interacted to form a structural and functional subunit complex. The data we show here takes this further to say that the regulation by KChIP2 on $I_{Na}$ occurs independently of $I_{to}$ and can influence $I_{Na}$ without the need to be part of a larger channel complex. This is supported by the observation that KChIP2 can coimmunoprecipitate with Nav1.5\textsuperscript{75}. Notably, our studies were conducted on dissociated myocytes and therefore take away the ability to study impulse propagation within native tissue, but it is intriguing to consider the influence this might have in an intact tissue.

Ultimately, our goal in this study was to characterize the changes in the cardiac action potential following KChIP2 loss, independent of any influences by $I_{to}$. Indeed, we were able to reveal a more profound significance for KChIP2 by identifying regulation on $I_{Ca,L}$ and $I_{Na}$. These influences are especially critical in cardiac pathologies where KChIP2 loss is so common and where these currents are also modified. There are numerous electrical remodeling events in the diseased heart, and the more we understand of the multiple roles of KChIP2, the more it seems KChIP2 might be responsible in mediating those changes. Together, this establishes KChIP2 as essential in the maintenance of
cardiac repolarization and depolarization, even independently from its role as a potassium channel interacting protein.

**Acknowledgments**

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2.6 Figures

(A) Protein sequence alignment across multiple mammals shows a high degree of sequence homology for KChIP2. (B) Relative expression of *kcnip2* (KChIP2) mRNA isolated from ventricular tissue (presented as means ± SEM, n = 3, guinea pig n = 5). Guinea pig expression is significantly less than other species but comparable to neonatal rat tissue where $I_{to}$ is present.
Figure 1.2: Knockdown of KChIP2 in isolated ventricular myocytes of the guinea pig prolongs the cardiac action potential. (A) Immunoblot for KChIP2 of whole cell lysate isolated from ventricular cells treated for 24 hrs with adenovirus encoding either GFP (control) or an mRNA antisense sequence for KChIP2 (KChIP2 KD). Beta-actin was used as a loading control. (B) Representative action potential tracings at 1000 ms cycle length from isolated ventricular cells following 24 hrs incubation with Ad.KChIP2-KD. (C) Summary data of APD<sub>90</sub> during 1000 ms and 400 ms cycle lengths. KChIP2 KD cells at 1000 ms (n = 16) show a significant prolongation of APD, compared to control cells (n = 14). KChIP2 KD at 400 ms (n = 11) was also significantly prolonged, compared to control cells (n = 9). (D) APD<sub>50</sub> following 1000 ms and 400 ms cycle lengths for the same treatment groups. Data presented as mean ± SEM; *P < 0.05; two-tailed Student’s t-test.
Figure 2.3: Action potential parameters. (A) Action potential amplitude and (B) resting membrane potential between control (n = 14) and Ad.KChIP2-KD (n = 16) isolated cardiomyocytes at 24 hrs shows no significant difference. (C) Left panel shows representative peak $I_{K1}$ elicited by a voltage step to -100 mV from a holding potential of -40 mV. Right panel shows summary data of these peak averages, indicating no significant change between control (n = 11) and KChIP2 KD (n = 10) cardiomyocytes. Data presented as mean ± SEM; two-tailed Student’s $t$-test performed.
Figure 2.4: APD prolongation observed following KChIP2 silencing is not defined by compromised repolarization. (A) Upper panel shows representative current traces for $I_{Ks}$ for both control and KChIP2 KD cardiomyocytes following 24 hrs incubation. Currents were generated from a holding potential of -40 mV with depolarizing voltage pulses from -30 mV to 60 mV, and then a return to -40 mV to generate outward tail currents in the presence of E4031. Lower panel shows the resulting I/V curve summary data between control (n = 19) and KChIP2 KD (n = 10) yielding no significant difference between treatment groups. (B) Upper panel shows representative current traces for $I_{Kr}$ for both control and KChIP2 KD cardiomyocytes following 24 hrs incubation. Currents were isolated as E4031 sensitive. Lower panel shows the resulting I/V curve summary data for tail currents between control (n = 9) and KChIP2 KD (n = 4). There was no significant difference between the two groups. Data presented as mean ± SEM; two-tailed Student’s t-test performed.
Figure 2.5: KChIP2 knock down enhances density of L-type Ca2+ current. (A) Representative $I_{\text{Ca,L}}$ current traces of control and KChIP2 KD treated cells following 24 hrs incubation. Currents were elicited from a holding potential of -40 mV and a depolarizing step to 10 mV. (B) I/V curve summary data of $I_{\text{Ca,L}}$ displays significant enhancement of current density following KChIP2 KD ($n = 12$) compared to control cardiomyocytes ($n = 20$), explaining the prolonged plateau phase of the cardiac action potential. (C) Steady-state activation of $I_{\text{Ca,L}}$ in control and KChIP2 KD treated myocytes. The data, depicted as normalized conductance, was fit with a Boltzmann curve but no significant difference was detected at any test pulse. (D) Evaluation of current decay
kinetics between control and KChIP2 KD treated myocytes at several membrane voltages. \( \tau \)-values were derived from single exponential fits of \( I_{\text{Ca,L}} \) decay following channel activation, also showing no significant difference at any test pulses. (E) Left panel shows representative immunoblots for Cav1.2 protein from whole cell lysates for control and KChIP2 KD treated myocytes. Protein expression was normalized to pan-cadherin expression. Right panel shows summary data depicting the normalized, relative densitometry of Cav1.2 expression, resulting in significantly increased expression following KChIP2 KD (\( n = 4 \)). Data presented as mean ± SEM; \*\( P < 0.05 \), **\( P < 0.01 \); two-tailed Student’s t-test performed for I/V plot, paired two-tailed Student’s t-test performed for western blot.
Figure 2.6: KChIP2 knock down attenuates $I_{Na}$ density. (A) Representative traces for $I_{Na}$ in cardiomyocytes in control and KChIP2 KD cells following 24 hrs incubation. (B) Summary data of the I/V curve for control (n = 13) and KChIP2 KD (n = 12) cardiomyocytes, showing reduced current density in response to KChIP2 loss. (C) Left panel shows representative immunoblots for Nav1.5 protein from whole cell lysates for control and KChIP2 KD treated myocytes. Protein expression was normalized to pan-cadherin expression. Right panel shows the average fold change from KChIP2 KD treated cardiomyocytes from control, which resulted in significantly decreased expression following KChIP2 KD (n = 3). Data presented as mean ± SEM; *$P < 0.05$ two-tailed
Student’s $t$-test performed for I/V plot, paired two-tailed Student’s $t$-test performed for western blot.
Chapter 3

KChIP2 Regulates the Cardiac Ca2+ Transient and Myocyte Contractility by Targeting Ryanodine Receptor Activity


In Review, Plos one

Conceived and designed the experiments: DMN XW KRL ID. Performed the experiments: DMN XW HL KRL. Analyzed the data: DMN XW KRL. Contributed reagents/materials/analysis tools: XW HL KRL ID. Wrote the paper: DMN XW ID.
KChIP2 Regulates the Cardiac Ca\textsuperscript{2+} Transient and Myocyte Contractility by Targeting Ryanodine Receptor Activity

**Short title:** KChIP2 Regulates the Cardiac Ca\textsuperscript{2+} Transient and Myocyte Contractility

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3.1 Abstract

Pathologic electrical remodeling and attenuated cardiac contractility are featured characteristics of heart failure. Coinciding with these remodeling events is a loss of the K+ channel interacting protein, KChIP2. While, KChIP2 enhances the expression and stability of the Kv4 family of potassium channels, leading to a more pronounced transient outward K+ current, \( I_{to,f} \), the guinea pig myocardium is unique in that Kv4 expression is absent, while KChIP2 expression is preserved, suggesting alternative consequences to KChIP2 loss. Therefore, KChIP2 was acutely silenced in isolated guinea pig myocytes, which led to significant reductions in the Ca\(^{2+}\) transient amplitude and prolongation of the transient duration. This change was reinforced by a decline in sarcomeric shortening. Notably, these results were unexpected when considering previous observations showing enhanced \( I_{Ca,L} \) and prolonged action potential duration following KChIP2 loss, suggesting a disruption of fundamental Ca\(^{2+}\) handling proteins. Evaluation of SERCA2a, phospholamban, RyR, and sodium calcium exchanger identified no change in protein expression. However, assessment of Ca\(^{2+}\) spark activity showed reduced spark frequency and prolonged Ca\(^{2+}\) decay following KChIP2 loss, suggesting an altered state of RyR activity. These changes were associated with a delocalization of the ryanodine receptor activator, presenilin, away from sarcomeric banding to more diffuse distribution, suggesting that RyR open probability are a target of KChIP2 loss mediated by a dissociation of presenilin. Typically, prolonged action potential duration and enhanced Ca\(^{2+}\) entry would augment cardiac contractility, but here we see KChIP2 fundamentally disrupts Ca\(^{2+}\) release events and compromises myocyte contraction. This novel role
targeting presenilin localization and RyR activity reveals a significance for KChIP2 loss that reflects adverse remodeling observed in cardiac disease settings.

**Key words:** KChIP2, Ryanodine Receptor, Presenilin, Calcium induced calcium release

**List of abbreviations**

K+ channel interacting protein (KChIP)

Ryanodine receptor (RyR)

Action Potential Duration (APD)

L-type Ca\(^{2+}\) current (\(I_{Ca,L}\))

SR Ca\(^{2+}\) ATPase (SERCA2a)

Sodium Calcium Exchanger (NCX)

Calcium induced calcium release (CICR)

Inositol 1,4,5-trisphosphate receptor (IP\(_3\)R)

\(N\)-methyl-D-aspartate receptor (NMDA)
3.2 Introduction

The development of heart failure, whether from atrial fibrillation, hypertrophy, or myocardial infarction, culminates in compromised contractility and the insufficient ability to pump blood for the demands of the body. While current therapeutics have done much to stem and treat the progression of heart failure, we are still unable to stop the progression, let alone reliably reverse adverse remodeling. Given the ever growing rise in number of heart failure patients, the need for greater understanding of the molecular targets for disease onset and progression are required.

The potassium channel interacting protein 2 (KChIP2) is a protein that consistently experiences degradation and sustained loss early in hypertrophy and heart failure\textsuperscript{64, 106}. This reproducibility suggests its loss may not just be symptomatic, but relevant to the progression and ailments of heart failure. KChIP2 is well-established as an accessory subunit and modulator of the Kv4 family of potassium channels, responsible for encoding the fast transient outward potassium current, $I_{to,f}$, critical for early phase 1 repolarization during the cardiac action potential\textsuperscript{65}. This current serves as the primary repolarizing current in rodents, directly contributing to action potential duration (APD), whereas in larger mammals it establishes the membrane potential for Ca$^{2+}$ entry and indirectly influences APD. Through interaction with the N-terminus of Kv4, KChIP2 can lead to the enhanced trafficking and stability of these channels\textsuperscript{70}, while also enhancing the duration of channel opening and recovery from inactivation, resulting in more pronounced $I_{to,f}$\textsuperscript{65, 91}. With the loss of KChIP2, there is an associated loss of Kv4, and the disappearance of $I_{to,f}$. However, this loss in $I_{to,f}$ does not appear to be a precipitating event
in heart failure, as animal models with the Kv4 gene removed and resulting APD prolongation, do not display signs of heart failure remodeling\textsuperscript{71}.

Supporting the prospect of additional roles for KChIP2 that may be relevant for cardiac disease remodeling is the guinea pig myocardium, which expresses KChIP2 but not Kv4\textsuperscript{98,111}. We previously showed that acute loss of KChIP2 in guinea pig myocytes led to prolonged action potential duration (APD) through increased L-type Ca\textsuperscript{2+} current ($I_{\text{Ca,L}}$) density\textsuperscript{111}. Indeed, we and others show that KChIP2 loss yields increased Cav1.2 protein expression\textsuperscript{73,111}, which in the mouse, resulted in diminished $I_{\text{Ca,L}}$ density due to a secondary interaction with an N-terminal fragment not conserved in the guinea pig. Additionally, KChIP2 appears to interact with Nav1.5, the Na\textsuperscript{+}-channel alpha subunit, as part of a macromolecular interaction that includes Kv4\textsuperscript{75}. Beyond these secondary roles for KChIP2, other members of the KChIP family (KChIPs 1-4), which are absent from the myocardium, have more diverse roles ranging from transcriptional regulation\textsuperscript{84,96} to the interaction and modulation of presenilin\textsuperscript{77,92}, a protein responsible for amyloid beta processing in the brain and more recently implicated in the potentiation of Ca\textsuperscript{2+} release from ryanodine receptors (RyR)\textsuperscript{81-83}. However, these roles have not been investigated for KChIP2, the only KChIP member expressed in the heart.

Given that we previously showed acute silencing of KChIP2 in guinea pig myocytes led to enhanced $I_{\text{Ca,L}}$ and APD prolongation\textsuperscript{111}, paired with the consistent KChIP2 loss observed in cardiac pathologies, we wanted to evaluate what influence acute silencing of KChIP2 has on Ca\textsuperscript{2+} handling and contractility. Unexpectedly, Ca\textsuperscript{2+} transients were reduced following KChIP2 knock-down, which further led to compromised contractile performance in the myocytes. Moreover, the attenuated Ca\textsuperscript{2+}
release was associated with a significant decline in RyR activity. While no changes were observed in the expression of Ca$^{2+}$ handling proteins, there was significant redistribution of the RyR modulator, presenilin, from repetitive sarcomeric localization to more disorganized and diffuse expression, potentially disrupting normal RyR Ca$^{2+}$ release. Much like the neuronal KChIP isoforms, this suggests KChIP2 can modulate presenilin, which in turn regulates RyR activity, illustrating a novel role for KChIP2 in cardiac disease.
3.3 Materials and Methods

Guinea Pig Ventricular Myocytes: Isolation and Short-term Culture

Single ventricular myocytes were isolated from adult guinea pigs as described previously\textsuperscript{99}. Briefly, guinea pigs were anesthetized by injection of fatal plus. Hearts were quickly removed and perfused via the aorta with a physiological salt solution (PSS) containing (in mmol/L) NaCl 140, KCl 5.4, MgCl\textsubscript{2} 2.5, CaCl\textsubscript{2} 1.5, glucose 11, and HEPES 5.5 (pH 7.4). After 5 minutes, perfusate was switched to a nominally calcium-free PSS with collagenase (Roche, 0.5 mg/mL) being added after an additional 5 minutes. After 15-20 minutes of digestion, hearts were perfused with a high K\textsuperscript{+} solution containing (in mmol/L) potassium glutamate 110, KH\textsubscript{2}PO\textsubscript{4} 10, KCl 25, MgSO\textsubscript{4} 2, taurine 20, creatine 5, EGTA 0.5, glucose 20, and HEPES 5 (pH 7.4). Ventricles were minced in high K\textsuperscript{+} solution, and single myocytes were obtained by filtering through a 115-μm nylon mesh. Myocytes were left to settle for 2 hours at room temperature before being collected in a low-speed spin. Cell pellets were resuspended in M199 medium supplemented with antibiotic and plated on uncoated 10 cm dishes. Cultures were treated with GFP adenovirus or adenovirus with a kcnip2 (KChIP2) mRNA antisense coding sequence in IRES with GFP. Cultures were then incubated in 5%CO\textsubscript{2} at 37°C for 24 hrs.

Viral Constructs

Control GFP and KChIP2 antisense adenoviruses were used as previously described\textsuperscript{111}. cDNA of the identified guinea pig KChIP2 antisense sequence was created and cloned into an adenoviral vector under the regulation of a CMV promoter (KChIP2-KD). The
vector coexpressed GFP through an internal ribosomal entry site. A control adenoviral vector was used that omitted the antisense sequence and expressed GFP alone.

*Calcium transients, SR Ca\(^{2+}\) load, and contractions in guinea pig myocytes*

Myocytes were bathed in a chamber with Tyrode’s solution composed of (mmol/L) NaCl 137, KCl 5.4, CaCl\(_2\) 2, MgSO\(_4\) 1, Glucose 10, HEPES 10, pH to 7.35 with NaOH.

Intracellular Ca\(^{2+}\) transient and sarcomere shortenings were initiated by field stimulation at 2Hz at 35°C. Ca\(^{2+}\) transient was measured using the fluorescent Ca\(^{2+}\) indicator indo-1\(_{AM}\) as described previously\(^{17}\). Cells were loaded with indo-1\(_{AM}\) by incubating them in Tyrode containing indo-1\(_{AM}\) (2\(\mu\)M) (Molecular Probes) and 0.025% (wt/wt) Pluronic F-127 (Molecular Probes) for 20 min at room temperature. The indicator was excited at 350 nm and the emitted signals were measured simultaneously at 405 nm and 485 nm. The emission field was restricted to a single cell with the aid of an adjustable window. The background fluorescence recorded from a cell without indicator loaded at both wavelengths was subtracted from the signal recorded from the cell before the fluorescence ratio was calculated. The 405nm/485nm fluorescence ratio was used to monitor changes in [Ca\(^{2+}\)]\(_i\) produced by stimulation. Ca\(^{2+}\) transient parameters were defined referring to the methods described previously\(^{112}\). Diastolic Ca\(^{2+}\) was defined as cytosolic Ca\(^{2+}\) level just prior to the onset of the Ca\(^{2+}\) transient or just prior to the action potential upstroke in the cases where there was no obvious Ca\(^{2+}\) transient. Amplitude of intracellular Ca\(^{2+}\) transient was calculated from the difference between peak and diastolic Ca\(^{2+}\). Time to peak was measured from the onset to the peak of Ca\(^{2+}\) transient. The duration of intracellular Ca\(^{2+}\) transient was measured as the onset of the Ca\(^{2+}\) transient to
the point of time when the transient decayed by 50%. SR content was compared between groups of myocytes after 10 mM Caffeine pulse induced SR Ca release using high speed solution exchange system. Sarcomere shortening was assessed using a video-based sarcomere length detection system (IonOptix Corporation)\textsuperscript{113}. Data acquisition was operated with an Axopatch 200B patch clamp amplifier controlled by a personal computer using a Digidata 1200 acquisition board driven by pCLAMP 7.0 software (Axon Instruments, Foster City, CA).

**Immunoblotting**

Freshly isolated guinea pig ventricular myocytes were cultured for 24 h at 37 °C in M199 medium under control GFP or with KChIP2 antisense virus. Upon collection, cardiomyocytes were washed in ice-cold PBS and resuspended in a RIPA lysis buffer (150 mM sodium chloride, 1.0% NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulphate), 50 mM Tris, pH 8.0, plus Roche Inhibitor tablet) and then sonicated on ice to disrupt cell membranes. 30-40 μg of whole cell extract was loaded into SDS-PAGE gels, transferred to nitrocellulose membrane, and western blotting performed using antibodies against RyR (1:800, Affinity Bioreagents), Serca2a (1:1000, Dr. Periasamy, Ohio State University), Phospholamban (1:1000, Santa Cruz), NCX (1:1000, Swant), GAPDH (1:4000, Santa-Cruz), beta-actin (1:1000, Sigma-Aldrich), pan-cadherin (1:1000, Cell Signaling), and presenilin 1 (1:500, Assay Biotech). Protein concentrations were determined by the BCA method (Pierce).

**Ca\textsuperscript{2+} Spark Recordings**
Line scan $\text{Ca}^{2+}$ transients were recorded by confocal line scanning (Leica DMi8). Myocytes were preloaded with 10 $\mu$M Rhod-3 dye (Thermofisher) in a tyrode solution consisting of (mmol/L) NaCl 137, KCl 5.4, CaCl2 2, MgSO4 1, Glucose 10, HEPES 10, pH to 7.35 with NaOH and 0.1% Pluronic® F-127 (Thermofisher). Myocytes were paced at 1 Hz for 10 beats and then the pacing was halted to record $\text{Ca}^{2+}$ spark activity at rest. Cells were scanned longitudinally along the long axis of the myocyte. Line scan images were processed and analyzed with ImageJ software utilizing the SparkMaster plugin\textsuperscript{114}.

**Immunohistochemistry and image quantitation**

Isolated guinea pig myocytes were plated on laminin coated coverslips for 2 hrs, after which the media was changed with fresh M-199 and treated with Ad.GFP or Ad.KChIP2 KD for 24 hrs. Cells were fixed in 4% formaldehyde diluted in PBS for 15 minutes. Cells were permeabilized for 10 min in PBS + 0.03% Triton X-100 and blocked for 2 hrs in a solution of PBS, 5% normal goat serum, and 1% BSA. Cells were incubated overnight with primary antibody (Presenilin 1, Assay Biotech at 1:50 and RyR, 1:50 Affinity Bioreagents) in PBS with 2% normal goat serum and 1% BSA. Cells were rinsed 3x in PBS then incubated with secondary antibody (Alexa-568 1:1000 against Presenilin 1 and Alexa-647 1:500 against RyR) in PBS with 2% normal goat serum and 1% BSA for 2 hrs at room temperature. Labeled cardiomyocytes were scanned with a Leica DMi8 confocal microscope. Fluorescence intensity profiles were generated by ImageJ using the plot profile function for the declared regions of interest. A single region of interest was evaluated per myocyte. The peaks of all resulting plot profiles within each treatment group were aligned to create a representative average trace.
Statistical Analysis

The experimental data were expressed as mean ± SEM. A Student’s t-test or a paired Student’s t-test in the case of immunoblot analysis was performed. The paired test was applied given that each set of Ad.GFP or Ad.KChIP2 KD myocytes were derived from the same cell isolation. All tests were two-sided and a significance level of p < 0.05 was defined as statistically significant (SPSS 18.0 software, SPSS, Chicago, IL).

Ethics Statement and Tissue Acquisition

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol for tissue isolation from adult guinea pig (Protocol Number: 2012-0175) samples was approved by the Committee on the Ethics of Animal Experiments of Case Western Reserve University. 14 male Hartley guinea pigs 5-6 weeks old from Charles River were used to conduct our studies.
3.4 Results

We isolated adult guinea pig ventricular myocytes, which were subsequently treated with a control virus encoding GFP (control) or an antisense sequence for KChIP2 (KChIP2 KD) to acutely suppress expression as previously confirmed\textsuperscript{111}. Following 24 hrs incubation, we evaluated the cells for changes to Ca\textsuperscript{2+} transients and contractility. In myocytes treated with KChIP2 KD we observed a significant reduction in Ca\textsuperscript{2+} transient amplitude by 18.8\% (Ca\textsuperscript{2+} transient amplitude: control 1.11 ± 0.06 vs KChIP2 KD 0.90 ± 0.07 AU) (Fig. 3.1A, B) at 0.5 Hz field stimulation. This change was accompanied by a significant prolongation of the Ca\textsuperscript{2+} transient duration (Fig. 3.1C) but no change in the time-to-peak (Fig. 3.1D). Remarkably, this reduction in Ca\textsuperscript{2+} transient amplitude and preservation of SR Ca\textsuperscript{2+} content occurred despite the presence of an increase in $I_{Ca,L}$\textsuperscript{111}, suggesting a potential impact of KChIP2 on calcium induced calcium release (CICR).

Coinciding with the reduction in calcium release, myocyte contractility was also compromised. With KChIP2 loss we observed a decrease in fractional shortening from 5.47 ± 0.50 in control cells to 3.63 ± 0.44 \% in KChIP2 KD (Fig. 3.2A, B). No changes were seen to the duration or the rate of contraction between treatment groups (Fig. 3.2C, D). Yet, we clearly see that with KChIP2 loss, Ca\textsuperscript{2+} release and contraction are weakened. Notably, SR Ca\textsuperscript{2+} content was assessed by caffeine stimulation showing no change between treatment groups (Fig. 3.3A, B), indicating the reduction in transient amplitude is not from a loss in SR Ca\textsuperscript{2+}.

To address how an increase in $I_{Ca,L}$ could lead to suppressed Ca\textsuperscript{2+} transients and reduced contractility, we evaluated the canonical players in Ca\textsuperscript{2+} handling machinery.
Ryanodine receptor (RyR2), SR Ca\(^{2+}\) ATPase (SERCA2a), phospholamban, and sodium calcium exchanger (NCX) expression, however, were all unchanged in response to KChIP2 KD (Fig. 3.4A, B). The observation of no change in the SR Ca\(^{2+}\) content (Fig. 3.3) is consistent with a lack of change in SERCA2a and phospholamban expression and regulation. While we did observe a significant prolongation of the Ca\(^{2+}\) transient, it does not appear that Ca\(^{2+}\) reuptake is responsible. Therefore, we next determined if Ca\(^{2+}\) release was instead affected by the loss in KChIP2.

While we saw no change in the amount of RyR expression, we wanted to evaluate a possible change in its function. To address this, we performed confocal line scan imaging to determine Ca\(^{2+}\) spark activity and, thus, assess RyR activity. Cells were paced for 10 beats at 1 Hz, immediately after which measurements for spontaneous Ca\(^{2+}\) release events were taken. We observed that cells with KChIP2 KD had a spark frequency 46.2% lower than control cells (Fig. 3.5A, B), suggesting reduced open probability for RyR receptor in myocytes without KChIP2. Notably, this reduced activity of Ca\(^{2+}\) release can explain the reduced Ca\(^{2+}\) transient amplitude and resulting contraction events. Additionally, no change in the time-to-peak (Fig. 3.5C) but a significant prolongation of spark decay (Fig. 3.5D) following KChIP2 KD are consistent with the conditions observed for the overall Ca\(^{2+}\) transient. There was also no change in the full width at half maximum amplitude between treatment groups (Fig. 3.5E), suggesting preservation of RyR clustering.
We were next curious how a loss in KChIP2 could lead to a modulated state of RyR activity. Recent investigations into the protein presenilin have shown its ability to modulate Ca\textsuperscript{2+} release activity through RyR\textsuperscript{81-83}. At the same time, KChIP3 was previously discovered to regulate presenilin activity and influence the degree of regulation it has on RyR\textsuperscript{115}. Therefore, we sought to determine if KChIP2 loss could impact presenilin expression, and thereby RyR activity. To this effect, we saw that KChIP2 KD led to no change in the amount of presenilin protein (Fig. 3.6A-B). However, evaluation of presenilin localization by immunocytochemistry revealed significant reorganization of expression (Fig. 3.6C, D). While control cells showed strong presenilin alignment with sarcomeric structure, KChIP2 KD led to significant disruption of this banded pattern. Presenilin became dispersed away from sarcomeric bands towards a more unstructured state. This observation was quantitated by evaluating regions of interest of the fixed myocytes using the plot profile function within ImageJ software\textsuperscript{116}. This revealed a reduction in peak signal intensity and an increase in minimum signal intensity following KChIP2 loss, depicting a decreased heterogeneity in the plot profile expression for presenilin (Fig. 3.6E). At the same time, the average fluorescence intensity for the regions of interest remained unchanged, reflecting the preserved protein expression shown by immunoblot (Fig. 3.6G). Notably, this redistribution was specific to presenilin, as RyR staining remained unchanged between treatment groups (Fig. 3.6D, F, H). As presenilin has previously been observed to enhance the degree of Ca\textsuperscript{2+} release through RyR, this relocalization of presenilin away from sarcomeric structure suggests a removal of this augmented state, yielding a compromised state of Ca\textsuperscript{2+} release and contraction following KChIP2 loss.
3.5 Discussion

In the present study we sought to determine the role of KChIP2 in \( \text{Ca}^{2+} \) handling and contractility in the guinea pig, particularly given our previous findings of enhanced \( I_{\text{Ca,L}} \) following acute KChIP2 loss\(^{111} \). Despite this observation, we found that with the same loss in KChIP2, the \( \text{Ca}^{2+} \) transient amplitude was diminished, and with it came an attenuated state of myocyte contractility. Notably, these changes occurred without an altered state of SR \( \text{Ca}^{2+} \) content or protein expression of the canonical \( \text{Ca}^{2+} \) handlers in the sarcolemma space. Instead, we observed reduced activity of the ryanodine receptor, coinciding with relocalization of presenilin, an established activator of RyR activity. Together, this implicates the loss of KChIP2 as a potential mediator of compromised cardiac contractility.

The shape and regulation of the cardiac action potential is intimately associated with the initiation and coordination of \( \text{Ca}^{2+} \) release events. In particular, the transient outward potassium current, \( I_{\text{to}} \), coordinates both the magnitude and duration over which \( \text{Ca}^{2+} \) can enter the cell, influencing both SR \( \text{Ca}^{2+} \) load and \( \text{Ca}^{2+} \) priming for CICR\(^9 \). However, the guinea pig does not encode \( I_{\text{to}} \) yet KChIP2 expression is maintained, suggesting that any potential influence KChIP2 might have over \( \text{Ca}^{2+} \) handling and contractility would have to come independently from its established regulation over \( I_{\text{to}} \). To this effect, we saw that KChIP2 silencing led to a significant increase in \( I_{\text{Ca,L}} \) through increases in Cav1.2 protein expression, producing a significant prolongation of the action potential\(^{111} \). Given that enhanced \( I_{\text{Ca,L}} \) would provide more activating \( \text{Ca}^{2+} \) in the CICR response, we anticipated this phenotype would have augmented CICR and contractility. However, we instead observed a dramatic loss in \( \text{Ca}^{2+} \) transient amplitude (Fig. 3.1A, B)
and cell shortening (Fig. 3.2A, B), indicating a fundamental disruption to the normal CICR events. As there were no changes in the expression of canonical Ca\textsuperscript{2+} handling proteins (Fig. 3.4), or to SR Ca\textsuperscript{2+} content (Fig. 3.3) to explain the smaller transient amplitude, this suggested a modulation to RyR activity. Such an effect was further supported by the observed reduction in Ca\textsuperscript{2+} spark frequency following KChIP2 KD (Fig. 3.5A, B).

Precedence for the ability of KChIP to modulate Ca\textsuperscript{2+} release events independent of its contributions to \(I_{lo}\) and APD have previously been observed, only not in the heart. KChIP3, also known as calsenilin, another member of the KChIP family with significant expression in the brain but absent in the heart, was previously identified to bind to and modulate a class of proteins known as presenilins [13, 14]. These transmembrane proteins have garnered much attention due to mutations that have closely associated with the development of Alzheimer’s Disease. Serving as part of a \(\gamma\)-secretase complex, these proteins can become deregulated leading to the formation of neurotoxic amyloid-beta peptides\textsuperscript{78}. Notably, these same mutations to presenilin 1 are strongly associated with alterations to cytosolic Ca\textsuperscript{2+} concentrations,\textsuperscript{78, 117, 118} which is believed to contribute directly to disease pathogenesis. In line with this, presenilin 1 has been shown to bind to several proteins critical to Ca\textsuperscript{2+} release mechanisms, including the inositol 1,4,5-trisphosphate receptor (IP\textsubscript{3}R)\textsuperscript{79}, the \(N\)-methyl-D-aspartate (NMDA) receptor\textsuperscript{80}, and most relevant to our investigation, the RyR receptor\textsuperscript{81-83}. As a consequence of its interaction with the RyR receptor, presenilin 1 enhances RyR open probability and mean Ca\textsuperscript{2+} current. Moreover, these gain of function mutations to presenilin 1 are shown to enhance the amplitude of Ca\textsuperscript{2+} release through RyR, and have also been associated with the
development of dilated cardiomyopathy and heart failure. Together, this suggests that loss of presenilin 1 at sarcomeric structures would lead to reduced RyR activity and thereby Ca$^{2+}$ release, as we see in our investigation.

Notably, KChIP2 shares a high degree of sequence homology with KChIP3/calsenilin, further supporting that its ability to modulate presenilins is a conserved function. Indeed, KChIP3 is capable of modulating Kv4 channel expression and kinetics in the same manner as KChIP2, and we have shown that KChIP2 much like KChIP3/DREAM can act as a transcriptional repressor, reinforcing the notion of shared roles between these family of proteins. Therefore, while KChIP3 has been shown to associate with presenilin in vitro through yeast-2-hybrid assays and natively through co-immunoprecipitation studies, we can speculate that such an interaction exists for KChIP2 and presenilin in the heart. Furthermore, it is not yet clear if this interaction serves to modulate the influence presenilin has over RyR activity, as previously shown in a heterologous system for KChIP3, or if this interaction is also significant for mechanisms of presenilin trafficking and/or localization, as implicated in this investigation. Considerably, KChIP2 loss leads not only to the destabilization and relocalization of Kv4 channel expression but also shifts Kv4 channel kinetics, showing an established utilization of multimodal regulation as a consequence of KChIP2 interactions. Therefore, it is entirely plausible that the redistribution of presenilin 1 expression and activity could be affected following KChIP2 loss.

While we show no change in the expression of a panel of calcium handling proteins, it is entirely possible that post-translational modifications to RyR may mediate the changes in CICR. In particular, phosphorylation of RyR can lead to a gain in CICR
through an increase in open probability. Therefore, a decline in the steady state level of RyR phosphorylation could be a relevant change consistent with this phenotype. However, phosphorylation of RyR has also been associated with an accelerated rate of SR Ca$^{2+}$ release as well as slowed channel inactivation. Here, in response to KChIP2 loss, we observe no change in the rate of Ca$^{2+}$ release, reflected in our time-to-peak measurements (Fig. 1D, 5C), while also producing significant delays in the decay rate for our Ca$^{2+}$ transient and spark events (Fig. 1C, 5D). The shifts we observe to RyR open probability and channel kinetics are therefore less likely to be compatible with the modifications contributed by a potentially reduced state of phosphorylation. At the same time, preliminary data using western blot evaluated two samples of guinea pig myocytes silenced for KChIP2, which revealed no change in phosphorylation status of RyR compared to control treated cells at the S2814 residue (data not shown). Therefore, it is unlikely that phosphorylation changes are responsible for the observed phenotype.

Overall, cardiac disease progression is defined largely by a continuing decline in cardiac performance. Investigations into the underlying phenotype has shown a multitude of gene changes and protein modifications can be responsible, including changes to $I_{\text{Ca,L}}$, loss of SERCA2a expression and function leading to depletion of SR Ca$^{2+}$ content, as well as modifications to the stability and activity of RyR Ca$^{2+}$, influencing CICR response. Presenilin so far has only been implicated in hypertrophic remodeling associated with primary mutations, however, this establishes precedence that disruption to normal presenilin activity contributes to cardiac remodeling through its influence over RyR. Therefore, the relationship between KChIP2 loss and disruption of
presenilin localization represents a novel pathway meaningful for acquired states of heart
disease as well.

KChIP2 depletion continues to be one of the most consistent across multiple lines
of pathogenesis. Critically, our data highlight the involvement of KChIP2 in contributing
to the state of compromised cardiac output, establishing it as much more than a modifier
of cardiac repolarization and depolarization. These contributions are critical when trying
to understand the culmination of events leading to and worsening heart failure, where
compromised contractility is paramount. Together, these data support KChIP2 as a
potential new target for prevention of cardiac disease.
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Figure 3.1: KChIP2 knock down reduces Ca^{2+} transient amplitude. (A)

Representative Ca^{2+} transient recordings taken from isolated adult guinea pig myocytes loaded with Indo-1 and paced at a 500 ms cycle length. Recordings were taken following 24 hrs treatment with an adenovirus encoding GFP (control, n = 17) or an mRNA antisense sequence for KChIP2 (Ad.KChIP2 KD, n = 17). Summary data for the (B) Ca^{2+} transient amplitude, (C) Ca^{2+} transient duration at 50% peak amplitude, and (D) the transient time-to-peak. Data presented as mean ± SEM; *P < 0.05, **P < 0.01; two-tailed Student’s t-test.
Figure 3.2: KChIP2 knock down reduces myocyte contractility. (A) Representative tracing of the change in distance between two consecutive sarcomeres during contraction. Cells were paced at a 500 ms cycle length for Ad.GFP (n = 26) or Ad.KChIP KD (n = 28) treated myocytes. Tracings were normalized to control cells. Summary data for (B) fractional shortening, (C) contraction duration at 50% amplitude, and (D) the time to peak contraction. Data presented as mean ± SEM; *P < 0.05, **P < 0.01; two-tailed Student’s t-test.
Figure 3.3: Reduced Ca$^{2+}$ transients following KChIP2 KD are not associated with reduced SR Ca$^{2+}$ load. (A) Examples of caffeine induced Ca$^{2+}$ transients measured in cells loaded with Indo-1. Ca$^{2+}$ transients preceding the caffeine trace were not collected in the same capture as the caffeine induced transient but were assessed with the same standard curve used in the transient recordings. (B) Summary data for the amplitude of caffeine induced Ca$^{2+}$ transients for Ad.GFP (n = 13) and Ad.KChIP2 KD (n = 13). Data presented as mean ± SEM.
Figure 3.4: KChIP2 KD is not associated with a change in expression in the major Ca\(^{2+}\) handling proteins. (A) Representative immunoblots from guinea pig myocyte whole cell lysates for Ryanodine receptor (RyR), Serca2a, Phospholamban (Pln), Sodium Calcium Exchanger (NCX), and glyceraldehyde-3-phospohate (GAPDH) following treatment with Ad.GFP or Ad.KChIP2 KD for 24 hrs. (B) Summary data for the densitometry fold change between paired samples of each protein target normalized to GAPDH or beta-actin in Ad.KChIP2 KD (n = 3-5) treated cells compared to Ad.GFP (n = 3-5). There are no significant differences between treatment groups. Data presented as mean ± SEM.
Figure 3.5: KChIP2 KD results in a significant reduction in spark frequency. (A)
Left panel (Ad.GFP) and right panel (Ad.KChIP KD) show representative recordings of confocal line scan imaging evaluating spontaneous Ca\textsuperscript{2+} spark activity following 1 Hz field stimulation. Summary data between control (n = 47 cells) and KChIP KD (n = 44 cells) for the (B) spark frequency, (C) Ca\textsuperscript{2+} spark time-to-peak, (D) decay time of the Ca\textsuperscript{2+} spark, and (E) full width at half maximum (FWHM) amplitude.
Figure 3.6: KChIP2 KD associates with a relocalization of presenilin 1 away from sarcomeric structures. (A) Representative immunoblot for presenilin 1 protein from whole cell lysates for Ad.GFP and Ad.KChIP2 KD treated myocytes. Protein expression was normalized to pan-cadherin expression. (B) Summary data for the average fold change in KChIP2 KD (n = 4) from control (n = 4) showing no reduction in presenilin 1 protein. (C) Representative immunostaining for presenilin 1 protein in Ad.GFP (left panel) and Ad.KChIP2 KD (right panel). Control cells show a strong sarcomeric pattern of expression that is disrupted in KChIP2 KD treated cells. White box represents region of interest selected for evaluating fluorescence intensity in ImageJ. (D) Representative immunostaining for RyR between Ad.GFP (left panel) and Ad.KChIP2 KD (right panel), showing preserved distribution. (E) Average plot profile for presenilin 1 from regions of interest (white boxes) showing the decreased peak intensity at sarcomeres and increased intensity between sarcomeres in Ad.KChIP2 KD (n = 25) compared to Ad.GFP (n = 25) myocytes. Plot profiles were created by the plot profile function within ImageJ. (F) Average plot profile for RyR in the same regions of interest assessed for presenilin 1,
shows no change in distribution. **(G)** Summary data for the average staining intensity for presenilin 1 (left panel) and the variance (standard deviation) in the plot profile (right panel) in the regions of interest. Preservation of the average intensity reflects no change in the amount of presenilin protein, however, a decrease in variance reflects the loss in organization for presenilin 1 protein expression upon KChIP2 KD. **(H)** The same parameters used to evaluate presenilin 1 distribution but now for RyR, show no change in average intensity or heterogeneity, reflecting no change in RyR localization.
Chapter 4

KChIP2 is a Core Transcriptional Regulator of Cardiac Excitability


KChIP2 is a Core Transcriptional Regulator of Cardiac Excitability

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4.1 Abstract

Arrhythmogenesis from aberrant electrical remodeling is a primary cause of death among patients with heart disease. Amongst a multitude of remodeling events, reduced expression of the ion channel subunit KChIP2 is consistently observed in numerous cardiac pathologies. However, it remains unknown if KChIP2 loss is merely a symptom or involved in disease development. Using rat and human derived cardiomyocytes, we identify a previously unobserved transcriptional capacity for cardiac KChIP2 critical in maintaining electrical stability. Through interaction with genetic elements, KChIP2 transcriptionally repressed the miRNAs miR-34b and miR-34c, which subsequently targeted key depolarizing ($I_{Na}$) and repolarizing ($I_{Io}$) currents altered in cardiac disease. Genetically maintaining KChIP2 expression or inhibiting miR-34 under pathologic conditions restored channel function and moreover, prevented the incidence of reentrant arrhythmias. This identifies the KChIP2/miR-34 axis as a central regulator in developing electrical dysfunction and reveals miR-34 as a therapeutic target for treating arrhythmogenesis in heart disease.
4.2 Introduction

Cardiac excitability is controlled by a combination of depolarizing and repolarizing currents, whose dysregulation during heart failure (HF) or myocardial infarction (MI) play a significant role in clinically relevant arrhythmias\(^{55,125}\). Aberrant remodeling culminates in altered Ca\(^{2+}\) current (\(I_{Ca}\))\(^{5,6}\), Na\(^{+}\) current (\(I_{Na}\))\(^{126,127}\), and a host of outward K\(^{+}\) currents (\(I_{K}\))\(^{44}\), creating impaired cardiac excitability and performance, accounting for high rates of mortality in HF patients\(^{16,128}\). However, large variability and breadth of electrical changes present challenges in determining which mechanisms are critical in driving arrhythmias and disease progression. Intriguingly, loss of the Potassium Channel Interacting Protein 2 (KChIP2) has proven to be a consistent event following cardiac stress, sparking interest into understanding its contribution in disease remodeling\(^{44,45,106}\).

It is well described that KChIP2 associates with and modulates the Kv4 family of potassium channels, which together define the fast transient outward potassium current (\(I_{to,f}\)), maintaining early cardiac repolarization\(^{41,65}\). However, emerging evidence suggests KChIP2 may not be limited to this role\(^{73-75}\). Investigations following KChIP2 knock-down show reduced transcript expression for the cardiac sodium channel gene, SCN5A, and its accessory subunit SCN1B, in addition to Kv4.3 protein, prompting the loss of both \(I_{to,f}\) and \(I_{Na}\)\(^{75}\). Considerably, these changes reflect conditions observed in the diseased heart, but more importantly implicate potential transcriptional significance for KChIP2 at the center of that remodeling. Indeed, other members of the KChIP family not expressed in the myocardium behave as transcriptional repressors, while also maintaining the ability to interact with Kv4 channels\(^{65,84,129-131}\). Therefore, we sought to identify the existence of...
cardiac KChIP2 transcriptional activity and its significance in electrical remodeling and arrhythmia susceptibility. Here, we find KChIP2 transcriptionally represses a set of miRNAs known as miR-34b and-34c. Through KChIP2 loss, miR-34b/c are elevated, subsequently targeting other ion channel genes defining $I_{Na}$ and $I_{to}$ densities. Either restoring KChIP2 expression or blocking miR-34b/c activity during cardiac stress reverses this remodeling and completely negates the occurrence of re-entrant arrhythmias. Together, this work unveils a novel, transcriptional mechanism for KChIP2, and defines it as a central mediator of cardiac electrical activity.
4.3 Results

*KChIP2 as a transcriptional repressor of miRNAs*

This study was approached with the knowledge that acute KChIP2 loss affected the *SCN5A* (Nav1.5), *SCN1B* (Navβ1), and *KCND3* (Kv4.3) genes in a manner suggesting miRNA activity. We therefore performed a miRNA microarray following KChIP2 silencing in neonatal rat ventricular myocytes (NRVMs), resulting in the induction of a number of miRNAs (Figure 4.1A). We evaluated the miRNAs that achieved at least a 2 fold increase (Figure 4.1B) using TargetScan to identify potential targeting to the mRNAs *SCN5A*, *SCN1B*, and *KCND3*. Ultimately, we identified miR-34b and -34c as the only miRNAs predicted to target not just one of these ion channel genes, but notably target all three collectively (Figure 4.1C). Notably, we also observed 14 miRNAs decreased greater than 2 fold (Figure 4.1B). However, a loss in miRNA expression is not consistent with the role of KChIP2 as a transcriptional repressor, and also would not lead to a decrease in ion channel mRNA expression. Real-time qPCR was used to confirm the array results, showing elevation in the mature transcripts for miR-34b and -34c (Figure 4.1D). Importantly, we also performed overexpression of three different cardiac KChIP2 isoforms which reduced the expression of miRs-34b/c (Figure 4.1D). Together, these changes are consistent with the novel idea that KChIP2 behaves as a transcriptional repressor.

Because KChIP2 is dominantly known as a modulator of Kv4 channels, with cytoplasmic localization, we addressed whether it could also localize to the nucleus where it could act as a transcriptional regulator. Indeed, fractionation of adult rat cardiomyocytes into nuclear fractions reveals endogenous KChIP2 nuclear expression in...
the absence of contaminating cytosolic (lactate dehydrogenase) and membrane associated proteins (Serca2a) (Figure 4.1E). This is reinforced in the localization patterns of adult cardiomyocytes, showing marked endogenous KChIP2 colocalization in the nucleus in the absence of the cytosolic marker lactate dehydrogenase (Figure 4.1F).

**KChIP2 interaction with the miR-34b/c promoter**

To assess whether the transcriptional changes seen in miR-34b/c were the consequence of KChIP2 activity on the promoter, a luciferase assay was conducted containing the cloned minimal miR-34b/c promoter in the presence of KChIP2. Notably, both miR-34b and -34c are transcribed in tandem under the regulation of a shared, intergenic promoter\(^{133}\). To identify potential DNA binding locations for KChIP2, we borrowed from what is known about the putative nucleotide binding sequence for the transcriptional repressor DREAM (KChIP3). This member of the KChIP family shares a high degree of homology with KChIP2, but more importantly has known transcriptional activity occurring through interaction with a nucleotide sequence known as the DRE motif\(^84\). MatInspector software\(^{134}\) was used to evaluate the miR-34b/c promoter for occurrences of this motif, revealing a potential site beginning 254 bp upstream of the miR-34b stem-loop (Figure 4.2A). A region of the promoter 500 bp to 191 bp upstream of the miR-34b stem-loop was cloned into the pGL4.10 luciferase vector and co-transfected with several KChIP2 isoforms into cos-7 cells. When compared to a GFP transfected control without KChIP2, we observed significant repression in the presence of KChIP23, 2.4, and 2.6 (Figure 4.2A), showing that KChIP2 can directly act on the miR-34b/c promoter to impart repressive action. To determine if physical KChIP2 interaction
with the promoter mediates the repressive state, native adult rat cardiomyocytes were used to perform chromatin immunoprecipitation, followed by qPCR with a primer set flanking the identified DRE site. KChIP2 pull-down resulted in significant enrichment of the DRE-containing PCR fragment when compared to an IgG control (Figure 4.2B).

To identify if the DRE site within the promoter fragment is responsible for the repression caused by KChIP2, the core nucleotide sequence was deleted from the promoter (Figure 4.2C). This attenuated the repressive action of KChIP2, implying that KChIP2 is capable of recognizing the same putative DNA binding motifs as DREAM and uses it to induce repressive action. Additionally, it is known that transcriptional derepression of DREAM is regulated through Ca\(^{2+}\) binding to EF-hand motifs\(^84\).

Therefore, to further characterize KChIP2 activity, the reporter assay was conducted following incubation with 10 mM caffeine to induce global elevations in Ca\(^{2+}\). This led to significant activation of the promoter (Figure 4.2D), reinforcing the transcriptionally repressive nature of KChIP2 and its conserved mechanisms with DREAM. Together, this data demonstrates that KChIP2 behaves as a transcriptional repressor on the promoter of miR-34b/c by direct binding to the putative DRE motif.

**SCN5A, SCN1B, and KCND3 targeted by miR-34b/c**

Previous studies identified reduction in Nav1.5, Navβ1, and Kv4.3 following KChIP2 silencing\(^75\). Having observed that KChIP2 knock-down elevates miR-34b/c, we next sought to determine whether miR-34b/c targets these ion channel transcripts to mediate their loss in expression. Precursor miRNAs for miRs-34b/c were transfected into
NRVMs to directly elevate their expression. Assessment of the resulting transcripts showed reduced mRNA for *SCN5A* and *SCN1B* following miR-34 expression, compared to a non-targeting control miR (Figure 4.3A). While *KCND3* levels remained unchanged (Figure 4.3A), Kv4.3 protein experienced significant reduction that reinforces the miRNA mode of translational inhibition without mRNA degradation previously noted²⁵ (Figure 4.3B and C).

To determine if the changes in channel expression was the consequence of miR-34 targeting to the 3’-UTR of these genes, and not the result of an indirect pathway, fragments of the 3’ region containing the seed sequence were fused to the end of a luciferase reporter construct. This construct was co-expressed with the miR-34b/c precursors in HEK293 cells, resulting in reduced activity in all three constructs when compared to a control miR-precursor (Figure 4.3E). Subsequently, mutations were made within the seed region where miR-34 targeting is predicted to bind (Figure 4.3D), which significantly attenuated the repressive action (Figure 4.3E). This suggests that miR-34b/c are indeed targeting the predicted seed region in the *SCN5A*, *SCN1B*, and *KCND3* genes and directly influencing their expression.

**miR-34b/c functionally regulates *I*$_{Na}$ and *I*$_{to}$ density**

Functional assessment of changes to *I*$_{Na}$ and *I*$_{to}$ were determined through patch clamp recordings in NRVM. Reflecting the changes in mRNA and protein, expression of miR-34b/c precursor produced a significant decline in *I*$_{Na}$ (Figure 4.3F). *I*$_{to}$, however, while having trended reductions, did not produce significant loss despite the loss in
Kv4.3 protein levels (Figure 4.3G). This can be attributed to a number of reasons. The current evaluation was conducted in rodent myocytes, where $I_{to}$ is comprised of the shared alpha subunits Kv4.2 and Kv4.3, which comprise a fast component of $I_{to}$ referred to as $I_{to,f}$. Additionally, there are the contributions of Kv1.4, another potassium channel subunit, which encodes a slow component, referred to as $I_{to,s}$. These descriptions are attributed to the respective rates of recovery from inactivation for each of these channels$^{41}$. Notably, our patch protocol in Figure 3G took into account the contributions of all three subunits, or $I_{to,total}$. Therefore, despite reductions in Kv4.3 protein expression the change in current resists as it is not the predominant channel contributing to $I_{to}$.

Importantly, Kv4.2 and Kv1.4 do not contain a miR-34 seed region. In fact, in response to miR-34b/c expression, mRNA levels for Kv4.2 actually experienced a trended elevation (Figure 4.3.1 – figure supplement 1) which could also contribute to the lack of reduction in $I_{to}$. We therefore modified our patch protocol to probe just $I_{to,f}$ and remove the contribution of Kv1.4, which now revealed a significant reduction in the $I_{to,f}$ density (Figure 4.3H). To further identify if the presence of Kv4.2, Kv4.3, and Kv1.4 in rats could explain the resisted change in $I_{to}$, cardiomyocytes derived from human induced pluripotent stem cells (iCells®) were used, since in the human background, Kv4.3 is the dominant contributor to $I_{to}$$^{41}$. Expression of miR-34b/c precursors now produced a significant loss in $I_{to}$ density (Figure 4.3J), while also maintaining reductions in $I_{Na}$ (Figure 4.3I). Importantly, this not only satisfies why $I_{to}$ loss was resistant in the NRVMs, but identifies conservation of miR-34 activity across species, implicating the importance of miR-34s in human cardiac ion channel regulation.
KChIP2 regulates miR-34b/c expression during cardiac stress

To begin to understand the pathogenic importance of this pathway, NRVMs were cultured in 100 μM phenylephrine (PE) for 48 hrs to mimic neuro-hormonal overload in a stressed myocardium. PE stimulation resulted in a dramatic decline of KCNIP2 (KChIP2), while also yielding significant elevation in miR-34b/c (Figure 4.4A and B). These conditions resulted in reductions in expression for SCN5A, SCN1B, and KCND3 transcripts (Figure 4.4C). Critically, maintaining KChIP2 levels through use of adenovirus encoding KChIP2 (Ad.KChIP2) normalized the expression of miRs-34b/c while reversing the loss in SCN5A and SCN1B; however, KCND3 levels remained suppressed (Figure 4.4B and C). Functional evaluation on both $I_{Na}$ and $I_{to,f}$ shows significant loss in density following PE treatment (Figure 4.4D and E), reflecting the changes we see in transcript expression and mimicking ion channel remodeling observed in HF. However, Ad.KChIP2 treatment restored the current density for both currents, despite KCND3 transcript expression being unaffected by KChIP2 expression. These observations strongly implicate a role for KChIP2 in maintaining proper electrical expression during pathological remodeling in the stressed heart. Moreover, we were able to observe significant reduction of KChIP2 and elevation of miR-34b/c within failing human heart tissue compared to non-failing (Figure 4.5A). Reinforcing this conservation was the identification of a predicted DRE motif proximal to the transcriptional start site, in the human miR-34b/c promoter as evaluated by MatInspector (Figure 4.5B). At the same time, significant loss of SCN5A and KCND3 transcripts in failing tissue (Figure 4.5C) also show conservation of miR-34b/c targeting within their 3’-UTRs (Figure 4.5D). Interestingly, SCN1B does not preserve its target site in humans, however, we also
observed no significant reduction in transcript expression from failing heart tissue (Figure 4.5C). Together, this reinforces the concept of KChIP2 as a core transcriptional regulator of electrical activity under normal and pathologic conditions.

To address the specific activity of miR-34b/c in mediating these changes in ion channel expression, NRVMs and iCells® were transfected with miR-34b/c antimir molecules during the duration of PE treatment. Much like KChIP2 delivery which reduced miR-34b/c expression, directly blocking miR-34b/c activity maintained $I_{Na}$ in both rat and iCells® (Figure 4.6A and D), further implicating miR-34b/c in the direct regulation of these ion channel transcripts. However, $I_{to,total}$ density in the NRVMs did not observe the same rescue (Figure 4.6B). We believe this is once again explained by the contributions of Kv1.4 and Kv4.2, in addition to Kv4.3 in defining rodent $I_{to}$. In fact, by probing just $I_{to,f}$, we revealed a significant, but incomplete restoration following miR-34b/c block (Figure 4.6C). Notably, the same experiment conducted in iCells® where Kv4.3 is the dominant subunit, resulted in the full restoration of $I_{to}$ (Figure 4.6E). To be more certain the restoration of current density was specific to miR-34b/c targeting the underlying subunits encoding $I_{Na}$ and $I_{to}$, rather than a general rescue in the molecular state of the cell, the repolarizing current $I_{Kt}$ was assessed in iCells®. PE successfully reduced this current, which is known to be reduced by cardiac stressors, however, it was unable to be rescued by miR-34b/c block (Figure 4.6.1 – figure supplement 1). Critically, this shows that KChIP2 regulation of $I_{Na}$ and $I_{to}$ is enacted through specific targeting of miR-34b/c activity, while the use of iCells® displays mechanistic conservation in human derived cells.
Inhibition of miR-34b/c blocks arrhythmia induction

Dysregulation of $I_{Na}$ and $I_{to}$ have been previously associated with arrhythmogenesis.$^{64,135}$ Therefore, in order to test the consequence of $I_{Na}$ and $I_{to}$ loss and the involvement of miR-34b/c in regulating susceptibility to arrhythmic events, optical mapping was performed in NRVM monolayers. As before, cells were exposed to 100 μM PE for 48 hrs following treatment with either a control or miR-34b/c antimir. Using point stimulation, we submitted the monolayers to baseline pacing (S1) followed by a single premature stimulus (S2) over a range of S1-S2 coupling intervals. Immediately following S2 capture, the occurrence of rapid, non-paced activity (arrhythmia) was assessed.

Figure 7A shows representative activation maps during S1 (top) and S2 (bottom) pacing. In all conditions, activation during S1 pacing shows uniform wavefront propagation, with evidence of conduction slowing following PE + control antimirs, consistent with reduced $I_{Na}$ density. Compared to S1 pacing, propagation during S2 pacing was slower in all conditions; however, in PE + control antimirs significant impulse slowing (isochrone crowding) and block (solid line) were observed. Critically, this block was sufficient to cause sustained reentrant excitation in 5 of 7 monolayers (Figure 4.7B and C). Remarkably, PE + miR-34b/c inhibition prevented conduction block and mitigated conduction slowing, protecting all monolayers from sustained re-entry (Figure 4.7C).

To determine the electrophysiological substrate responsible for the reentrant activity observed, monolayers were evaluated for changes in APD and conduction velocity. Reflecting the changes in $I_{Na}$ and $I_{to}$ expression, exposure to PE for 48 hrs significantly prolonged APD and slowed conduction velocity compared to control dishes.
across multiple pacing cycle lengths (Figure 4.7D and E). APD prolongation from PE was unresponsive to miR-34b/c inhibition; however, this was anticipated as we previously determined $I_{to}$ is not restored by miR-34b/c block in NRVM due to the additional Kv1.4 and Kv4.2 mediated current. However, treatment with miR-34b/c antimir, which maintained $I_{Na}$ density in isolated myocytes (Figure 4.6A), produced a trend towards restoration of conduction velocity, suggesting other mechanisms of conduction slowing following PE treatment that are uninfluenced by miR-34b/c activity.

Therefore, to more precisely assess changes in cellular excitability, we determined the effective refractory period (ERP) under each condition. Reflecting the prolonged APD and reduced $I_{Na}$, PE treated cells displayed a significantly longer ERP (Figure 4.7F) than control cells. However, treatment with the miR-34 antimir significantly shortened ERP towards control. Notably, this recovery occurred in the absence of a shortened APD, suggesting a significant recovery of $I_{Na}$ excitability. Thus, even without being able to rescue $I_{to}$, we were still able to restore cellular excitability through miR-34b/c inhibition and limit the occurrence of conduction block and reentry. Overall, the observation KChIP2 can normalize electrical remodeling in a setting of myocardial stress highlights a much expanded and multimodal role in establishing the cardiac electrical state.
4.4 Discussion

This study established a novel transcriptional role for cardiac KChIP2, whereby it maintains a repressive influence over the miR-34b/c promoter. KChIP2 loss either by direct silencing or pathologic means, removes repression over miR-34b/c expression. Consequentially, reductions in transcript and protein expression for Nav1.5, Navβ1, and Kv4.3 are observed as an outcome of miR-34b/c targeting to seed regions present in the 3’-UTR of these genes, allowing KChIP2 to manipulate functional expression of a host of critical cardiac ion channel genes, ultimately acting as a key regulator of cardiac excitability and arrhythmia susceptibility.

While we evaluated a discrete pathway targeted by KChIP2 transcriptional activity, there are doubtless many other gene targets. To begin to address this discussion, a gene expression array was performed on NRVM following 48 hrs of KChIP2 silencing. Evaluation of genes that experienced at least a 2 fold change revealed an increase in expression for 293 genes and a decrease in expression for 407 genes in response to KChIP2 silencing (Supplemental Table 1). Notably, of the genes experiencing increased expression, 192 of them (65.5%) were predicted to contain a DRE motif within promoter elements, implicating the potential of KChIP2 transcriptional activity directly mediating these changes. Additionally, of the genes responding with reduced expression, 71 of them (17.4%) were predicted to contain a miR-34b/c target site within their 3’-UTR. Importantly, we see significant reduction to SCN5A and SCN1B, consistent with previous data and the mechanisms investigated here. Considerably, whether these changes are the direct consequence of KChIP2 transcriptional repression, or more indirect KChIP2 dependent mechanisms, including prolonged APD from loss in Ito density contributing to
altered Ca\textsuperscript{2+} handling, the results are still relevant to cardiac remodeling, particularly
given the associated loss of KChIP2 in cardiac disease states. Notably, a diverse range of
gene pathways were implicated in response to KChIP2 loss, including cardiovascular
signaling, regulation to G-protein coupled receptor pathways, relaxation and contraction,
TGFβ signaling, apoptotic, and NFκB dependent signaling mechanisms, all of which
have a relevance in disease remodeling (Supplemental Table 1). Importantly, these data
are supportive of our conclusion that KChIP2 is a key regulator of cardiac pathology.
While our study focused on the transcriptional pathway by which KChIP2 could exert a
concerted regulation of $I_{Na}$ and $I_{to}$, further investigations pursuing some of the targets
highlighted from this gene expression array will likely reveal a broader role for KChIP2
as a transcriptional regulator of cardiac physiology much like is seen for KChIP3
(DREAM) in the brain.

Indeed, the role of KChIP2 as a multimodal regulator of cardiac ion channels has
been an emerging topic. Recent work has identified KChIP2 regulation of Ca\textsubscript{v}1.2 through
direct interaction with an inhibitory N-terminal domain on the channel, effectively
reducing $I_{Ca,L}$ in the absence of KChIP2\textsuperscript{70,73}. Our previous work has also suggested that
KChIP2 is part of a larger macromolecular complex that includes subunits for both
Na\textsubscript{v}1.5 and Kv4 channels that lead to functional increases in both currents when
coexpressed with KChIP2\textsuperscript{75}. In the same study we also identified transcriptional changes
in SCN5A and SCN1B following acute knockdown of KChIP2 in NRVM, which provided
the motivational basis for the work presented here. Taken together, these observations
suggest a highly promiscuous nature for KChIP2.
Notably, another member of the KChIP family, KChIP3, has also been discovered
to interact with multiple membrane proteins, including regulation of Kv4 channels\textsuperscript{77, 136},
while also displaying Ca\textsuperscript{2+} regulated transcriptional repression\textsuperscript{84}. Even the role of
KChIP3 as a transcriptional repressor is multimodal, including direct binding to DRE
motifs, in addition to interacting with and suppressing the activity of the cAMP response
element-binding protein (CREB), an established transcriptional activator\textsuperscript{137}. Both of these
processes are Ca\textsuperscript{2+} regulated, due to three functional high affinity EF-hand motifs
residing in the protein\textsuperscript{84}. Occupancy of these sites upon increases in intracellular Ca\textsuperscript{2+}
lead to conformational changes that cause DNA binding release \textsuperscript{84} or dissociation from
CREB\textsuperscript{137}, causing de-repression of downstream gene targets. Given that the entirety of
the KChIP gene family displays strong conservation around these EF-hand residues,
suggests conservation of these Ca\textsuperscript{2+} regulated responses. Indeed, we observed that
caffeine stimulation produced increased activity of the miR-34b/c promoter (Figure 4.2D)
in the presence of KChIP2. Additionally, when we deleted the DRE element in the miR-
34b/c promoter, we observed an incomplete removal of suppression (Figure 4.2C).
However, as KChIP3 represses gene expression through alternative CREB dependent
regulation, the partially retained repressive activity may be attributed to this secondary
function. Further analysis of the promoter by MatInspector revealed several potential
sites of predicted CREB binding that may have allowed for partially maintained KChIP2
suppression, even in the absence of the DRE site. Given that KChIP2 and KChIP3 share a
high degree of homology only reinforces the observation of multiple activities for
KChIP2 as well.
The physiologic implications of KChIP2 targeting miR-34b/c expression is one of tremendous significance for many cardiac pathologic states. Rapid depletion of KChIP2 protein is a widespread event that underlies remodeling in many cardiac diseases, including chronic HF, MI, and atrial fibrillation\(^\text{16}\). Considerably, these diseases also present with reductions in \(I_{to}\) and \(I_{Na}\). The relationship between KChIP2 and \(I_{to}\) has been heavily studied, frequently identifying that KChIP2 loss induces the destabilization of Kv4.2/4.3 channels and mediates the decline in current density\(^\text{70}\). However, the work presented here offers the unique alternative that translation block through miRNA interaction mediates the decline in Kv4.3. Given that Kv4.2 does not contain a miR-34 target region in its 3’-UTR, but still experiences degradation following KChIP2 loss, it is likely that both mechanisms contribute to the resulting loss in \(I_{to,f}\). However, it is also observed that reduced KChIP2 expression stimulated by phenylephrine + propranolol in \textit{in vitro} cultures of NRVM experienced increased Kv4.2 protein while KChIP2 and Kv4.3 levels were reduced\(^\text{138}\), supporting the opportunity for miRNA dependent translational block targeting Kv4.3, rather than just destabilization of all Kv4 channels.

In the same settings of cardiac disease where KChIP2 is down, there are also observations of \(I_{Na}\) depletion\(^\text{139,140}\). Our data of miR-34 targeting Na\(_{v}1.5\) provides a means for describing this loss in activity. Notably, others have shown a loss in the full length transcript for Nav1.5 mRNA and a corresponding increase in a truncated isoform without the miR-34b/c target region present\(^\text{24}\), reinforcing the observations for miR-34b/c mediating the decline of \(SCN5A\). Overall, the consequential loss of both \(I_{Na}\), and \(I_{to}\), suggests KChIP2 loss during cardiac stress may be a nodal event in a cascade of gene regulation defining electrical remodeling in the stressed myocardium. Indeed, earlier
work was done that sought to determine the significance of KChIP2 in the development of hypertrophic remodeling. In a rat TAC banding model, it was observed that maintaining KChIP2 expression attenuated hypertrophy and pathogenic remodeling that otherwise lead to a worsening myocardium during pressure overload. This reverse in remodeling was attributed to changes in intracellular Ca$^{2+}$ signaling brought on by restoration of an abbreviated APD. Yet, we were able to observe that inhibition of miR-34b/c could also attenuate adverse remodeling without influencing APD (Fig 7) implicating multiple pathways of KChIP2 intervention. Indeed, the miR-34 family has recently been implicated in the development and progression of hypertrophy and heart failure, in rodent models of both MI and pressure overload. Critically, these studies, combined with our own data, show that blockade of the miR-34 family can attenuate pathologic remodeling, expanding the significance of KChIP2 and miR-34 in cardiac pathogenesis.

There are still some challenges in understanding the role of KChIP2 in the progression of hypertrophy and heart failure. Investigations conducted in KChIP2 null mice have shown that when submitted to TAC banding, there is no worsened phenotype when compared to wild type mice. In fact, arrhythmia susceptibility was lowered in the KChIP2 null mice during heart failure, believed to be the result of reduced dispersion of repolarization. At the same time, there were no observed changes to $I_{Na}$. While our current understanding is unable to account for this disparity, it may be that compensatory regulation exists in these mice as a consequence of constitutive KChIP2 absence during development, fundamentally changing its regulatory significance. Evidence for this is observed when restoring KChIP2 expression in myocytes isolated from KChIP2 null
mice, which resulted in no rescue of Kv4.2 protein expression or recovery of $I_{to, f}$. However, restoration of KChIP2 following acute loss from pathologic consequences in a rat model was able to rescue $I_{to, f}$, consistent with what we see in our own maintenance of KChIP2 following prolonged PE exposure (Fig 4E). The significance of this begins to suggest deviations in KChIP2 regulatory impact depending on acute versus constitutive loss.

Ultimately, our endpoint was to determine whether electrical dysregulation brought on by KChIP2 loss was able to influence arrhythmia susceptibility through the activity of miR-34b/c. Despite only rescuing $I_{Na}$ and not $I_{to}$ in the NRVMs, as evidenced by the shortened ERP with sustained APD prolongation (Fig 7D and F), we found this was sufficient to rescue arrhythmia induction following PE treatment (Fig 7C). Indeed, previous studies have revealed the relationship between changes in Na$^+$ channel density and arrhythmia induction. As $I_{Na}$ becomes compromised, it begins to resolve an expanding interval of premature stimuli declared the vulnerability period. Within this interval, reentry is more likely to occur as a result of non-uniform conduction block surrounding the point of excitation. Both theoretical and experimental studies show that when Na$^+$ channel availability is reduced, the vulnerable period increases. Therefore, by restoring Na$^+$ channel through miR-34b/c inhibition, we are effectively minimizing the vulnerable period and making unidirectional conduction block less likely to occur.

Care must still be taken before translating these mechanisms to the clinical setting. Our investigated pathway was developed using cultured rodent myocytes, differing from human electrophysiology in its APD and the impact of underlying
currents. We must also understand the electrical impact of miR-34 inhibition \textit{in vivo}.

However, we know from this investigation that miR-34b/c are elevated in native human HF tissue (Figure 4.5A), and that functionally, the inhibition of miR-34b/c in human derived cardiomyocytes following stress can achieve restoration of both $I_{Na}$ and $I_{to}$ (Figure 4.6C and 6D), reinforcing species dependent conservation. At the same time, conduction block due to compromised cellular excitability has long been understood to be important for clinically relevant arrhythmias\textsuperscript{147}. These observations together suggest strong therapeutic potential for targeting miR-34 in the treatment of electrical instabilities. Currently, the use of locked nucleic acids and related technologies have been used to successfully target miRNA activity \textit{in vivo}\textsuperscript{148}. While miR-34b/c is also expressed outside the heart, it is unclear what long-term consequences its inhibition will have as a therapeutic. However, these outcomes will have to be weighed against the potential therapeutic advantage it will have in alleviating cardiac events.

Overall, this newly identified KChIP2/miR-34 pathway reflects electrical remodeling observed within multiple cardiac pathologies. Moreover, the events brought on by KChIP2 loss are critical in initiating electrical instabilities and arrhythmias implicated in sudden cardiac death. The identification of KChIP2 transcriptional capacity significantly reshapes its role in cardiac biology as a core mediator of cardiac electrical activity and reveals KChIP2 and miR-34 as therapeutic targets for managing arrhythmogenesis in heart disease.
4.5 Materials and Methods

Isolation and cell culture of neonatal rat ventricular myocytes and human derived cardiomyocytes (iCells®)

Rat neonatal ventricular myocytes were isolated 1-2 days after birth as previously described. Briefly, hearts were minced in HBSS, and tissue fragments were digested overnight with trypsin at 4 °C. Trypsinized fragments were treated repeatedly for short periods of time with collagenase at 37 °C followed by trituration. Dissociated cells were pre-plated for 2 hrs at 37 °C in DMEM supplemented with 5% fetal bovine serum (FBS) and penicillin/streptomycin. NRVMs were collected and replated in DMEM/5% FBS/penicillin/streptomycin with 0.1 mM bromodeoxyuridine (BrdU) to suppress fibroblast growth and maintained at 37 °C, 5% CO2. These conditions were maintained for 24-36 hrs, after which culture conditions deviated based on application of cells.

Human-induced pluripotent stem cell (hiPSC)-derived cardiomyocytes (iCell Cardiomyocytes; Cellular Dynamics International, Madison, WI) were cultured in iCell Cardiomyocytes Maintenance Medium (Cellular Dynamics International) in an atmosphere of 93% humidified air and 7% CO2 at 37°C. For electrophysiological recordings, 20 000–40 000 cardiomyocytes were plated on glass coverslips coated with 0.1% gelatin as described.

Rat ventricular myocyte isolation

Single ventricular myocytes were isolated from adult rat hearts. Briefly, rats were anesthetized by injection of ketamin. Hearts were quickly removed and perfused via the
aorta with a physiological salt solution (PSS) containing (in mmol/L) NaCl 140, KCl 5.4, MgCl2 2.5, CaCl2 1.5, glucose 11, and HEPES 5.5 (pH 7.4). After 5 minutes, perfusate was switched to a nominally calcium-free PSS with collagenase (Roche, 0.5 mg/mL) being added after an additional 5 minutes. After 15-20 minutes of digestion, hearts were perfused with a high K+ solution containing (in mmol/L) potassium glutamate 110, KH2PO4 10, KCl 25, MgSO4 2, taurine 20, creatine 5, EGTA 0.5, glucose 20, and HEPES 5 (pH 7.4). Ventricles were minced in high K+ solution, and single myocytes were obtained by filtering through a 115-μm nylon mesh. Myocytes were then plated on laminin coated coverslips for 1.5 hrs before fixing with 4% formaldehyde in PBS to be used for immunohistochemistry. Alternatively, cells were resuspended in a 1% formaldehyde/PBS solution to be used for ChIP studies.

Transfection for KChIP2 overexpression, siRNA treatment, or miRNA-precursor and inhibitor delivery

NRVM cultures used for transfection and total RNA and protein collection were conducted on 35 mm dishes seeded with 1.5 x 10^6 cells. Following the initial 24-36 hrs of plating, NRVMs were transfected with KChIP2.3 (NM_173192.2), KChIP2.4 (NM_173193.2), or KChIP2.6 (NM_173195.2) for the overexpression of KChIP2, which was inserted into the pIRES2-EGFP plasmid from Clontech as previously conducted. The plasmid without the KChIP2 insert was used as the control. Lipofectamine 2000 reagent (Invitrogen) was used to deliver the constructs according to the manufacturer’s instructions. Following the transfection period, media was changed to DMEM/5%
FBS/penicillin/streptomycin. Cells were cultured for 72 hrs total before collection for total RNA, with a media change once after 48 hrs of culture.

Knockdown of KChIP2 was conducted by transfecting with siRNA for KChIP2 (Ambion, Cat#: 4390771, ID: s132782), or a scrambled siRNA control (Ambion, Cat#: 4390843). 180 pmol of siRNA was transfected using 15 μL of Lipofectamine 2000 reagent according to the manufacturer’s instructions. Following the transfection period, media was changed to DMEM/5% FBS/penicillin/streptomycin. Cells were cultured for 72 hrs total before collection for total RNA, with a media change once after 48 hrs of culture.

NRVM were also transfected with 180 pmol of miR-34b/c precursors (miR-34b MC12558, miR-34c MC11039, Invitrogen) or a non-targeting control (negative control 4464058, Invitrogen) using 15 μl lipofectamine RNAi Max (Invitrogen) according to the manufacturer’s instructions. Cells were left for 48-72 hrs and then collected for RNA. NRVM were also used for patch clamp recordings to measure $I_{Na}$ and $I_{to}$. These were plated at 100,000 cells/dish in 35 mm dishes and the miR-precursors were modified with an attached FAM reporter to visualize transfected cells. 25 pmol of miR-34 precursor with 2 μl Lipofectamine RNAiMax was used according to the manufacturer’s instructions.

Transfection of control or miR-34b/c antimirs were also used during the phenylephrine induction assays for evaluation with patch-clamp recordings in NRVM and iCells® and
optical mapping in NRVM only. NRVM seeded at 100,000 cells/35 mm dish for patch-clamping received 22.5 pmol of miR-34b inhibitor (Invitrogen, MH12558) with 22.5 pmol of miR-34c inhibitor (Invitrogen, MH11039) or 45 pmol of a non-targeting miR-inhibitor (Invitrogen, 4464076) using 3.75 μl Lipofectamine RNAi Max (Invitrogen). NRVM used for optical mapping were seeded at 1.5 x 10^6 cells/35 mm dish and 225 pmol each of the miR-34b and -34c inhibitor or 450 pmol of the non-targeting control miR-inhibitor were delivered using 22.5 μl of Lipofectamine RNAi Max.

miR-inhibitors were also delivered in iCells®, for cells seeded at 20,000-40,000 cells per each well of a 12-well for patch-clamp studies. 5 pmol each of the miR-34b and -34c inhibitor or 10 pmol of the non-targeting control miR-inhibitor were delivered using 1.0 μl of Lipofectamine RNAi Max.

RNA preparation, quantitative RT-PCR of miRNA/mRNA targets, miRNA array, and whole-transcriptome microarray

Total RNA was isolated from NRVM using Trizol Reagent (Invitrogen) according to the manufacturer’s instructions. RNA was also collected from human control and heart failure tissue samples. Tissue samples were first pulverized using liquid N2 and mortar and pestle to assist in the homogenization with Trizol. Subsequent RNA was used as a template for cDNA synthesis in reverse transcriptase reactions using the Multiscribe Reverse Transcriptase kit (Invitrogen) for detecting both mRNAs and miRNAs. The quantitative PCR reactions were performed with the ABI 7500 Real-Time PCR system using either SYBR green technology for coding genes or Taqman reagent for detecting
mature miRNAs. mRNAs were normalized with GAPDH or ribosomal protein 27 (RPL27) and miRNAs with small nucleolar RNA U87 or U6. All miRNA primer sets were designed and provided by Invitrogen Taqman Assays. Real-time PCR reactions were conducted using TaqMan® Universal Master Mix II (Invitrogen). miRNA primer sets for real-time PCR detection were as follows:

Rat miR-34b: Assay name, mmu-miR-34b-5p; Assay ID, 002617; Catalogue #, 4427975

Human/Rat miR-34c: Assay name, hsa-miR-34c; Assay ID, 000428; Catalogue #, 4427975

Rat U87 (housekeeping gene): Assay name, U87; Assay ID, 001712; Catalogue #, 4427975

Human miR-34b: Assay name, hsa-miR-34b; Assay ID, 000427; Catalogue #, 4427975

Human U6 (housekeeping gene): Assay name, U6 snRNA; Assay ID, 001973; Catalogue #, 4427975

Primer sets used in the detection of mRNA transcripts were designed in Primer 3 Plus and specificity to the intended target verified using Primer Blast (NCBI).

Rat Scn5a

Forward primer, 5’-TCAATGACCCAGCCAATTACCT-3’, Reverse primer, 5’-CCCGGCATCAGAGCTGTT-3’

Rat Scn1b

Forward primer, 5’-ACGTGCTCATTGTGGTGAAGC-3’, Reverse primer, 5’-CCGTGGCAGCAGCAATC-3’
Rat Kcnd3
Forward primer, 5’-GCCTTCGAGAACCCACA-3’, Reverse primer, 5’-GATCACCAGACCGCAATG-3’

Rat Kcnip2
Forward primer, 5’-ACTTTGTGGCTGGTTTGTCG-3’, Reverse primer, 5’-TGATACAGCCGTCTTTGTTGAG-3’

Rat GAPDH
Forward primer, 5’-AGTTCAACCGGCACAGTCAAG-3’, Reverse primer, 5’-ACTCCACGACATACTCAGCAC-3’

Rat Rpl27
Forward primer, 5’-GCTGTCGAAATGGGCAAGTT-3’, Reverse primer, 5’-GTCGGAGGTGCCATCATCAA-3’

Human Kcnip2
Forward primer, 5’-TGTACCGGGGCTTCAAGAAC-3’, Reverse primer, 5’-GGCATTGAAGAGAAAAGTGGCA-3’

Human Scn5a
Forward primer, 5’-CTGCGCCACTACTACTTCACCAACA-3’, Reverse primer, 5’-TCATGAGGGCAAAGAGCAGCGT-3’

Human Scn1b
Forward primer, 5’-GACCAACGCTGAGACCTTCA-3’, Reverse primer, 5’-
TCCAGCTGCAACACCTCATT-3’

Human Kcnd3

Forward primer, 5’-TCAGCACGATCCACATCCAG-3’, Reverse primer, 5’-
CTCAGTCCGTCGTCGTCTGCTTT-3

Human GAPDH

Forward primer, 5’-TCCTCTGACTTCAACAGCGA-3’, Reverse primer, 5’-
GGGTCTTACTCCTTGAGGC-3’.

RNA collected from NRVM following KChIP2 and control siRNA treatment were
submitted to miRNA microarray analysis to determine miRNAs regulated by the loss of
KChIP2. The array was performed by the Gene Expression and Genotyping Facility at
Case Western Reserve University using the Affymetrix GeneChip® miRNA 4.0 array.
The resulting .CEL files were used with ExpressionConsole to conduct RMA analysis to
derive the relative intensities of the miRNA probe set. The raw datasets are available
from the Gene Expression Omnibus (Accession GSE75806).

Additionally, RNA collected from NRVM following KChIP2 silencing with an adeno-
shRNA expression system with non-targeting (control) and KChIP2 targeting constructs
were used to assess global gene changes following KChIP2 loss. A total of 1.5 x 10^6 cells
were plated on 35 mm dishes. Cells were cultured in DMEM/5%
FBS/penicillin/streptomycin with 0.1 mM BrdU for 24 hrs. After 24 hrs, media was
replaced with fresh DMEM/5% FBS/penicillin/sptreptomycin and the corresponding control and KChIP2 shRNA virus. Cells were cultured for 48 hrs (with a media change after 24 hrs) and collected for total RNA and evaluated using a whole-transcriptome microarray. The array was performed by the Gene Expression and Genotyping Facility at Case Western Reserve University using the Affymetrix rat Clariom™ S Assay. The resulting .CEL files were used with Expression Console and the Transcriptome Analysis Console provided by Affymetrix (available here: http://www.affymetrix.com/support/technical/software_downloads.affx) to derive the relative changes in gene expression. The raw datasets are available from the Gene Expression Omnibus (Accession GSE94623)

*Design of shRNA viral construct*

The design of non-targeting (control) and KChIP2 shRNAs was conducted as described with modifications. shRNA inserts were optimally designed using a compilation of the RNAi Consortium and Invitrogen design algorithms. To begin the design, oligos were ordered that contained the shRNA sequence for control: 5’-GTTGACAGTGAGCGATCTCGCTTGGGCGAGAGTAAGTAGTGAAGCCACAGATGTACTTACTCTCGCCCAAGCGAGAGTGCCTACTGCCTC-3’ and KChIP2: 5’-GTTGACAGTGAGCGCGAGCTGGGCTTTCAACTTATATAGTGAAGCCACAGATGTATATAAGTTGAAAGCCCAGCTCATGCCTACTGCCTC-3’. These oligos were modified in a PCR reaction to add on cloning sites for insertion into the pSM2 vector using the primer set: 5’-CAGAAGGCTCGAGAAGGTATATGCTGTTGACAGTGAGCG-3’ and 5’-CAGAAGGCTCGAGAAGGTATATGCTGTTGACAGTGAGCG-3’ and 5’-
CTAAAGTAGCCCTTGAATTCCGAGGCAGTAGGCA-3’. Underlined are the XhoI and EcoRI restriction sites used for cloning. Following the insertion of this sequence into the pSM2 vector, the insertion was cloned out again using the primer set 5’-GAGCTCGCTAGCGCTACCGGTCGCCACCATGGTGAGCAAGGGCGAGG-3’ and 5’-GATTGCCAAGCTTCTAGATAAACGCATTAGTCTTCCAATTG-3’. Underlined are the NheI and HindII restriction sites, which were then used to clone the inserts into the adenovirus construct, Ad.CGI. During insertion, the GFP encoded by the viral vector was digested out as the shRNA system expression GFP in tandem with the shRNA. The modified Ad.GFP construct was transfected with the psi5 vector into CRE8 cells for the production and amplification of packaged viral constructs to then be used for silencing studies.

**Adult rat heart tissue fractionation to assess nuclear localization of KChIP2**

Fractionation of adult rat heart tissue was performed as described with slight modifications. Briefly, freshly isolated heart tissue was minced in ice cold PBS. Tissue was washed several times to remove residual blood from sample. Approximately 300 mg of tissue was weighed out and suspended in cytosolic lysis buffer, consisting of 150 mM NaCl, 50 mM HEPES (pH 7.4), 25 µg/mL Digitonin, and 10% Glycerol. Tissue pieces were homogenized then filtered through a QIAshredder homogenizer column (Qiagen, 79656). Filtered lysate was then incubated at 4°C on an end-over-end rotator for 10 min. Samples were then centrifuged at 4000 x g for 10 min at 4°C. Supernatant was collected as the cytosolic fraction. The remaining pellet was resuspended in membrane lysis buffer consisting of 150 mM NaCl, 50 mM HEPES (pH 7.4), 1% IGEPAL, and 10% glycerol.
Sample was incubated for 30 min in end-over-end rotator at 4°C, followed by centrifugation at 6000 x g for 10 min at 4°C. The supernatant was collected as the membrane associated fraction, while the remaining cell pellet was resuspended in the nuclear lysis buffer consisting of 150 mM NaCl, 50 mM HEPES (pH 7.4), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 10% glycerol. Lysate was placed on an end-over-end rotator for 10 min at 4°C, which was then followed by brief sonication. The lysate was then centrifuged at 6800 x g for 10 min at 4°C. The supernatant was collected as the nuclear fraction. Roche protease inhibitor tablets were added fresh before the addition of each lysis buffer.

**Immunoblotting**

In order to perform western blot experiments looking at KChIP2 nuclear expression, cytosolic, membrane, and nuclear extracts were isolated as described above. 20-30 μg of protein extracts were loaded into SDS-PAGE gels, transferred to nitrocellulose membranes, and western blotting performed using lactate dehydrogenase (Abcam Cat# ab52488 RRID:AB_2134961, 1:1000) to represent the cytosolic fraction, Lamin-B1 (Abcam Cat# ab16048 RRID:AB_443298, 1:1000) representing the nuclear fraction, Serca2a (1:1000, Dr. Periasamy, Ohio State University) and KChIP2 (UC Davis/NIH NeuroMab Facility Cat# 75-004 RRID:AB_2280942, 1:50) to observe localization.

Western blot performed on NRVM was conducted to assess Kv4.3 protein expression following miR-34 precursor treatment. NRVM were rinsed with PBS then scraped and
collected. Cell pellets were re-suspended in RIPA Buffer (150 mM sodium chloride, 1.0% NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulphate), 50 mM Tris, pH 8.0, plus Roche Inhibitor tablet) and then sonicated on ice to disrupt cell membranes. 30-40 μg of whole cell extract was loaded into SDS-PAGE gels, transferred to nitrocellulose membrane, and western blotting performed using Kv4.3 (UC Davis/NIH NeuroMab Facility Cat# 75-017 RRID:AB_2131966, 1:500), and actin (Sigma-Aldrich Cat# A4700 RRID:AB_476730, 1:1000).

**Immunofluorescence**

Freshly isolated adult rat ventricular myocytes were plated on laminin coated coverslips for 1.5 hrs to allow for attachment. Cells were quickly rinsed with room temperature PBS before being fixed by 4% formaldehyde in PBS for 15 min. Cells were permeabilized for 10 min in PBS + 0.03% Triton X-100 and blocked for 2 hrs in a solution of PBS, 5% normal goat serum, and 1% BSA. Cells were incubated overnight with primary antibody lactate dehydrogenase (Abcam Cat# ab52488 RRID:AB_2134961, 1:100) and KChIP2 (UC Davis/NIH NeuroMab Facility Cat# 75-004 RRID:AB_2280942, 1:50) in PBS with 2% normal goat serum and 1% BSA. Cells were rinsed 3x in PBS then incubated with secondary antibody (Alexa-568 Thermo Fisher Scientific Cat# A11036 RRID:AB_10563566 1:500 against LDH and Alexa-647 Thermo Fisher Scientific Cat# A-21236 RRID:AB_2535805 1:500 against KChIP2) in PBS with 2% normal goat serum and 1% BSA for 2 hrs at room temperature. Coverslips were mounted onto glass slides with mounting media containing DAPI. Labeled cardiomyocytes were scanned with a Leica DMI8 confocal microscope.
Reporter Assays and Designing Reporter Constructs

miR-34b/c promoter reporter assays

miR-34b/c promoter reporter assays were performed in COS-7 cells (ATCC Cat# CRL-1651, RRID:CVCL_0224). 0.4 x 10^5 cells were plated in 24-well plates. 24 hrs later cells were transfected with Polyfect transfection reagent (Qiagen) according to manufacturer’s instructions. 75 ng of either the pGL4.10 promoter-less control, the pGL4.10+miR-34b/c promoter, or the pGL4.10+500bp DRE deleted construct was transfected with 225 ng of KChIP2.3, KChIP2.4, KChIP2.6, or GFP control vectors. 5 ng of pGL4.74 renillin construct was co-transfected as a normalizing control. Cells were cultured for an additional 48 hrs before they were prepared for measuring luciferase activity normalized to renillin.

3’-UTR reporter assays

3’-UTR reporter assays were performed in HEK293 cells (ATCC Cat# CRL-1573, RRID:CVCL_0045). 0.4 x 10^5 cells were plated in 24-well plates. 24 hrs later cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cells were transfected with 50ng of the pmirGlo construct containing either the 3’-UTR for SCN5A, SCN1B, or KCND3. Reporter constructs were co-expressed with the mno-miR-34b and -34c precursors together (0.75 pmol each precursor) or the non-targeting control precursor (1.5 pmol). The cells were cultured for an additional 48 hrs, after which they were prepared for measurement of luciferase and renillin activity for normalization.
All reporter assays made use of the Promega Dual-Luciferase Reporter Assay system (Promega). Reagents and cells were prepared according to the manufacturer’s instructions. Data was collected using a Perkin-Elmer EnSpire 96-well plate reader.

**Designing miR-34b/c promoter**

The miR-34b/c promoter was cloned from chromatin isolated from rat liver. Forward (5’-GAGCTCGCTAGCTAAACGTGTTCACATTTTGTTGCC-3’) and reverse (5’-TGCCAAGCTTCAGTCCCCGGAGACCCTC-3’) primers containing NheI and HindIII restriction sites respectively, indicated by underlined regions, were used to amplify the promoter region and allow cloning into the Promega pGL4.10 promoterless luciferase vector. Deletion of the putative DRE site identified by MatInspector software (RRID:SCR_008036)\(^{134}\) was conducted using the QuickChange II Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer’s instructions using the primers listed: 5’-TTAACGGAGACGGGACCACGCGTGAG-3’ and 5’-CTCACGCCGGGTCCCGTCTCCGTTAA-3’. All plasmids were sequenced to confirm the presence and integrity of inserted elements. MatInspector is a commercially available software from the Genomatix software suite (https://www.genomatix.de/).

**Designing 3’-UTR constructs**

3’-UTRs for SCN5A, SCN1B, and KCND3 genes were also cloned from rat liver chromatin. A series of primers were used to clone genomic fragments that flanked the region of the 3’-UTR containing the miR-34b/c target site into the pmirGLO Dual-Luciferase miRNA target expression vector (Promega). Scoring and identification of the target sites was done using TargetScan 7.1 (RRID:SCR_010845)\(^{132}\) (available here:
Forward and reverse primers for *SCN5A*, with XhoI and XbaI sites underlined, were: 5’-
GCTAGCCTCGAGGCAGAGTTCCGCGTCTCTGT-3’ and 5’-
GGGGCAGCTCTCTAGAGCTTTTAATTCTGGC-3’. Forward and reverse primers for *SCN1B*, with NheI and XbaI sites underlined, were: 5’-
CTCGCTAGCTTCCCACACGCACTGCCA-3’ and 5’-
GAGTCTAGAGATGAGGCCAGAACC-3’. Forward and reverse primers for *KCND3* with the NheI and XbaI sites underlined, were: 5’-
CTCGCTAGCGGTAGGTCACCTTAGCCG-3’ and 5’-
GAGTCTAGACCAGGCACAAGTCTGCAGTA-3’. Mutagenesis was conducted on the identified miR-34b/c seed region to disrupt miRNA interaction. The following primers were used with the QuickChange II Site-Directed Mutagenesis kit. *SCN5A*: 5’-
AACATCTTTTTTCATGAACACATCAGGAGTGCTCGTGCTCTCTAAACCCTGAGC-3’, 5’-
GCTCAGGTTAAGGAGACCGACTCTGAACTGCTGATGTTCATGGAAAAAGAGTGTT-3’; *SCN1B*: 5’-GCTTCCACACGCTCGGGCAGGCCAGCCGGC-3’, 5’-
GCCGCTGGCCTGCCCGAGCGTGTGGGAAGC-3’; *KCND3* site 1: 5’-
ACCTTAGCAGGCGCCCTGAGTGCGGACCTGACCAG-3’, 5’-
CTGTGCAGGTCAGCTGCAGGTCAGGAGTGCTGAGT-3’; *KCND3* site 2: 5’-
GGACAGTAAATCCTTCTCCGTGAGTCGGAAGTACTGCAGACTTGTGCCT-3’, 5’-
AGGCACAAGTCTGACTTCCGAGTACTCAGGGAGGATTTACTGTCC-3’.

All plasmids were sequenced to confirm the presence and integrity of inserted elements.
Chromatin Immunoprecipitation

Chromatin Immunoprecipitation was performed as described with minor modifications. Briefly, freshly isolated adult rat cardiomyocytes were fixed in a 1% formaldehyde solution in PBS for 14 min and quenched with 0.125 M glycine for 5 min. Cells were treated with a 0.05% trypsin/0.02% EDTA 1x PBS solution for 8 min at 37°C to partially digest the cells aiding in removal of cytoplasmic extract and purification of nuclear extract during cell lysis steps. Trypsin was inactivated by the addition of 10% FBS, and the cell pellet was rinsed 3x in ice cold PBS. Chromatin was extracted by the treatment with several lysis buffers. Lysis buffer 1 (50 mM Hepes-KOH, pH 7.5; 140 mM NaCl; 1 mM EDTA; 10% Glycerol; 0.5% Igepal; 0.25% Triton-X) was added to the cells for 10 min with rocking, followed by 15-20 dounces with a glass teflon douncer on ice. This cell lysate fraction was discarded and the remaining cell pellet was resuspended in Lysis buffer 2 (10 mM Tris-HCl, pH 8.0; 200 mM NaCl; 1 mM EDTA; 0.5 mM EGTA) for 5 min with rocking. This was again followed by 15-20 dounces with a glass teflon douncer on ice. Lastly, remaining cell pellet was resuspended in Lysis buffer 3 (10 mM Tris-HCl, pH 8.0; 100 mM NaCl; 1 mM EDTA; 0.5 mM EGTA; 0.1% Na-Deoxycholate; 0.5% N-lauroylsarcosine). Cell suspension was split in half to be used for IgG or KChIP2 ChIP. Samples were then sheared on a BioRuptor (Diagenode, total 18 cycles, hi-power, 30 sec on/off). The sonicated chromatin was immunoprecipitated with 15 ug of antibody (either α-KChIP2 or IgG control) bound to Dynabeads (Invitrogen) followed by washing and elution. Immuno precipitate and input chromatin samples were then reverse crosslinked followed by purification of genomic DNA. Target and nontarget regions of genomic
DNA were amplified by qRT-PCR using SYBR Green. Data were analyzed by calculating the immunoprecipitated DNA enrichment normalized to a region 8kb upstream of the target site in the KChIP2-IP compared to the IgG-IP. Antibodies used in ChIP were KChIP2 (UC Davis NeuroMab 75-004) and IgG (Millipore Cat# 12-371 RRID:AB_145840) ChIP-PCR primer sequences were: miR-34b target site: forward 5’-GGTCACCTCGCCAGTAGGA-3’, reverse 5’-GGAGTCCTGCTCTCCTCCTCA-3’. miR-34b 8kb upstream: 5’-CCACCCTCTCAGTAGCTTGCA-3’, reverse 5’-CAGTGCCAGGGATAGGAAG-3’

**Phenylephrine stimulation of myocytes with Adenovirus or antimir treatment**

Phenylephrine stimulation experiments were performed to evaluate gene expression changes, functional changes in ionic current by patch-clamp technique, or conduction properties by optical mapping. RNA studies for gene expression changes were conducted in 6-well plates with 1.5 x 10^6 cells plated per well for the collection of RNA. For patch-clamp recordings, NRVMs were on coverslips coated with laminin (Sigma, L2020) inside of 35 mm dish at a density of 100,000 cells/well. For optical mapping 1.5x10^6 NRVMs were plated on aclar coverslips (Electron Microscopy Sciences) coated with fibronectin (BD Biosciences, 356008) in a 35 mm dish. Following the initial 24-36 hrs of plating, media was switched to 1:1 DMEM:F12 (without serum or BrdU) and supplemented with 1x insulin-transferrin-selenium-X (Invitrogen), 1% PS, and 142 μM Na^+ Ascorbate for an additional 24-36 hrs. After this time, treatment media was applied, consisting of the same DMEM:F12 media with supplements and 100 μM phenylephrine. At the same time, control cells without phenylephrine were transduced with adeno.GFP, while
phenylephrine treated cells received either the adeno.GFP or adeno.KChIP2.6 to restore KChIP2 expression during phenylephrine treatment. Alternatively, cells were transfected using Lipofectamine RNAi Max using manufacturer’s protocol to deliver a control or combination of miR-34b and -34c antimir. In the case of transfected cells, the transfection was performed prior to the initiation of phenylephrine treatment. Phenylephrine treatment was sustained for 48 hrs (fresh media was swapped after 24 hrs, maintaining phenylephrine treatment, but no more virus was applied). Phenylephrine studies were also performed on iCells®. iCell Cardiomyocyte Maintenance Medium was supplemented with 142 μM Na⁺ Ascorbate. iCells® were only treated with the antimirs and submitted to patch-clamp recordings with the same treatment conditions applied to the NRVM. Notably, cells used for patch clamp recordings or optical mapping were washed at least 3 times over a minimum of 20 minutes in media without phenylephrine present for washout.

**Patch Clamp Experiments**

Macroscopic $I_{Na}$ and $I_{to}$ were recorded using the whole-cell configuration of the patch clamp technique. $I_{Na}$ was recorded in the solution containing 50 mM NaCl (for NRVM) and 25 mM (for iCells®), 80 or 105 mM N-methyl D-glucamine, 5.4 mM CsCl, 1.8 mM MgCl₂, 1.8 mM CaCl₂, 10 mM glucose, 10 mM HEPES, pH 7.3. 1 μM of nisodipine was used to block L-type Ca currents. $I_{Na}$ was elicited from a holding potential of -80 mV with depolarizing voltage pulses from -60 mV to 45 mV for 16 ms. To measure $I_{to}$, cells were placed in the Tyrode's solution containing (mmol/L) NaCl 137, KCl 5.4, CaCl₂ 2.0, MgSO₄ 1.0, Glucose 10, HEPES 10, CdCl₂ 0.3, and TTX 100 mM, pH to 7.35 with
NaOH. Patch pipettes were pulled from borosilicate capillary glass and lightly fire-polished to resistance 0.9-1.5 MΩ when filled with electrode solution composed of (mmol/L) aspartic acid 120, KCl 20, MgCl₂ 2, and HEPES 5, NaCl 10, EGTA 5, Na-GTP 0.3, Phosphocreatine 14, K-ATP 4, Creatine phosphokinase 2 and brought to a pH of 7.3. $I_{\text{to, total}}$ amplitude was measured as the difference between peak current and steady-state current during a 400-ms voltage step ranging from –30 to +60 mV from a holding potential of –70 mV. Recording $I_{\text{to,f}}$ used a modified protocol to kinetically isolate the current. A 150 ms voltage step to -80 mV from a holding potential of -20 mV was used to allow recovery of $I_{\text{to,f}}$ but not $I_{\text{to,s}}$. This was followed by a 50 ms prepulse to -20 mV to eliminate $I_{\text{INa}}$. $I_{\text{to,f}}$ amplitude was then measured as the difference between peak current and steady-state current during 500-ms voltage steps ranging from -30 to +40 mV. Ionic current density (pA/pF) was calculated from the ratio of current amplitude to cell capacitance. All experiments were performed at 35°C except $I_{\text{INa}}$ (room temperature).

Low-resistance electrodes (<2 MΩ) were used, and a routine series resistance compensation was performed to values of >80% to minimize voltage clamp errors. The uncompensated Rseries was therefore <2 MΩ. Command and data acquisition were operated with an Axopatch 200B patch clamp amplifier controlled by a personal computer using a Digidata 1200 acquisition board driven by pCLAMP 7.0 software (Axon Instruments, Foster City, CA). Current densities, cell capacitance, current-voltage relationship, and conductance, were measured as previously described¹⁵⁵.

*Optical mapping studies*
Following 48 hrs of PE treatment of the NRVM, cells were prepared for optical mapping studies. Prior to recordings, NRVMs were washed twice for 10 minutes each in DMEM:F12 treatment media without PE to wash out the PE and remove any acute effects. They were then transferred to Tyrodes solution (140 NaCl, 4.56 KCl, 0.73 MgCl₂, 10 HEPES, 5.0 dextrose, 1.25 CaCl₂) containing 10 µM Di4 (Sigma, D8064) for 20 minutes. Monolayers were then washed with normal Tyrodes solution before mounting on stage adapter to maintain cells at 34-35°C. Di4 fluorescence 685/80 nm was measured using an upright microscope (MVX10, Olympus) with a cooled CCD camera (Princeton Instruments). A solid-state light source (Sola Light Engine, Lumencore) was used for dye excitation (510/80 nm) over a 16 x 12 mm field of view. Cells were paced by point stimulation at cycle lengths of 1000 ms, 750 ms, 500 ms, 350 ms and 350 ms to obtain conduction velocity and APD restitution curves. Analysis of recordings were conducted via custom software developed in Matlab (MathWorks) as described previously (PMID: 12960954). Additional Matlab custom software (Rhythm) was also used for analysis. Arrhythmia data was collected using baseline pacing (S1, 750 ms) followed by a single premature stimulus (S2) with a coupling interval beginning at 150 ms and prolonged by 10 ms until either capture of a single beat or arrhythmia ensued.

**Ethics Statement and Tissue Acquisition**

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol for tissue isolation from neonatal rat (Protocol Number: 2013-0015) was approved by the Committee on the Ethics of Animal Experiments of Case Western
Reserve University. Tissue from the left ventricular free wall of non-failing and failing human heart samples were acquired from the Cleveland Clinic Foundation (CCF) tissue repository. All protocols were approved by the CCF Institutional Review Board (IRB# 2378). Samples were received coded and no identifying metrics were documented for the study.

Statistical testing

Results are expressed as mean ± SEM and represent data from at least three independent experiments. Statistical analysis for continuous data was performed using a two-tailed Student’s t-test. When multiple comparisons were evaluated, a Bonferroni correction was performed. The null hypothesis was rejected if $P < 0.05$. Statistical testing of non-continuous data, as seen with arrhythmia susceptibility measurements, was performed using the Mann-Whitney Test. Evaluation of samples sizes were initially performed using stringent conditions for expected molecular and functional changes. Assuming as a little as a 20% change in control to treated conditions, an error rate of 10%, and a power of 0.8 at a threshold of 0.05, provided a sample size of 4 per experimental condition. However, because of anticipated variability from the use of primary cells for many of the experiments and multiple comparisons in some datasets, larger sample sizes were used.

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### 4.6 Figures

**Figure 4.1: miR-34 regulation linked to changes in KChIP2 expression.** (A) Results of miRNA microarray showing the log2 of the fold changes in miR expression following 72 hrs of KChIP2 siRNA treatment. Arrow identifies miR-34b and -34c amongst the panel of altered miRNAs. Analysis of miRNAs for mRNA targets using TargetScan 7.1 was restricted to those above 2 fold induction (dashed line) (B) Tables showing the list of those miRNAs showing at least a 2 fold increase or decrease following KChIP2 silencing. (C) Alignment of the 3'-UTR of SCN5A, SCN1B, and KCND3 genes with miRs-34b/c from rat, showing hybridization of the seed region. Grayed letters indicate variation in sequence between miR-34b and -34c. A single site of interaction is indicated for SCN5A and SCN1B while two sites exist for KCND3. (D) Real-time qPCR analysis showing
percent change of miR-34b/c expression from control cells in NRVM transfected with KChIP2.3 (n = 5), KChIP2.6 (n = 6), KChIP2.4 (n = 4), or KChIP2 siRNA (n = 4-5). (E)

Cytosolic, membrane, and nuclear fractions of native adult rat heart tissue. KChIP2 nuclear localization was assessed by using lactate dehydrogenase (LDH), Serca2a, and Lamin-B as cytoplasmic, membrane, and nuclear markers respectively. (F)

Representative z-stack images of adult rat ventricular myocyte. Nuclear stained regions (DAPI, blue) show the absence of cytosolic protein LDH (green), while KChIP2 (red) staining reveals significant colocalization. Data presented as mean ± SEM. *P < 0.05; **P < 0.01, compared to control.
Figure 4.2: KChIP2 represses miR-34b/c expression by direct interaction with a putative DRE motif in promoter. (A) A region from -500 to -191 of the miR-34b/c promoter was cloned into the promoterless luciferase construct, pGL4.10. This construct was co-transfected into COS-7 cells in the presence of KChIP2.3 (n = 3), KChIP2.6 (n = 8), or KChIP2.3 (n = 3) and compared to GFP alone. Renillin (pGL4.74) was used as a normalization control. Results are depicted as a % change in activity compared to GFP alone. (B) IgG and KChIP2 ChIP-PCR conducted on native adult rat cardiomyocytes. The target primer site residing within the cloned promoter was evaluated for enrichment following pull down (n = 3), showing significant enrichment of the target region. (C) Luciferase assay conducted in COS-7 cells to evaluate the outcome of deleting the
putative DRE site in the miR-34b/c promoter. COS-7 cells were transfected with the same WT reporter construct inserted into the pGL4.10 vector or with the DRE motif deleted, both in the presence of KChIP2.6. Activity was normalized to renillin (pGL4.74). Deletion of a putative KChIP2 interaction site (DRE motif) partially abolished the repressive effect KChIP2.6 had over the miR-34b/c promoter (n = 4) compared to WT (n = 9). (D) COS-7 cells transfected with KChIP2.6 and the pGL4.10 containing the WT miR-34b/c promoter were treated with or without 10 mM caffeine for 6 hrs, leading to promoter activation (n = 4). Results were normalized to renillin activity. Data presented as mean ± SEM. *P < 0.05; **P < 0.01, as indicated or compared to control.
**Figure 4.3:** Cardiac ion channel directly regulated by miR-34a/b/c through interaction with their 3’-UTR. (A) NRVM over-expressing precursors for miR-34b/c were collected for mRNA transcript levels. Results (normalized to non-targeting miR) show down-regulation of SCN5A, and SCN1B, but unchanged levels for KCND3 (n = 7-8). (B) Protein levels from NRVM with over-expressed miR-34b showing reduced protein expression for Kv4.3 (KCND3). Multiple bands for Kv4.3 represent different glycosylation states of the protein. (C) Summary data of the immunoblot (n = 4). (D) Alignment of the 3’-UTR of SCN5A, SCN1B, and KCND3 genes with miRs-34b/c, with
mutations made to the seed regions (highlighted in red) to disrupt interaction at the seed region. (E) Reporter assay with the 3’-UTR cloned into pmiRGlo reporter construct. Luciferase activity in HEK cells transfected with WT or mutant 3’-UTRs. Results are presented as a percent change from a non-targeting miR precursor (n = 5) normalized to renillin activity. (F) I/V curves for $I_{Na}$ measured in NRVM over-expressing precursors for control (n = 24), miR-34b (n = 24), or miR-34c (n = 18). (G) I/V curves for $I_{I0,_total}$ measured in NRVM over-expressing precursors for control (n = 15), miR-34b (n = 12), or miR-34c (n = 11). (H) $I_{I0,f}$ was also assessed in NRVM through kinetic subtraction of $I_{I0,s}$. Resulting I/V curves now reveal a significant reduction in current density in miR-34b (n = 14) and miR-34c (n = 15) precursor treated cells compared to control (n = 16). (I) The same experiments conducted in human derived cardiomyocytes (iCells®) expressing miR-34b/c together, measuring $I_{Na}$ (control, n = 24; miR-34b/c, n = 21) and (J) $I_{I0, total}$ (control, n = 24; miR-34b/c, n = 25). Data presented as mean ± SEM. *$P < 0.05$; **$P < 0.01$, as indicated or compared to control. See also Figure 3 – figure supplement 1.
Figure 4.3.1 – figure supplement 1: Kv4.2 (kcnd2) expression in NRVM following expression of miR-34b/c precursor. RT-qPCR detection of Kv4.2 (kcnd2) following over-expression of miR-34b/c precursors expressed in NRVM. Results reflect fold changes relative to a control miR-precursor (n = 7). While the elevation in kcnd2 following miR-34b/c over-expression is not significant, a strongly trended elevation in suggests compensatory upregulation of Kv4.2, contributing to the minimal loss of $I_{to}$ in NRVM, despite significant Kv4.3 loss. Data presented as mean ± SEM.
Figure 4.4: In vitro cardiac disease signaling links KChIP2 loss with miR-34 elevation. (A) Real-time qPCR evaluation of relative kcnip2 following treatment with 100 μM PE for 48 hrs in NRVM (n = 6). Results normalized to ribosomal protein RPL27. (B) Evaluation of miR-34b (n = 8) and miR-34c (n = 7) relative expression in NRVM under control (no PE with Ad.GFP), 100 μM PE with Ad.GFP, or 100 μM PE with Ad.KChIP2 to maintain KChIP2 expression during the 48 hr treatment. Expression levels were normalized to small nucleolar RNA, U87. (C) The same treatment conditions in (B), evaluating relative mRNA expression for SCN5A (n = 10), SCN1B (n = 10), and KCND3 (n = 7). (D) Functional current-voltage measurements of INa from NRVM under control (n = 29), PE+Ad.GFP (n = 27), and PE+Ad.KChIP2 (n = 30). (E) I/V curve for Ito,f recordings in control (n = 7), PE+Ad.GFP (n = 9) and PE+Ad.KChIP2 (n = 9). Data presented as mean ± SEM. *P < 0.05, **P < 0.01, as indicated or compared to control, #P < 0.05, compared to PE+Ad.GFP.
Figure 4.5: Preservation of the KChIP2/miR-34b/c axis in human heart failure. (A) Human tissue taken from the left ventricle of non-failing (NF) (n = 8) and failing patients (n = 20) evaluating KChIP2 and miR-34b/c RNA expression. KChIP2 levels were normalized to GAPDH and miR expression to small nucleolar RNA U6. (B) Evaluation of the human miR-34b/c reveals a conserved DRE motif in proximity of the miR-34b stem loop (-242 bp), as predicted by MatInspector, suggesting conservation of KChIP2 activity in the regulation of miR-34b/c expression. (C) Human heart failure tissue evaluating RNA levels for SCN5A, SCN1B, and KCND3. Significant reductions in heart failure samples (n = 20) were observed for SCN5A and KCND3, but not for SCN1B, compared to non-failing tissue (n = 8). (D) Alignment of the 3'-UTR of SCN5A, SCN1B, and KCND3 genes with miRs-34b/c from human. Grayed letters indicate variation in sequence between miR-34b and -34c. A single site of interaction is indicated for SCN5A, matching observations in the rat, while KCND3 has 3 potential sites, compared to 2 observed in the rat. Notably, SCN1B miR-34b/c targeting is not conserved in human
shown by imperfect hybridization in the seed region. Data presented as mean ± SEM. \( *P < 0.05; **P < 0.01 \), as indicated or compared to control.
Figure 4.6: miR-34 block reverses loss of both $I_{\text{Na}}$ and $I_{\text{to}}$ in disease signaling. (A) $I_{\text{Na}}$ I/V curve measured in NRVM transfected with either non-targeting antimirs (control, n = 26), non-targeting miR + 100 μM PE (PE+control antimir, n = 20), or miR-34b/c antimirs + 100 μM PE (PE+miR-34 antimir, n = 21) for 48 hrs. (B) $I_{\text{to, total}}$ I/V measurements in NRVM showing current density is lost in PE+control (n = 16) and remains down in the PE+miR-34 antimir (n = 16), compared to control (n = 17) cells. (C) $I_{\text{to, f}}$ I/V measurements in NRVM. Cells treated with PE+control antimir (n = 22) have reduced current density, that is now partially restored in the PE+miR-34 antimir (n = 23) cells compared to control (n = 27). (D) I/V curve for $I_{\text{Na}}$ taken in iCells® showing that miR-34 antimirs (n = 6) can rescue current density back toward control (n = 6), when compared to PE+control (n = 6). (D) I/V curve for $I_{\text{to, total}}$ measurements in iCells® showing miR-34b/c antimir in the presence of PE (n = 15) can rescue current density towards control (n = 15) while PE+control (n = 15) remains reduced. Data presented as mean ± SEM. *$P < 0.05$ versus control, **$P < 0.01$, as indicated or compared to control antimir, # $P < 0.05$, ## $P < 0.01$ compared to PE+control antimir. See also Figure 6 – figure supplement 1.
Figure 4.6.1 – figure supplement 1: $I_{Kr}$ is insensitive to miR-34 block following PE stimulation. (A) $I_{Kr}$ I/V curve measured in iCells® transfected with either non-targeting antimers (control, n = 6), non-targeting miR + 100 μM PE (PE+control antimir, n = 6), or miR-34b/c antimers + 100 μM PE (PE+miR-34 antimir, n = 6) for 48 hrs. Lack of restoration suggests specificity of miR-34b/c targeting to specific ion channel transcripts. Data presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$, versus control.
Figure 4.7: miR-34 block retains excitability in NRVM monolayers following prolonged PE treatment. (A) Isochronal conduction maps of monolayers submitted to PE (100 μM) with either a non-targeting control or miR-34b/c antimir. Conduction maps on the top row represent the final S1 (750 ms) preceding the S2, showing no pre-existing abnormalities in propagation. The square function represents the site of pacing. The second row shows the first incidence of capture of the premature stimulus (S2). PE + control antimir results in significant conduction block around the pacing site (solid line). Conduction block was minimal in control and PE + miR-34b/c antimir groups. (B) Conduction map showing an example of sustained reentry for the PE + control antimir treated group shown in (A). (C) Summary data for the occurrence of sustained reentry
following S1S2 pacing. (D) Restitution curve of APD$_{80}$ in paced NRVM monolayers treated with either control antimir (n = 6-8), PE + control antimir (n = 6-11), or PE + miR-34b/c antimir (n = 7-12). (E) Conduction velocity restitution curve in paced NRVM monolayers treated with either control antimir (n = 6-8), PE + control antimir (n = 6-11), or PE + miR-34b/c antimir (n = 17-12). (F) Measurement of the effected refractory interval evaluated by identifying the shortest premature stimulus that would elicit capture or arrhythmia induction, under control (n = 6), PE+control antimir (n = 13), and PE+miR-34b/c antimir (n = 12). Data presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$, as indicated or compared to control antimir.
Chapter 5

Discussion and Future Direction
5.1 KChIP2 is more than an ion channel auxiliary subunit

Through the course of these investigations, we were able to identify a significantly expanded importance of the accessory subunit, KChIP2 in the heart, that not only redefines the limitations of its actions, but implicates the involvement of these mechanisms in the functional remodeling observed in cardiac disease development. Notably, these alternative roles were incentivized by previous discoveries for KChIP3 in tissues outside the heart. Given the highly conserved sequence homology of the KChIP family members, coupled with the observation that KChIP3/calsenilin/DREAM is capable of sustaining multiple functions simultaneously, suggested the preservation of multiple roles for KChIP2 as well. Such a hypothesis was further driven by the understanding investigated in the first manuscript (chapter 2), showing that KChIP2 expression is preserved in the guinea pig myocardium, where Kv4 and $I_w$ are absent, implicating the potential significance of alternative, unknown roles for KChIP2.

Ultimately, these investigations in the guinea pig following acute KChIP2 silencing showed a significant prolongation of the cardiac AP, which was the consequence of increased Cav1.2 protein, thereby enhancing depolarizing current during the plateau phase. In spite of what would have been considered an ionotropic effect on the heart, we actually saw compromised cardiac contractility secondary to suppressed Ca$^{2+}$ transients. While the expression of the classic Ca$^{2+}$ handling machinery was preserved, we observed a significant loss in RyR activity, accounting for the attenuated Ca$^{2+}$ transients. This effect appeared to be the result of the redistribution of the protein presenilin 1, a known enhancer of RyR activity$^{81-83}$, away from RyR localized regions of the myocyte. Notably, these observations are reflective of the established interaction of KChIP3/calsenilin with presenilin 1$^{77, 115}$. However, while previous work detailed the
ability of KChIP3 to modulate the effect presenilin 1 has over RyR, our data expands this relationship to also include KChIP2 and further details its mode of action to include the destabilization of normal presenilin 1 localization.

In addition to understanding the contributions of KChIP2 in the guinea pig, previous work had also revealed that acute KChIP2 silencing in neonatal rat ventricular myocytes led to transcriptional changes in the genes SCN5A/Nav1.5, SCN1B/Navβ1, and preserved mRNA but protein loss of KCND3/Kv4.375. Again, drawing inspiration from the established multimodal roles of KChIP3, or DREAM, we now sought to evaluate if a transcriptional capacity existed for KChIP2 that could explain these changes. Given the pattern of both mRNA degradation (for SCN5A and SCN1B) coupled with mRNA preservation and potential translational inhibition (for KCND3), we focused on the involvement of altered miRNA expression. As a result of these studies, we identified that indeed, KChIP2 maintained a transcriptional capacity that induced the repression of the miRNAs, miR-34b and -34c, consistent with the role of KChIP3 as a transcriptional repressor. The loss in KChIP2 expression therefore led to the activation of the shared promoter for miR-34b/c, resulting in enhanced levels of both miRNAs. These miRNAs subsequently targeted the ion channel genes described above, suppressing their expression and functional current densities. Investigations of this pathway under cardiac stress revealed further that either restoring KChIP2 or blocking miR-34b/c could rescue the losses in both \( I_{Na} \) and \( I_{to} \) from rat and human derived cells. Moreover, the regulation of these currents under the same stressors were sufficient to induce arrhythmias in myocyte monolayers, which could then be entirely suppressed by the blockade of miR-34b/c.
5.2 Phenotypic changes following KChIP2 loss recapitulate adverse reprogramming observed in HF

Most inspiringly, is that through the course of these investigations the observed phenotypes following acute KChIP2 loss recapitulate numerous states that have been well-established in cardiac disease remodeling. We saw significantly prolonged action potentials, a defining feature in the electrophysiology of HF\textsuperscript{125}, in both our guinea pig and rat studies as a consequence of reducing KChIP2 in the absence of disease signaling. While this was an expected phenotype in the rodent where $I_{\text{to}}$ is the primary repolarizing current\textsuperscript{157}, this was unanticipated and novel in the guinea pig where there is no encoded $I_{\text{to}}$.\textsuperscript{98} Notably, in larger mammals such as canine and human, where $I_{\text{to}}$ does contribute to the AP\textsuperscript{107}, its effect on terminal repolarization occurs indirectly by establishing the potential at which $I_{\text{Ca,L}}$ is activated. What this means is that decreases in $I_{\text{to}}$ have been shown to produce shortened APDs by affecting the driving force for Ca\textsuperscript{2+} entry and altering Cav1.2 open probability. Yet, reductions in KChIP2 and $I_{\text{to}}$ are some of the most reproducible observations in HF development, just as is APD prolongation\textsuperscript{2, 46, 125, 158}. The mechanism investigated in the guinea pig offers a novel mode of reconciling these changes happening simultaneously, with the common element being loss in KChIP2. Moreover, despite the increase in Ca\textsuperscript{2+} delivery, we also observed compromised contractility. Undeniably, cardiac decompensation and a continually worsening state of ejection fraction contribute to the overall morbidity of HF. It therefore becomes interesting to speculate from these studies just how much of that remodeling is contingent on just KChIP2 loss alone.
In addition to compromised cardiac performance, we also see influence on the underlying currents that contribute to the cardiac AP; specifically losses in $I_{Na}$ and $I_{to}$. As discussed previously, these currents are also well-known to experience reductions throughout the course of HF, and the implication of KChIP2 through miR-34b/c in enacting these changes offers significant insight and therapeutic potential. Indeed, reductions in $I_{Na}$ are relevant to arrhythmogenic potential in several ways. Most directly, $I_{Na}$ reflects the extent of cellular excitability, one of the parameters that contributes to conduction velocity. With a loss in $I_{Na}$, the rate of action potential firing is slowed and the propagation of signal from one electrically coupled myocyte to the next is likewise reduced. Critically, slowed conduction represents a powerful substrate in the development of re-entrant arrhythmias, particularly when paired with conduction block, by allowing for the sustainment of the re-entrant circuit.

At the same time, reduced $I_{Na}$ can also be a source of re-entrant initiation by reducing the amount of “excitation reserve” which becomes relevant during the relative refractory period. During this window, a depolarizing pulse of enough strength can re-elicit an AP before the cell restores its resting membrane potential. Premature stimuli from events such as an EAD or DAD under normal conditions might lead to uniform capture of the extra stimultus and result in a single premature ventricular contraction. However, the probability of achieving uniform capture with compromised $I_{Na}$ produces a more heterogeneous environment and therefore is more likely to promote unidirectional conduction block, leading to the formation of a re-entrant circuit.

Ultimately, what we have here are mechanisms associating the loss in KChIP2 with parameters that define both the morbidity (decline in cardiac output) and mortality
(increased arrhythmic risk) related to SCD from lethal ventricular arrhythmias, which are paramount in the phenotypic changes found in patients with HF. This combined with the observation that KChIP2 loss occurs early in the trajectory of cardiac disease remodeling, highlights KChIP2 loss as an extremely potent source in driving the adverse remodeling that defines a large percentage of the traits in cardiac disease.

5.3 Additional findings correlating KChIP2 loss with disease development

While these findings are recent, others have previously looked to KChIP2 loss as a potential driver of adverse remodeling. In a rat model of early cardiac hypertrophy induced by pressure overload from aortic constriction, the animals were treated with gene therapy delivering a KChIP2 construct in order to preserve its expression. As a consequence, the authors showed a significantly attenuated state of hypertrophy which they associated with a reduction in intracellular Ca\textsuperscript{2+} concentration provided by a shortened APD from sustained $I_{\text{to}}$. This led to the prevention in activation of both MAP kinase as well as calcineurin and nuclear factor of activated T-cell (NFAT) pathways, both of which are heavily implicated in driving the gene changes that promote hypertrophic remodeling. Interestingly, MAP kinase activation has also been shown to target the promoter of KChIP2 and silence its expression in response to cardiac stress. Similarly, other pathways activated with stresses on the heart including notch signaling and $\alpha_1$-adrenergic receptor activation of the transcription factor nuclear factor (NF)-κB lead to specific targeting of the KChIP2 promoter. Moreover, histone methylation, a signature of gene activation on chromatin, has been shown to be lost at specific loci following cardiac stress, with one such residue targeting the promoter for KChIP2.
Notably, the resulting loss in KChIP2 expression associated with a reduction not only in $I_{to}$, but also $I_{Na}$, as well as an increase in $I_{Ca,L}$ density. Encouragingly, these phenotypic changes recapitulate the mechanistic responses to KChIP2 loss we observe across our studies\cite{163}. Therefore, while gene therapy may not be a viable technique for translational therapeutics, pharmacological targeting of these pathways to preserve KChIP2 expression may offer an alternative means of capturing the mechanistic benefits provided by KChIP2.

5.4 Potency of miR-34b/c signaling and translational therapeutic potential

Through our investigations, however, we see that it may not be necessary to achieve KChIP2 preservation to capture its therapeutic potential. We show that blocking miR-34b/c activity could similarly rescue $I_{Na}$ and $I_{to}$, particularly in human derived cells where Kv4.3 is the predominant channel\cite{103,164-166}. Most interesting as well, was that miR-34b/c block did not rescue APD prolongation following PE treatment, yet evaluation of NRVM hypertrophy assessed by the change in capacitance of the cells during patch-clamp, revealed an attenuated state of cellular hypertrophy (data not shown). This suggests that APD influences over Ca$^{2+}$ activated pathways may not be the primary mechanism driving hypertrophy and may instead be associated with yet unidentified targets of miR-34b/c action. This concept is reinforced by recent investigations in a myocardial infarction and pressure overload mouse model of HF, where miR-34b/c blockade was utilized *in vivo*, leading to the reduction in total heart hypertrophy, improved systolic function, reduced cardiac fibrosis, and increased angiogenesis, amongst other positive physiologic metrics in line with improved heart outcomes\cite{141}. It is
believed many of these outcomes originate from a reduction in myocyte apoptosis, eliminating significantly the amount of inflammatory stress and fibrotic replacement that contributes to additive pathologic strain. Indeed, miR-34b/c was initially implicated in cancer biology, where miR-34b/c was shut off in tumor cells, promoting their survival\textsuperscript{167}. Clearly, there is a dramatic breadth of influence for both KChIP2 and miR-34b/c, supporting the dramatic phenotypic changes they have so far been shown to mediate.

Notably, recent advances in the understanding of miRNA biology and their involvement in disease pathways have provided some insight into the regulation of ion channel and the functional regulations that lead to arrhythmogenesis. Importantly, miRNAs are small non-coding RNAs approximately 22 nucleotides in length that regulate expression of target genes through sequence-specific hybridization to the 3’ untranslated region of coding mRNAs which results in either the degradation of the target mRNA or the translational inhibition of ribosomal complexes, both leading to the compromised expression of the target gene\textsuperscript{168, 169}. Because of this mode of action, a single miRNA can target numerous mRNAs, providing a wide breadth of regulatory complexity to gene expression programs. miRNAs themselves can be expressed as discrete genes under regulation of their own promoter elements, or alternatively can be processed from intronic sequences of coding genes, therefore having patterns of expression related to the processing of the given gene. Regarding this, miR-1 is one particular cardiac specific miRNA shown to be elevated in the diseased heart which has been identified to contribute to decreases in $I_{K1}$, $I_{Ca,L}$, as well as RyR2 through the targeting of the underlying subunits\textsuperscript{170, 171}. The inhibition of miR-1 activity through delivery of molecular mimics to the 3’-UTR targeting attenuated these remodeling events and relieved
ventricular arrhythmia susceptibility in the mice investigated for this mechanism, representing an alternative strategy with the potential to target a diverse range of pathways relevant to disease.

The attractiveness in utilizing antimir therapy is that delivery of miR blocking reagents offers a significantly more accessible therapeutic opportunity. These molecules can be delivered by simple subcutaneous injections and are small enough (12-16 nucleotides in length) to be taken up passively into the cell\textsuperscript{172}. Modifications to nucleotide chemistry allow these molecules to escape endogenous nuclease activity resulting in significantly extended half-lives, as well as highly stable thermodynamic binding to miRNA targets. Notably, this technology has been used in the treatment in a range of diseases\textsuperscript{173}, including current Phase II clinical trials in the treatment of Hepatitis C infection\textsuperscript{174}, showing their potency and viability.

5.5 Limitations and Future Directions

The most significant limitation of our current set of investigations is that all experiments were carried out \textit{in vitro}. However, given that the pathways and novel KChIP2 mechanisms we have investigated are potent contributors to both cardiac performance and electrical stability, it is most logical the next steps of investigation would take us to the \textit{in vivo} characterization of KChIP2 expression in animal models of HF progression. The challenge, however, lies in deciding which particular animal model to take advantage of for identifying the full potential of KChIP2 actions, as well as addressing the greatest translatability of its effects. As such, the guinea pig is not appropriate for its lack of $I_{to}$. Similarly, investigations in rodent models are also not ideal
given that $I_{to}$ contributes too much in defining the cardiac AP. At the same time, for reasons discussed prior, $I_{to, total}$ is comprised of the three underlying subunits Kv4.2, Kv4.3, and Kv1.4, where miR-34b/c targets only Kv4.3. For mice and rats, Kv4.2 represents the majority of the $I_{to}$ encoding subunits, reflected in our inability to modulate $I_{to, f}$ or APD when blocking miR-34b/c activity following myocyte stress. Alternatively, rabbit HF models have been used given their AP is much more reflective of human. However, investigations into the underlying components of $I_{to}$ have also shown Kv4.2 to be the dominant contributor. Lastly, we consider assessment of the KChIP2/miR-34b/c axis in a canine model of HF, which ultimately represents the closest reflection of human electrophysiology both in AP waveform, regional heterogeneities of underlying currents, and most importantly, dependence of Kv4.3 in defining its $I_{to}$. Ultimately, we would pursue the therapeutic potential of both maintaining KChIP2 expression with gene therapy or blocking miR-34b/c activity with a class of miR inhibitors known as LNA-antimirs. We would subsequently collect data regarding alterations to the underlying currents of the cardiac AP, with emphasis placed on $I_{Na}$ and $I_{to}$, as well as in vivo and ex vivo (optical mapping) arrhythmia susceptibility. Notably parameters in HF investigations such as changes in ejection fraction and cardiac hypertrophy will also be evaluated, particularly given the history of miR-34b/c blockade attenuating hypertrophic remodeling. It will be especially discerning as well to identify therapeutic differences in restoring KChIP2 versus just the block of miR-34b/c. While miR block represents a significantly more accessible translatable therapy, it is unlikely miR-34 would be the limit of KChIP2 contributions, when considering its potential effect on presenilin or even other transcriptional targets likely to be relevant in cardiac remodeling.
Another major avenue of continued investigation for KChIP2 would be a more comprehensive analysis of the genomic regions of KChIP2 interaction, accomplished through the method of ChIP-seq. This technique makes use of chromatin immunoprecipitation followed by next-generation high throughput sequencing which allows for the indiscriminate identification of all genomic fragments with which KChIP2 interacts. This contrasts with our previous analysis of the miR-34 promoter which evaluated KChIP2 binding through the use of a discrete primer set that was designed specifically for a region of interest. However, the potential gain in vast amounts of information comes at the cost of requiring more rigorous methodologies to achieve a successful experiment. Most notably, the experiment requires the use of a ChIP-grade antibody for performing the immunoprecipitation, which at this time does not exist for KChIP2. This challenge, however, can be overcome by making use of protein fusion tags that have high molecular stringency and binding affinity, allowing for specific pull-downs that yield highly enriched chromatin required for the downstream molecular processing. Ultimately, this would provide a collective list of genes which would be suggested to contain KChIP2 regulatory elements in their promoter. This list could subsequently be paired with gene array profiles collected from HF tissue to more fully understand the full spectrum of KChIP2 intervention in disease progression.

Lastly, a point of concerted interest in more fully understanding the mechanisms of KChIP2. Particularly, the delineation of how a single protein can contribute to such a diverse array of functions. It is well-established that KChIP2 contributes to the interactions with and biophysical modulation of Kv4 channels, but through our investigation we also see KChIP2 as capable of influencing presenilin localization, and a
capacity to bind DNA and enact transcriptional repression. Each of these actions requires a different set of localization parameters, and presumably a different set of binding profiles to satisfy its interactions. As was previously stated, KChIP2, as well as KChIP3, which also maintains multimodal functions, contain splice variants that yield a variable N-terminal region. Analysis of the protein sequence within these sites has shown the presence of myristoylation and palmitoylation residues which have been shown to affect the extent of membrane localization of KChIP2 protein. For example, these modifications are frequently observed for G protein coupled receptors to tether the underlying subunits to the inner service of the plasma membrane. Predisposing KChIP2 to precise patterns of localization would therefore represent a method by which a cell could establish boundaries of KChIP2 function simultaneously within a cell. However, we showed that three isoforms for KChIP2, with representative expression of these post-translational motifs, were equally capable of repressing miR-34b/c, suggesting separation of KChIP2 functions are not as simple as this. However, the ability of these sites to be modified does not necessarily indicate they are modified. It would be much more valuable instead to isolate membrane, cytosolic, and nuclear fractions and subsequently perform mass spec to evaluate the relative enrichment of the various KChIP2 splice variants residing in each of these regions. Utilization of splice variants specific antibodies could also provide visualization of these localization differences, however, such antibodies would first have to be made.

Alternative to the proposal that localization is a fixed event, other studies have shown that the relocalization of KChIPs from membrane-associated regions, to diffuse patterns of nuclear and cytosolic expression, is an inducible event. The inducibility is
most often associated with the sustained elevation in intracellular Ca^{2+} and could be probed further by introducing mutations to the EF-hands of the KChIP2 protein, rendering them Ca^{2+} insensitive. It would then be interesting to observe if this affects the potential induction of this relocalization. KChIP2 constructs creating protein fusions to fluorescent reporter tags would have to made to visualize the events in real-time. Additional follow up studies could then be used for native KChIP2 staining to observe such inductions on endogenous levels of KChIP2. Such a comprehensive study would allow for predictive understanding in the actions and regulations governing KChIP2 dependent mechanism and may even provide further insight into acute signaling during the course of HF remodeling.
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