Ankyrin-G Coordinates Intercalated Disc Signaling Platform to Regulate Cardiac Excitability In Vivo

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Abstract

**Rationale**—Na\textsubscript{v}1.5 (SCN5A) is the primary cardiac voltage-gated Na\textsubscript{v} channel. Na\textsubscript{v}1.5 is critical for cardiac excitability and conduction, and human SCN5A mutations cause sinus node dysfunction, atrial fibrillation, conductional abnormalities, and ventricular arrhythmias. Further, defects in Na\textsubscript{v}1.5 regulation are linked with malignant arrhythmias associated with human heart failure. Consequently, therapies to target select Na\textsubscript{v}1.5 properties have remained at the forefront of cardiovascular medicine. However, despite years of investigation, the fundamental pathways governing Na\textsubscript{v}1.5 membrane targeting, assembly, and regulation are still largely undefined.

**Objective**—Define the in vivo mechanisms underlying Na\textsubscript{v}1.5 membrane regulation.

**Methods and Results**—Here, we define the molecular basis of a Na\textsubscript{v} channel regulatory platform in heart. Using new cardiac-selective ankyrin-G\textsuperscript{−/−} mice (cKO), we report that ankyrin-G targets Na\textsubscript{v}1.5, and its regulatory protein, calcium/calmodulin-dependent kinase II (CaMKII) to the intercalated disc. Mechanistically, β\textsubscript{IV}-spectrin is requisite for ankyrin-dependent targeting of CaMKII\textsubscript{δ}, however β\textsubscript{IV}-spectrin is not essential for ankyrin-G expression. Ankyrin-G cKO myocytes display decreased Na\textsubscript{v}1.5 expression/membrane localization, and reduced \(I_{Na}\) associated with pronounced bradycardia, conduction abnormalities, and ventricular arrhythmia in response to Na\textsubscript{v} channel antagonists. Moreover, we report that ankyrin-G links Na\textsubscript{v} channels with broader...
intercalated disc signaling/structural nodes, as ankyrin-G loss results in reorganization of plakophilin-2 and lethal arrhythmias in response to beta-adrenergic stimulation.

**Conclusions**—Our findings provide the first in vivo data for the molecular pathway required for intercalated disc Na\textsubscript{v}1.5 targeting/regulation in heart. Further, these new data identify the basis of an in vivo cellular platform critical for membrane recruitment and regulation of Na\textsubscript{v}1.5.

**Keywords**

Nav1.5; protein trafficking; targeting; cell biology; ankyrin; sodium channels; cytoskeletal dynamics; mouse mutant; arrhythmia (mechanisms)

**INTRODUCTION**

SCN5A-encoded Na\textsubscript{v}1.5 is the principal voltage-gated Na\textsubscript{v} channel in heart. Na\textsubscript{v}1.5 regulates the rapid upstroke of the cardiac action potential, and human SCN5A mutations are linked with multiple forms of human cardiovascular disease including sinus node dysfunction, atrial fibrillation, conduction defects, and ventricular arrhythmias.\textsuperscript{1–3} Na\textsubscript{v}1.5 dysfunction is further linked with arrhythmias associated with acquired heart failure.\textsuperscript{4} Based on the role of Na\textsubscript{v}1.5 in health and disease, therapies to target select Na\textsubscript{v}1.5 properties have remained at the forefront of cardiovascular medicine.\textsuperscript{5} Unfortunately, the molecular pathways underlying Na\textsubscript{v}1.5 regulation remain largely undefined partially due to lack of essential in vivo data.

Na\textsubscript{v}1.5 is principally regulated by membrane voltage. However, more recent data demonstrate that Na\textsubscript{v}1.5 is secondarily modulated by the calcium/calmodulin-dependent kinase II\textsubscript{δ} (CaMKII\textsubscript{δ}) for acute action potential modulation and propagation.\textsuperscript{6,7} Importantly, elevated CaMKII activity in heart disease is associated with increased pro-arrhythmic Na\textsubscript{v}1.5-dependent late sodium current (I\textsubscript{Na,L}).\textsuperscript{6,7} The mechanisms underlying CaMKII\textsubscript{δ}-dependent regulation of Na\textsubscript{v}1.5 were unknown until recent work revealed phosphorylation of the channel (Na\textsubscript{v}1.5 S571) at baseline and hyper-phosphorylation of the channel in disease.\textsuperscript{8} However, the cellular pathways underlying this local regulation have remained undefined.

Ankyrin polypeptides play critical roles in ion channel and transporter targeting in excitable and non-excitable cells. Ankyrin-R (ANK1) links membrane transporters to the cytoskeleton in erythrocytes and human ANK1 mutations cause hereditary spherocytosis.\textsuperscript{9} Ankyrin-B (ANK2) is critical for ion channel and transporter targeting in heart, brain, and pancreas, and ankyrin-B dysfunction has been linked with sinus node disease, atrial fibrillation, ventricular arrhythmia, and diabetes.\textsuperscript{10–14} Finally, ankyrin-G (ANK3) plays central roles for ion channel targeting in neurons.\textsuperscript{15} While in vitro work supports an association between ankyrin-G and Na\textsubscript{v}1.5 in heart,\textsuperscript{16,17} little is known about the role of this *complex in vivo*. Further, a link between ankyrin-G/Na\textsubscript{v}1.5 and CaMKII\textsubscript{δ} is undefined. Finally, the functional pathophysiological consequences for disrupting any of these putative complexes are unknown.

We report the molecular basis of a novel signaling platform in heart that couples CaMKII\textsubscript{δ} to Na\textsubscript{v}1.5. Our in vivo data demonstrate that ankyrin-G serves as an intercalated disc
receptor for both Na\textsubscript{v}1.5 and β\textsubscript{IV} spectrin, a molecule originally identified in brain and linked with neurological disease.\textsuperscript{18} Mice harboring a conditional null allele for ankyrin-G in heart (cKO) are surprisingly viable, but display decreased Na\textsubscript{v}1.5 expression, membrane localization, and \(I_{\text{Na}}\) associated with bradycardia, conduction abnormalities, QRS prolongation, and ventricular arrhythmias in response to Na\textsubscript{v} channel antagonists. Further, ankyrin-G cKO mice show loss of β\textsubscript{IV} spectrin recruitment to the intercalated disc membrane. β\textsubscript{IV} spectrin C-terminal domain associates with CaMKIIδ, and ankyrin-G cKO mice, as well as β\textsubscript{IV} spectrin mutant mice lacking the C-terminal domain (\textsuperscript{qv}4J) show defects in CaMKIIδ targeting and CaMKIIδ-dependent regulation of \(I_{\text{Na,L}}\). Finally, we report that the ankyrin-G-dependent protein platform links Na\textsubscript{v} channels with broader intercalated disc signaling/structural nodes, as in vivo ankyrin-G loss results in remodeling of plakophilin-2, a resident desmosome protein critical for intercalated disc integration with the intermediate filament-based cytoskeleton. Together, our findings identify a novel molecular platform critical for the membrane recruitment and regulation of Na\textsubscript{v}1.5 in heart. These findings further provide new insight into the pathways underlying cardiac excitability in health and disease.

METHODS

Biochemistry

Immobilized GST-fusion proteins were incubated with 100 μg left ventricular heart lysate overnight in pull-down buffer at 4 °C.\textsuperscript{19} The samples were washed three times in pull-down buffer, eluted, and proteins were separated by SDS/PAGE. The gels were transferred to nitrocellulose and immunoblotted. Nitrocellulose blots were developed using standard protocols.

Additional Methods are found in Supplemental Materials.

RESULTS

Generation of mice with cardiac-specific deletion of ankyrin-G

Based on its role in assembly of excitable domains in the nervous system\textsuperscript{20} and in vitro links with Na\textsubscript{v}1.5 in myocytes\textsuperscript{21}, we hypothesized that ankyrin-G may serve as a molecular platform for cardiac Na\textsubscript{v}1.5 signaling. To test this role in vivo, we generated a conditional null mutant allele where exons 22–23 the mouse ankyrin-G gene (\textit{Ank3}) is flanked by LoxP sites (\textit{Ank3}\textsuperscript{fl/fl}) and therefore are deleted in the presence of Cre recombinase (Figure 1A–C). We selectively eliminated ankyrin-G in post-natal cardiomyocytes by utilizing αMHC-Cre\textsuperscript{knock-in mice}; homozygous conditional knockout mice are referred to as αMHC-Cre; \textit{Ank3}\textsuperscript{fl/fl} or cKO. αMHC-Cre; \textit{Ank3}\textsuperscript{fl/fl} WT age- and sex-matched littermates were utilized as control mice. Surprisingly, cKO mice were viable, displayed no gross differences in size, weight, feeding, grooming, and showed no apparent deficits in motor function, unlike mice harboring selective deletion of cerebellar ankyrin-G.\textsuperscript{20} Immunoblots from whole heart lysates showed elimination of ankyrin-G in cKO heart (Figure 1D). Selective loss of ankyrin-G in the heart was confirmed by immunoblot from cortex, cerebellum, skeletal muscle, and kidney of control and cKO mice where we observed no difference in ankyrin-G
expression (Figure 1E–F). At the level of the single ventricular myocyte, ankyrin-G is enriched at the intercalated disc along with N-cadherin (Figure 1G). Ankyrin-G expression at the intercalated disc (and minor population at transverse-tubule) was eliminated from cKO ventricular myocytes (Figure 1H).

**Ankyrin-G cKO mice display abnormal Na\(^v\) channel targeting and function**

Ankyrin-G is linked with voltage-gated Na\(^v\) channel function in cerebellum.\(^{20}\) We therefore examined Na\(_{v}\)1.5 expression, localization, and function in ankyrin-G cKO hearts. We observed a significant decrease in Na\(_{v}\)1.5 expression in cKO hearts by immunoblot (Figure 2A–B, p<0.05). While primarily localized to the intercalated disc, Na\(_{v}\)1.5 is also found in secondary populations at the peripheral sarcolemma\(^{23, 24}\). In line with immunoblot data, Na\(_{v}\)1.5 expression was significantly reduced in cKO compared with WT myocytes (Figure 2C–D). Moreover, consistent with the localization of ankyrin-G, we observed selective loss of Na\(_{v}\)1.5 from the intercalated disc in cKO myocytes (Figure 2C–F). Na\(_{v}\)1.5 immunostaining at the peripheral sarcolemma in cKO myocytes was unaffected (Figure 2C–F). However, we observed populations of intracellular Na\(_{v}\)1.5-positive puncta in ankyrin-G cKO myocytes (Figure 2F). This loss was specific for Na\(_{v}\)1.5 as expression levels of Ca\(_{v}\)1.2, Na/Ca exchanger 1 (NCX), and ankyrin-B were unchanged between WT and cKO hearts (Online Figure I). Consistent with these data, we observed ~50% reduction in peak \(I_{\text{Na}}\) in cKO myocytes compared with WT littermates (Figure 2G–J). While \(I_{\text{Na}}\) was reduced in cKO myocytes, we observed no difference in Na\(_{v}\)1.5 steady-state voltage-dependent inactivation or recovery from inactivation (Online Figure II). Finally, consistent with prior findings in \(\text{Scn5a}^{+/−}\) mice,\(^{25}\) loss of \(I_{\text{Na}}\) in cKO myocytes resulted in a significant decrease in action potential (AP) amplitude and maximum upstroke velocity (\(V_{\text{max}}\); Online Figure III). Together, these findings demonstrate an in vivo requirement of ankyrin-G for cardiac Na\(_{v}\)1.5 membrane expression and function.

**Ankyrin-G recruits βIV spectrin to the myocyte intercalated disc**

Ankyrins partner with spectrin polypeptides to bridge membrane and cytoskeletal structures.\(^{26}\) In the nervous system, while ankyrin-G and βIV spectrin are required for assembly of the axon initial segment, ankyrin-G appears to serve as the primary organizing protein. To investigate the functional relationship between ankyrin-G and βIV spectrin in heart, we tested the localization of βIV spectrin in ankyrin-G cKO mice. In WT myocytes and heart, βIV spectrin was concentrated at the intercalated disc membrane (Figure 3, Online Figure IV).\(^{8}\) However, βIV spectrin was nearly absent from the intercalated disc membrane of cKO myocytes (Figure 3, Online Figure IV). Instead we observed populations of βIV spectrin-positive puncta near the peri-nuclear region in cKO myocytes (Figure 3). Thus, ankyrin-G is required for the recruitment of βIV spectrin to the myocyte intercalated disc.

**βIV spectrin is not required for ankyrin-G or Na\(_{v}\)1.5 targeting in myocytes**

We tested the converse requirement of βIV spectrin for ankyrin-G and Na\(_{v}\)1.5 targeting in heart. For these experiments, we utilized a βIV spectrin mutant mouse model (\(\text{qv}^{A}\)J mice) harboring a premature stop codon in the 10th spectrin repeat resulting in a truncated polypeptide lacking ankyrin-G-binding activity (located in 15\(^{th}\) spectrin repeat; see Figure
As expected, a GST-βIV spectrin fusion protein harboring the qvJ1 mutation lacked binding activity for ankyrin-G and Nav1.5 (Figure 4B). In contrast to findings in neurons,27 qvJ1 myocytes displayed no significant difference in ankyrin-G or Na_v channel expression compared to control hearts by immunoblot (Figure 4C–D). Further, we observed no difference in intercalated disc proteins N-cadherin or β-catenin in qvJ1 hearts (Figure 4C–D). In line with immunoblot data, we observed no difference in ankyrin-G or Na_v1.5 localization at the intercalated disc between control and qvJ1 myocytes (Figure 4E–H). However, consistent with the role of the βIV spectrin C-terminus in CaMKIIδ targeting,8 qvJ1 myocytes displayed a significant decrease in CaMKIIδ expression (Figure 4C–D, p<0.05). In summary, our findings define a requirement of ankyrin-G for βIV spectrin targeting to the intercalated disc, whereas ankyrin-G is targeted to the disc independent of βIV spectrin-binding.

**Ankyrin-G recruits CaMKIIδ to the myocyte intercalated disc**

βIV spectrin, via a short C-terminal motif, associates with CaMKIIδ and CaMKIIδ displays aberrant CaMKIIδ intercalated disc targeting.8 Based on the loss of βIV spectrin in ankyrin-G cKO hearts (Figure 3), we hypothesized that ankyrin-G cKO hearts would display decreased CaMKIIδ expression and abnormal intercalated disc targeting. We observed reduced CaMKIIδ expression by immunoblot in cKO versus control hearts (Figure 5A–B). In cKO heart, we observed selective loss of CaMKIIδ at the intercalated disc (location of βIV spectrin) versus other myocyte membrane populations (i.e. transverse-tubules, Figure 5C–D; Online Figure V). Thus, ankyrin-G, via βIV spectrin, controls the intracellular targeting of CaMKIIδ to the intercalated disc.

**Ankyrin-G recruits CaMKIIδ to regulate Na_v1.5 phosphorylation**

In response to sympathetic stimulation, CaMKIIδ phosphorylates Na_v1.5, via serine 571 (S571) to regulate Na_v1.5-dependent late current (I_{Na,L}).8,28 Ankyrin-G recruits both Na_v1.5 and CaMKIIδ to the intercalated disc (Figures 2, 5). In line with these data, we observed reduced Na_v1.5 S571 phosphorylation by both immunoblot and immunostaining in cKO myocytes compared with control cells (Online Figure VI A–D). When corrected for peak I_{Na}, we did not observe differences in I_{Na,L} between WT and cKO myocytes at baseline (Online Figure VII). However, consistent with the loss of CaMKII-dependent regulation of I_{Na,L} in the absence of ankyrin-G, we observed a significant difference in Iso-induced I_{Na,L} between WT and cKO myocytes (Online Figure VII). Specifically, while WT myocytes displayed nearly a two-fold increase in I_{Na,L} in response to Iso, we observed no statistical change in I_{Na,L} in cKO myocytes ±Iso regardless of the voltage (Online Figure VII; p<0.05). Our combined data support an in vivo ankyrin-G-based platform with dual roles for Na_v channel recruitment and CaMKIIδ-dependent regulation.

**Ankyrin-G cKO mice display bradycardia, conduction defects, and arrhythmia**

Impaired Na_v1.5 function is associated with phenotypes of sinus node dysfunction, conduction defects, and ventricular arrhythmia.1–3 We therefore tested the role of the ankyrin-G-dependent disc platform for cardiac electrical regulation. Conscious ankyrin-G cKO mice monitored by electrocardiogram (ECG) telemetry exhibited significant reduction...
in resting heart rate compared with control mice (Figure 6A–C). Impaired atrioventricular (AV) conduction in cKO mice was also readily apparent in cKO mice as noted by an increase in PR interval compared with control mice (Figure 6D–F; intra-atrial conduction also reduced as evidenced by increased P wave duration, p<0.05). Moreover, cKO mice displayed a significant increase in the QRS interval, consistent with delayed intraventricular conduction (Figure 6D–E, G). In summary, in vivo findings strongly support a role of the ankyrin-G-based protein complex in regulation of Na⁺ channel function and cardiac excitability.

Flecainide induces bradycardia, QRS prolongation, and arrhythmia in cKO mice

Class 1C anti-arrhythmics (Na⁺ channel antagonists) may be utilized in clinical practice to identify individuals with Na⁺ channel abnormalities.³⁹ Moreover, these compounds (e.g. flecainide) have been used to uncover Na⁺ channel-based arrhythmia mechanisms in animal models.³⁰, ³¹ To test the functional relevance of Na⁺ channel dysfunction in the ankyrin-G cKO mouse, we analyzed ECGs of control and cKO mice following flecainide administration.³², ³³ At baseline, cKO mice displayed bradycardia compared to control mice (Figure 7A–B, E). Flecainide reduced heart rate in both control and cKO mice (Figure 7A–E). However, the effect of flecainide on heart rate was significant and potentially pathological in cKO mice as this intervention resulted in an overall ~30% decrease in heart rate compared with a normal mouse at baseline (Figure 7E; p<0.05). Further, in cKO but not control mice, flecainide caused AV block and ventricular arrhythmia (Figure 7F–H). Specifically, 10% of control mice displayed AV block in response to flecainide, whereas we recorded AV block in ~73% of cKO mice (Figure 7F–G; p<0.05). Of the mice showing AV block, the incidence of block was >140 fold greater in cKO mice (control: 0.019 ± 0.019 episodes/min; cKO: 2.75 ± 0.98 episodes/min; p<0.05). Moreover >80% of cKO mice showed arrhythmia including bigeminy and non-sustained ventricular arrhythmia, compared to 10% of control mice (Figure 7H, p<0.05). Consistent with conscious ECG recordings, anesthetized cKO mice displayed prolonged QRS interval compared with control mice (Online Figure VIII). This QRS interval difference between genotypes was further accentuated by flecainide administration (Online Figure VIII; p<0.05). Ankyrin-G cKO mice also showed increased PR interval duration following flecainide (Online Figure IX, p<0.05). Together, these data strongly support the role of ankyrin-G-protein complex in Na⁺ channel regulation in vivo, as well as link Na⁺ channel-based mechanisms with observed cardiac electrical phenotypes in the ankyrin-G cKO mouse.

Ankyrin-G cKO mice display defects in plakophilin-2 expression and cellular organization

Beyond membrane ion channels, in vitro work supports roles of ankyrin-G for membrane protein regulation at cell junctions.¹⁷, ³⁴ We therefore tested the in vivo requirement of ankyrin-G for expression and localization of key intercalated disc proteins as well as Na⁺ β-subunits that have been previously linked with ankyrin polypeptides.³⁵ Notably, we observed no difference in expression or localization of disc proteins N-cadherin and β-catenin, or Na⁺ channel β2 and β4 subunits between control and cKO hearts (Figure 8A–K; Online Figure X). In contrast, plakophilin-2 showed a 50% increase in expression in cKO hearts (Figure 8A–B). Further, we observed a striking redistribution of plakophilin-2 from the intercalated disc of cKO myocytes to the cytosolic regions of cKO myocytes (Figure
8K–L; Online Figure XI). While prior work proposed a link for ankyrin-G in targeting of connexin4317, we observed no difference in connexin43 or ZO-1 expression by immunoblot (Figure 8C–D). Together, our new in vivo data clearly support a role of ankyrin-G in intercalated disc protein organization.

Ankyrin-G cKO mice display catecholamine-induced arrhythmia and death

Ankyrin-G cKO mice display a number of pro-arrhythmic ECG phenotypes associated with reduced $I_{Na}$ (Figures 6–7). However, based on the additional observed molecular defects in ankyrin-G cKO animals (Figure 8), we tested cKO animals for arrhythmia susceptibility in response to elevated adrenergic stimulation. Ankyrin-G cKO mice showed arrhythmia phenotypes following a standard I.P. epinephrine injection protocol to mimic catecholaminergic stress (Online Figure XII). Specifically, the majority (~62.5%) of cKO mice displayed multiple instances of ventricular arrhythmia and ~38% of cKO mice died of malignant arrhythmias following this protocol (Online Figure XII; 3/8 cKO vs 0/6 control; p<0.05). The cKO mice also displayed decreased AV conduction compared to WT mice (increased PR interval; p<0.05; n=5/genotype) in these experiments. Examples of sustained premature ventricular contractions (PVCs) as well as polymorphic ventricular arrhythmia in cKO mice are shown in Online Figure XIB–C. We recorded no incidence of ventricular arrhythmia (0%) or death (0%; Online Figure XIIA, E) in WT animals using this protocol. In summary, we conclude that loss of the cardiac ankyrin-G-based protein platform results in pro-arrhythmic ECG phenotypes at rest, and arrhythmia and death in response to catecholaminergic stress. Of note, as catecholamine-based arrhythmias in cKO mice were observed in the absence of elevated CaMKIIδ or $I_{Na,L}$, we tested for potential alterations in myocyte calcium handling in cKO myocytes. Consistent with whole animal data (e.g. PVCs), cKO myocytes displayed increased incidence of spontaneous Ca waves compared with WT myocytes (Online Figure XIII; p<0.05).

Ankyrin-G cKO mice display cardiac structural phenotypes

Ankyrins are linked with assembly and maintenance of structural domains in erythrocytes, epithelia, and neurons.36–38 Further, our data, and prior data from Delmar and colleagues17 link ankyrin-G with plakophilin-2, a protein associated with cardiac structural remodeling in humans and mice.31, 39, 40 We therefore examined ankyrin-G hearts for changes in cardiac structure or function at baseline and in aging. At 8 weeks, we observed no difference in heart weight/body weight (or tibia length) or systolic or diastolic wall thickness between WT and cKO mice (Online Figure XIV). While we observed a non-significant trend for increased systolic and diastolic LV diameter in cKO mice (p=N.S.), cKO mice displayed a small, but significant decrease in ejection fraction compared to WT littermates (Online Figure XIV). Notably, cKO mice displayed obvious cardiac phenotypes with age (~9 months) as indicated by a further depression in ejection fraction, increase in systolic and diastolic chamber diameters, and reduction of both the anterior and posterior wall thickness compared to WT littermates (Online Figure XV).
DISCUSSION

Voltage-gated Na\textsubscript{v} channels are critical for the rapid upstroke of the cardiac action potential and cardiac conduction. Defects in Na\textsubscript{v} channel function are linked with a host of congenital and acquired forms of human disease including sinus node dysfunction, atrial fibrillation, conduction disorders, ventricular arrhythmia, and heart failure.\textsuperscript{1–3} Despite these compelling human disease linkages and decades of elegant Na\textsubscript{v} channel biophysical studies, the field still lacks significant fundamental knowledge of the regulatory mechanisms governing the function of this critical molecule. Here we use in vivo animal models to define the cellular pathway underlying the targeting of Na\textsubscript{v}1.5 and its regulatory molecule, CaMKII\delta to the intercalated disc membrane. Ankyrin-G, a cytoskeletal adapter protein, associates with Na\textsubscript{v}1.5 and recruits the channel to the myocyte membrane. Loss of ankyrin-G results in defects in Na\textsubscript{v} channel expression, localization, and function. We further show that ankyrin-G, via the recruitment of \beta\textsubscript{IV} spectrin, also targets CaMKII\delta to the intercalated disc. As CaMKII\delta phosphorylates Na\textsubscript{v}1.5 to modulate cardiac myocyte excitability in health and disease\textsuperscript{8, 28}, our findings provide data that support the mechanisms underlying the biogenesis of this membrane signaling domain. Finally, our findings confirm in vivo protein pathways for ankyrin-G and Na\textsubscript{v}1.5 in heart. Specifically, as described by Delmar and colleagues in cultured myocytes\textsuperscript{17}, our in vivo data link ankyrin-G with the desmosomal protein plakophilin-2. Further, our data associate ankyrin-G with the disc protein \beta\textsubscript{IV} spectrin.

Ankyrin-G cKO mice were surprisingly viable, but display bradycardia, AV conduction defects, QRS prolongation, and/or arrhythmia associated with flecainide or epinephrine. Consistent with our proposed mechanism of \textit{I}_{Na} dysfunction in these animals, Na\textsubscript{v}1.5 is linked with impulse propagation through the sinoatrial node (SAN\textsuperscript{2}), the AV node\textsuperscript{41}, and the ventricular myocardium.\textsuperscript{42} Prior in vivo work links Na\textsubscript{v}1.5 dysfunction with bradycardia, and reduced SAN pacemaker potential.\textsuperscript{42} Moreover, consistent with our findings, flecainide has been previously shown to evoke ventricular arrhythmias in multiple mouse models of Na\textsubscript{v} channel deficiency.\textsuperscript{30, 31} While our findings clearly link ankyrin-G with Na\textsubscript{v}1.5 targeting, they reveal new ankyrin-G associated pathways that are relevant to cardiac electrical, signaling, and structural roles in both health and disease. Genetic mutation in the ankyrin-binding motif of Na\textsubscript{v}1.5 has been previously linked with loss of myocyte \textit{I}_{Na}, abnormal Na\textsubscript{v} channel targeting, and human Brugada syndrome.\textsuperscript{21} However to date, ankyrin-G (ANK3) variants have not yet been linked with human arrhythmia. Based on past findings in other tissues, as well as work in this study, a pure loss-of-function ANK3 allele may be incompatible with life. Mice simply lacking \textit{Ank3} in the cerebellum are frail and display severe neurological defects. Moreover, human ANK3 variants have been linked with bipolar disease, schizophrenia, and autism.\textsuperscript{43–45} Ankyrin-G is required for normal retinal protein trafficking, and essential for lateral membrane biogenesis in columnar epithelia.\textsuperscript{37, 46} Based on our findings, we predict that more subtle variants may cause sinus node disease, arrhythmia, and even structural heart disease due to defects in intercalated disc infrastructure.

As noted above, our data demonstrate that ankyrin-G recruits \beta\textsubscript{IV} spectrin to the intercalated disc. In other cell types, \beta-spectrins, through their association with \alpha-spectrins and actin
form critical submembrane cytoskeletal infrastructure. In fact, defects in spectrins have been linked with a host of pathologies in humans and animals. Moreover, \( \beta_{IV} \) spectrin associates with, and targets a subpopulation of CaMKII\( \delta \) to the intercalated disc to phosphorylate Na\( \text{v}_{1,5} \). Defects in CaMKII\( \delta \) phosphorylation of Na\( \text{v}_{1,5} \) have now been linked with multiple forms of heart failure in humans and animal models.

Our in vivo data support a role of ankyrin-G in organization of the intercalated disc. Plakophilin-2 is a key disc protein, linking desmosomal cadherins with desmoplakin and the intermediate filament system. Human plakophilin-2 loss-of-function variants are linked with arrhythmogenic right ventricular cardiomyopathy (ARVC) and Brugada syndrome. While cardiac phenotypes in the cKO model are distinct from human ARVC (increased levels of PKP2 in cKO model versus reduced PKP2 levels in ARVC), the unexpected new data on structural defects in ankyrin-G cKO hearts and association with plakophilin-2 alterations are noteworthy. However, the specific relationship between ankyrin-G and plakophilin-2, as well as the potential link between these pathways and the observed structural defects in cKO mice, will require additional investigation. Interestingly, unlike plakophilin-2, we did not observe alterations in expression of intercalated disc proteins N-cadherin, connexin43, ZO-1, or \( \beta \)-catenin in cKO mice by immunoblot. Notably, Delmar and colleagues previously showed reduced connexin43 expression in ankyrin-G siRNA transfected neonatal myocytes. While future experiments will be important in defining the relationship between these molecules (i.e. compensatory changes versus direct protein partners), our findings clearly implicate ankyrin-G as a multifunctional regulatory molecule in the heart. Further, our work demonstrates that phenotypes observed in the ankyrin-G cKO mouse likely extend far beyond simple \( I_{Na} \) deficiency.

Our in vivo findings demonstrate a critical role for ankyrin-G for intercalated disc Na\( \text{v}_{1,5} \) targeting. However, Na\( \text{v}_{1,5} \) targeting to non-intercalated disc membranes has been proposed as ankyrin-independent, relying instead on unique cellular machinery including syntrophin/dystrophin, SAP97, caveolin-3, MOG1, and FGF12. Based on the essential role of Na\( \text{v} \) channels for myocyte excitability, we propose that the vertebrate has evolved multiple mechanisms for Na\( \text{v}_{1,5} \) membrane targeting. Future studies that explore the specific roles of ankyrin-G versus other targeting proteins in ion channel trafficking versus membrane scaffolding will be important to explore the relative contribution of each protein in dictating cardiac excitability and function using in vivo models. Additionally, it will be important to define the identity of populations of Na\( \text{v}_{1,5} \)-positive puncta in cKO myocytes (blue arrows in Figure 2F, i.e. endosomes, T-tubule populations, etc.). Further, based on the in vivo link between ankyrin-G and plakophilin-2, it will be important to further investigate the mechanistic roles of ankyrin-G in regulation of intermediate filaments at the intercalated disc. Finally, it will be important to investigate the role of ankyrin-G for targeting other Na\( \text{v} \) channels in heart (e.g. ‘brain’ Na\( \text{v} \) channel gene products).

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.
Acknowledgments

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Nonstandard Abbreviations and Acronyms

<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>CaMKII</td>
<td>calcium/calmodulin-dependent kinase II</td>
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<td>cKO</td>
<td>conditional knock-out mouse</td>
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<td>GST</td>
<td>glutathione-S-transferase</td>
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<td>Iso</td>
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<td>AV</td>
<td>Atrioventricular</td>
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References


Novelty and Significance

What Is Known?

- Cardiac voltage-gated Na\textsubscript{v} channels (Na\textsubscript{v}1.5) are critical for ventricular depolarization and cardiac conduction.
- Defects in Na\textsubscript{v}1.5-based pathways have been linked with both congenital and acquired forms of human cardiovascular disease.
- The molecular pathways underlying Na\textsubscript{v}1.5 regulation remain largely undefined partially due to lack of essential in vivo data.

What new information does this Article Contribute?

- Ankyrin-G, associates with Na\textsubscript{v}1.5 and recruits the channel to the myocyte intercalated disc membrane.
- Ankyrin-G, via the recruitment of β\textsubscript{IV} spectrin, targets the critical cardiac signaling molecule CaMKII\textsubscript{δ} to the intercalated disc to regulate Na\textsubscript{v}1.5 activity.
- In vivo loss of ankyrin-G results in defects in cardiac excitability and arrhythmia.

Na\textsubscript{v}1.5 is the primary cardiac voltage-gated Na\textsubscript{v} channel (SCN5A). Nav 1.5 is central for initiation of the ventricular action potential and both loss- and gain-of-function mutations in SCN5A have been linked with diverse cardiovascular pathologies including ventricular arrhythmia, conduction defects, sinus node dysfunction, and atrial fibrillation. Based on critical role of Na\textsubscript{v}1.5 in human disease, therapies to target select Na\textsubscript{v}1.5 properties have remained at the forefront of cardiovascular medicine. Unfortunately, the molecular pathways underlying Na\textsubscript{v}1.5 regulation remain largely undefined partially due to lack of essential in vivo data. Ankyrin-G, associates with Na\textsubscript{v}1.5 and recruits the channel to the myocyte membrane. Loss of ankyrin-G results in defects in Na\textsubscript{v} channel expression, localization, and function. We Ankyrin-G, via the recruitment of β\textsubscript{IV} spectrin, also targets CaMKII\textsubscript{δ} to the intercalated disc. Mice harboring a conditional null allele for ankyrin-G in heart (cKO) display decreased Na\textsubscript{v}1.5 expression, membrane localization, and I\textsubscript{Na} associated with bradycardia, conduction abnormalities, QRS prolongation, and ventricular arrhythmias in response to Na\textsubscript{v} channel antagonists. Finally, in vivo ankyrin-G loss results in remodeling of plakophilin-2. Together, our findings identify a novel molecular platform critical for the membrane recruitment and regulation of Na\textsubscript{v}1.5 in heart.
Figure 1. Generation of cardiac-specific ankyrin-G null mouse

(A) Scheme for generation of cardiac conditional ankyrin-G null mouse (cKO). LoxP sites were inserted to flank exons 22–23 in ankyrin-G gene (Ank3). Mice homozygous for loxP insertion (f/f) were crossed with mice expressing Cre recombinase under control of the αMHC promoter. Cre-mediated excision of exons 22–23 resulted in the production of a premature stop codon after exon 21. (B) Flox (f) allele (434 bp) and WT (+) allele (366 bp) identified by PCR. (C) Cre expression (+ Cre) evidenced by band at 300 bp not observed in WT animals (− Cre) and in no template control (Ctrl). (D–E) Ankyrin-G expression in WT and cKO mouse tissue lysates. Note that unlike heart, other tissues examined express multiple molecular weight forms of ankyrin-G. (F) Data from experiments in D–E were quantified and expressed relative to WT tissue (corrected for actin expression; n=4/genotype, p<0.05). (G–H) Expression of ankyrin-G (green) and N-cadherin (red) in WT and cKO myocytes (Bar=10 microns; arrow notes intercalated disc). DAPI (blue) was included to stain nuclei.
Figure 2. Ankyrin-G is required for myocyte Na\textsubscript{v}1.5 expression and localization

(A–B) Immunoblots of Na\textsubscript{v}1.5 in WT and cKO heart. We observed reduced Na\textsubscript{v}1.5 protein levels in cKO heart. In B, Na\textsubscript{v}1.5 expression levels are normalized to GAPDH loading control (n=4/genotype; p<0.05). (C–D) Na\textsubscript{v}1.5 immunostaining (red) is significantly reduced at the intercalated disc of cKO myocytes (Bar=10 microns). Myocytes were co-labeled for N-cadherin (green) and nuclei (blue DAPI). (E–F) Magnified images of Nav1.5 immunostaining of peripheral sarcolemma and intercalated disc of WT and cKO myocytes. Note in E that Na\textsubscript{v}1.5 is localized at both intercalated disc (white arrow) and peripheral sarcolemma membrane (yellow arrows). While disc staining is reduced in cKO myocytes, the sarcolemmal staining is unaltered (F). In cKO myocytes, we also observed intracellular Nav1.5-positive puncta (blue arrows). (G–H) Representative $I_{Na}$ traces from WT and cKO mouse myocytes. (I–J) Reduced $I_{Na}$ current in cKO versus WT myocytes (WT: n=10; cKO: n=9; p<0.05).
Figure 3. Ankyrin-G targets βIV spectrin to the intercalated disc

(A–B) βIV spectrin intercalated disc targeting is altered in cKO myocytes. βIV spectrin expression is clustered in the peri-nuclear region of cKO myocytes (arrows).
Figure 4. Ankyrin-G targets Na\(_{\text{v}1.5}\) to the intercalated disc independent of β\(_{\text{IV}}\) spectrin

(A) Diagram of β\(_{\text{IV}}\) spectrin domains and location of q\(_{v}^{\text{4J}}\) truncation upstream of ankyrin-G-binding site. (B) GST-β\(_{\text{IV}}\) spectrin associates with Na\(_{\text{v}1.5}\) (via ankyrin-G). β\(_{\text{IV}}\) spectrin representing the q\(_{v}^{\text{4J}}\) mouse truncation lacks binding activity for ankyrin-G and thus Na\(_{\text{v}1.5}\).

(C–D) WT and q\(_{v}^{\text{4J}}\) hearts display no difference in expression of ankyrin-G or Na\(_{\text{v}1.5}\) by immunoblot. CaMKIIδ levels are reduced in cKO hearts consistent with q\(_{v}^{\text{4J}}\) allele lacking the CaMKIIδ-binding site (see A). Levels in C are normalized for actin expression (n=4/genotype; p<0.05). (E–H) WT and q\(_{v}^{\text{4J}}\) myocytes display no difference in ankyrin-G or Na\(_{\text{v}1.5}\) localization. Bar=10 microns.
Figure 5. Ankyrin-G targets CaMKIIδ to the intercalated disc via βIV spectrin

(A–B) CaMKIIδ expression and localization is altered in cKO myocytes. Levels in A are normalized for actin expression (n=4/genotype; p<0.05). (C–D) CaMKIIδ expression is selectively reduced at the intercalated disc of cKO myocytes (yellow arrows in C versus white arrows in D. Asterisk notes no change in CaMKIIδ localization at T-tubules between genotypes. Bar=10 microns.
Figure 6. Cardiac-specific ankyrin-G null mice exhibit bradycardia and conduction abnormalities

(A–C) Ankyrin-G cKO mice display bradycardia compared with WT littermates (WT: n=5; cKO: n=7; p<0.05). Bar in B, C = 200 ms. Representative ECG traces for (D) WT and (E) cKO mice. cKO mice show increased PR (F) and QRS (G) intervals compared with WT mice (WT: n=6; cKO: n=7; p<0.05).
Figure 7. Ankyrin-G cKO mice display conduction defects and arrhythmia in response to Na\textsubscript{v} channel antagonist

(A–B) ECG traces from WT and ankyrin-G cKO mouse 30 seconds prior to flecainide administration. (C–D) ECG traces from same mice (in A–B) observed 10 minutes post-flecainide administration (20 mg/kg, I.P.; bar=200 ms). Examples of AV block (arrowheads) and ventricular phenotypes (brackets) are noted in D. (E–H) Ankyrin-G cKO mice show significant decrease in heart rate and increase in frequency of AV block and ventricular arrhythmia following flecainide compared with WT littermates (p<0.05).
Figure 8. Plakophilin-2 organization is disrupted in cKO myocytes

(A–B) Immunoblots of myocyte intercalated disc proteins in WT versus cKO heart. Note that plakophilin-2 levels are significantly increased in cKO hearts (n=4/genotype; p<0.05).

(C–K) Confocal imaging of WT (left) and cKO (right) myocytes labeled with antibodies for resident intercalated disc proteins. Note that while primarily localized to the disc of WT myocytes, plakophilin-2 is clustered in the peri-nuclear region of cKO myocytes (arrowheads; Bar=10 microns).