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Dysfunction in the β II Spectrin-Dependent Cytoskeleton Underlies Human Arrhythmia

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Abstract

Background—The cardiac cytoskeleton plays key roles in maintaining myocyte structural integrity in health and disease. In fact, human mutations in cardiac cytoskeletal elements are tightly linked with cardiac pathologies including myopathies, aortopathies, and dystrophies. Conversely, the link between cytoskeletal protein dysfunction in cardiac electrical activity is not well understood, and often overlooked in the cardiac arrhythmia field.

Methods and Results—Here, we uncover a new mechanism for the regulation of cardiac membrane excitability. We report that β II spectrin, an actin-associated molecule, is essential for the post-translational targeting and localization of critical membrane proteins in heart. β II spectrin recruits ankyrin-B to the cardiac dyad, and a novel human mutation in the ankyrin-B gene disrupts the ankyrin-B/ β II spectrin interaction leading to severe human arrhythmia phenotypes. Mice lacking cardiac β II spectrin display lethal arrhythmias, aberrant electrical and calcium handling phenotypes, and abnormal expression/localization of cardiac membrane proteins. Mechanistically, β II spectrin regulates the localization of cytoskeletal and plasma membrane/sarcoplasmic reticulum protein complexes that include the Na/Ca exchanger, RyR2, ankyrin-B, actin, and α II spectrin. Finally, we observe accelerated heart failure phenotypes in β II spectrin-deficient mice.

Conclusions—Our findings identify β II spectrin as critical for normal myocyte electrical activity, link this molecule to human disease, and provide new insight into the mechanisms underlying cardiac myocyte biology.

Keywords

protein trafficking arrhythmia; cytoskeleton; arrhythmia (heart rhythm disorders); cytoskeletal dynamics; cardiovascular physiology; catecholaminergic polymorphic ventricular tachycardia

Introduction

Life for the vertebrate requires sustained and rhythmic beating of the heart for delivery of oxygen and nutrients to the brain and other organs. The vertebrate cardiomyocyte has evolved elegant membrane regulatory pathways to maintain efficient excitation-contraction coupling at baseline or in the face of acute and chronic stress. Central to this membrane regulation is the cardiac cytoskeleton. In the heart, the cytoskeletal network is comprised of a highly ordered array of structural and accessory proteins spanning from the plasma membrane to the nucleus.

Over the past two decades, the cardiac cytoskeleton has emerged as a central governing factor in the control of cardiac membrane integrity, and dysfunction in cytoskeleton and cytoskeletal-associated proteins has been directly linked with a host of human cardiac pathologies, most notably cardiac myopathies and dystrophies. In fact, human mutations in cardiac cytoskeletal or cytoskeletal-associated genes that alter myocyte signal transduction, myocardial mechanics, and force transmission are now directly linked with dilated cardiomyopathy, muscular dystrophy, and arrhythmogenic cardiomyopathy.¹⁻⁴

In contrast to myopathy and dystrophy fields, the role of the cytoskeleton in normal cardiac electrical function is not well resolved. Further, until the past decade, human arrhythmia mechanisms were primarily limited to mutations in cardiac ion channels.⁵ However, while literally hundreds of human variants in cardiac Na⁺, K⁺, and Ca²⁺ channel alpha- and beta-subunits have been linked with sinus node disease, atrial fibrillation, conduction disease, and ventricular fibrillation, a second class of human arrhythmias has emerged due to mutations in ion channel-associated proteins including α -syntrophin, ankyrin-G, caveolin-3, FGF12, and ankyrin-B.⁶ Mechanistically, dysfunction in these proteins is linked with diverse cellular pathologies including defects in channel synthesis and membrane targeting, channel gating, and channel post-translational modifications. While this information has been important for new disease diagnosis and fundamental cardiac cell biology, there remain large cohorts of phenotype positive/genotype negative patients with familial forms of cardiac arrhythmia. Further, there remain pressing unanswered questions regarding the role for the cardiac cytoskeleton for the local organization of membrane ion channel complexes *in vivo*.

Based on genetic findings in a proband with severe ventricular arrhythmia and cardiac arrest, we uncovered a new and essential cytoskeletal-based pathway critical for cardiac electrical function. We identify β II spectrin as an integral regulatory node for the organization of critical myocyte membrane and membrane-associated proteins. β II spectrin is critical for the regulation of ankyrin-B and α II spectrin, and defects in this assembly result in severe arrhythmia associated with aberrant calcium phenotypes. Moreover, we link dysfunction in this pathway with accelerated heart failure phenotypes. In summary, our findings provide a new mechanism for human excitable cell disease, as well as uncover new roles for the cardiac cytoskeleton in human cardiovascular disease.

Methods

Statistics

Data are presented as mean \pm SEM. For the comparison of two groups, we performed Wilcoxon-Mann-Whitney *U* tests. For the comparison of greater than two groups, we applied a Kruskal-Wallis test. When we obtained a significant P value, we continued with pair-wise comparisons utilizing Wilcoxon-Mann-Whitney *U* tests according to the closed testing principle. For our study, a value of $P < 0.05$ was considered statistically significant.

Human Studies

Approval for use of human subjects was obtained from the Institutional Review Board of Ohio State University, subjects provided informed consent.

Animal Studies

Procedures followed were approved and in accordance with institutional guidelines (Ohio State University)

Additional methods are provided in the Data Supplement.

Results

Identification of a new class of ANK2 human arrhythmia mutation

Human *ANK2* variants cause cardiac arrhythmia phenotypes including sinus node disease, atrial fibrillation, conduction block, ventricular arrhythmia, syncope, and sudden cardiac death.⁷⁻¹¹ We identified a new class of *ANK2* variant in a proband with severe history of recurrent sudden cardiac arrest due to ventricular fibrillation (VF) (Figure 1A). The proband is a 36-year-old female with prolonged QT_c on her electrocardiogram (ECG) who suffered out of hospital cardiac arrest due to VF. Following resuscitation, she underwent implantation of a transvenous dual chamber implantable cardioverter-defibrillator (ICD). Since her first event, she has had recurrent VF resulting in syncope and ICD shocks, with ICD interrogation demonstrating premature ventricular complexes (PVCs) preceding episodes of VF. In addition to prolonged QT_c interval (Figure 1B), the individual displays regular PVCs (Figure 1C), both harbingers of potential arrhythmic events. Initial genetic testing for variants in *KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1*, and *KCNE2* was negative for deleterious mutations. Subsequent genetic testing utilizing an extended sequencing panel including 5 additional genes (*ANK2*, *KCNJ2*, *CAV3*, *RYR2*, and *CASQ2*) revealed an *ANK2* c.2969G>A change resulting in the substitution of Arg to Gln at position 990 (p.R990Q). Exon array of *ANK2* and other genes previously tested by sequencing analysis did not detect any deletions or duplications. The c.2969G>A variant is rare across multiple populations with a minor allele frequency of ~0.007% (0/4406 African-American alleles, 1/8599 European-American alleles; NHLBI ESP). Notably, R990 is highly conserved from human to zebrafish, roundworm, and fruit fly (Figure 1D), and structural modelling reveals that the p.R990Q variant is juxtaposed to the central ZU5 binding surface for β II spectrin (Figure 1A, E¹²). This region of ankyrin has not previously been linked with disease and in fact is >1,500 base pairs from any previously identified variant (Figure 1A,D-E).

Ankyrin-B and β II spectrin are molecular partners in human heart

A requisite function of a canonical ankyrin polypeptide is association with the α/β spectrin hetero-tetramer. This complex, by association with actin (via spectrin) functionally couples integral membrane proteins (ion channels, receptors, transporters) with the cytoskeletal infrastructure. Ankyrins associate with β spectrin gene products through conserved residues in the N-terminal ZU5 (Zu5N) domain.¹² Based on the location of the human variant, we tested the functional relationship between ankyrin-B and β II spectrin. β II spectrin is normally localized at myocyte T-tubules (Figure 2A and Supplemental Figure 1) with ankyrin-B.¹³ Ankyrin-B directly associates with radiolabelled β II spectrin (Figure 2B), and β II spectrin co-immunoprecipitates with ankyrin-B from detergent-soluble lysates from adult mouse heart as well as non-failing human heart (Figure 2C-D). We observed a larger macromolecular complex between ankyrin-B and β II spectrin with membrane proteins including the Na⁺/K⁺ ATPase and Na⁺/Ca²⁺ exchanger (ankyrin-binding partners) as well as the cytoskeletal element actin by co-immunoprecipitation experiments from both mouse and human heart (Figure 2C-D). These data demonstrate the presence of a β II spectrin/ankyrin-B cytoskeletal complex in heart.

We hypothesized that the human p.R990Q variant confers susceptibility to cardiac arrhythmia by altering ankyrin-B-binding for β II spectrin, thus affecting integration of membrane proteins with the cytoskeleton.^{12, 14} While wild-type ankyrin-B robustly associated with β II spectrin, we observed a significant decrease in binding of ankyrin-B p.R990Q with β II spectrin (Figure 2E-F). *In silico* modelling for the human p.R990Q variant using the co-structure for ankyrin-B and β II spectrin revealed that the R990 forms several hydrogen bonds and salt bridges with surrounding residues, a property strictly conserved in all ankyrins (Figure 2G). Moreover, modelling revealed that p.R990Q results in loss of salt bridge interactions with surrounding residues (Figure 2G). Ankyrin-B p.R990Q may directly disrupt β II spectrin-binding. Alternatively, based on the localization of the residue, this mutation may destabilize the folding of the ZU5 domain, resulting indirectly in the loss of spectrin-binding and overall loss of ankyrin-B function.

Analysis of human variants that block β II spectrin-binding in myocytes

We tested the relationship between human ankyrin-B variants and β II spectrin in primary myocytes. As mice lacking global ankyrin-B expression die *in utero* or shortly after birth thus limiting the ability to study ankyrin-dependent mechanisms past post-natal day 2, we generated a new mouse model homozygous for a conditional cardiac ankyrin-B null allele (α MHC-Cre; ankyrin-B conditional knockout (cKO) mouse; Supplemental Figure 2). The null allele was created by targeting *Ank2* exon 24 disrupting all known *Ank2* splice products. The mutant allele was confirmed by Southern blot and PCR strategies (Supplemental Figure 2B), and myocytes from ankyrin-B cKO mice display loss of ankyrin-B protein by immunoblot and immunostaining (Supplemental Figure 2C-F). Using well-differentiated primary neonatal ankyrin-B cKO myocytes (post-natal day 7), we tested activity of the p.R990Q variant. WT ankyrin-B-GFP or human ankyrin-B variant p.R990Q were introduced into ankyrin-B cKO myocytes. Notably, compared with control WT myocytes (Supplemental Figure 3A-B), ankyrin-B cKO myocytes display loss of targeting of $\text{Na}^+/\text{Ca}^{2+}$ exchanger, a known binding partner in heart (Supplemental Figure 3C-D). GFP-ankyrin-B was striated when expressed in ankyrin-B cKO myocytes, and was sufficient to rescue the localization of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Supplemental Figure 3E-F). In contrast, ankyrin-B p.R990Q, similar to two ankyrin-B mutants previously identified to lack spectrin-binding (A1000P, DAR976AAA)¹³, was not appropriately targeted (diffuse cytoplasmic expression) and failed to rescue the localization of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Supplemental Figure 3G-L).

β II spectrin cKO mice display sinus node dysfunction and ventricular arrhythmia

The functional role for β II spectrin for post-natal cardiac function *in vivo* is unknown and untested. We therefore examined cardiac electrical phenotypes in a new mouse model with conditional deletion of cardiac β II spectrin (Figure 3A; β II spectrin cKO). β II spectrin cKO mice lack β II spectrin in heart but not in other tissues (Figure 3B-F). Telemetry studies of conscious mice show reduced heart rate, increased heart rate variability, atrioventricular (AV) block, and pro-arrhythmic ECG phenotypes (widened QRS complexes, prolonged QT interval) in β II spectrin cKO mice compared with WT littermates at baseline (Figure 4A-H). Notably, catecholaminergic stress (exercise or low dose epinephrine) resulted in pronounced ventricular arrhythmia (both non-sustained and sustained episodes) and death (Figure 4L-N).

We observed no difference in maximal heart rate in response to low or high dose epinephrine (Supplemental Figure 4) and under no circumstances were ECG or arrhythmic anomalies observed in control littermates. While cardiac electrical dysfunction may arise secondary to structural heart disease (hypertrophy, heart failure), we observed no significant differences in mean left ventricular ejection fraction (LVEF), cardiac output, wall thickness, or other significant structural phenotypes between control and β II spectrin cKO mice (Supplemental Figure 5A-B and Supplemental Figure 6A-M).

β II spectrin cKO myocytes display afterdepolarizations and abnormal calcium waves

Based on severity of observed arrhythmias in β II spectrin cKO mice, we examined β II spectrin cKO mouse ventricular myocyte action potentials. Unlike myocytes from control littermates, β II spectrin cKO myocytes displayed chaotic electrical behavior even in the absence of catecholamine stimulation (Figure 5A,C). Mean APD₉₀ was not different between genotypes (Figure 5I). However, we observed frequent spontaneous afterdepolarizations in β II spectrin cKO myocytes at baseline (Figure 5C-D). Afterdepolarizations were present independent of pacing frequency and increased in both frequency and duration following superfusion with isoproterenol (Figure 5B, D, F). We tested whether spontaneous calcium release potentially underlies the chaotic electrical behavior of β II spectrin cKO myocytes by examining action potentials in the presence of ryanodine, an inhibitor of Ca²⁺ release from the SR. Notably, ryanodine (100 nM) prevented afterdepolarizations in myocytes \pm isoproterenol (Figure 5G-H, J). Together, these data strongly support a critical role for β II spectrin in regulating myocyte electrical behavior and suggest a role of intracellular Ca²⁺ as a potential mechanism underlying the electrical arrhythmogenic phenotypes.

Spontaneous Ca²⁺ waves (SCaW) at the level of the myocyte may mediate Ca²⁺-dependent afterdepolarizations, known cellular triggers for arrhythmia.^{15, 16} Therefore, we compared the relative propensity of control and β II spectrin cKO myocytes to generate SCaWs. β II spectrin cKO myocytes were significantly more likely to generate SCaWs compared with control myocytes (Figure 5M-Q). SCaWs were observed in ~56% of β II spectrin cKO myocytes versus only ~18% of control myocytes (Figure 5K), and we observed <0.2 waves/WT myocyte versus an average of ~2 waves/ β II spectrin cKO myocyte ($p < 0.05$). Importantly, spontaneous unified Ca²⁺ release events (indicated by a perpendicular spontaneous dye front in the confocal line-scans) were frequently observed in β II spectrin cKO myocytes but not in control myocytes (Figure 5O-Q). Such perpendicular waves are indicative of a unified Ca²⁺ release within the myocyte, and provide evidence that the cell spontaneously reached threshold that initiated an action potential. Such a unified spontaneous Ca²⁺ release was not observed in control myocytes; rather only slowly propagating waves were observed (Figure 5M). This SCaW-dependent phenotype provides a mechanism for the afterdepolarizations observed in β II spectrin cKO myocytes.

β II spectrin is required for organization of cardiac membrane proteins

We hypothesized that loss of normal calcium cycling and arrhythmias in β II spectrin cKO mice reflected the central role for β II spectrin in organizing cardiac membrane proteins. To test this hypothesis, we first examined the status of T-tubule and SR membrane proteins

critical for calcium release. While we observed no difference in T-tubule L-type calcium channel localization or T-tubule structure between genotypes (Figure 6A-B), we observed heterogeneity in the subcellular localization of ryanodine receptor 2 (RyR₂), the primary cardiac SR calcium release channel in β II spectrin cKO myocytes (Figure 6D and Supplemental Figure 7). To further examine potential RyR₂ heterogeneity, we analysed RyR₂ localization by total internal reflection fluorescence (TIRF) and super-resolution imaging (see Supplemental Methods). In agreement with previous super-resolution imaging of Soeller and colleagues¹⁷, RyR₂ is localized by these techniques to discrete clusters of irregular shapes/sizes (Figure 6E). In contrast, RyR₂ imaging of heterogeneous regions of interest (i.e. red * in Figure 6D, right) in β II spectrin cKO myocytes showed reduced size/intensity of RyR₂ clusters as well as irregular intensity patterning compared with control myocytes (Figure 6F). Consistent with these findings RyR₂ levels were significantly reduced in β II spectrin cKO hearts (Figure 6G-H). This loss was selective for RyR₂ versus other SR proteins as we observed no difference in the expression or localization of the SR calcium ATPase (SERCA2) or in the intercalated disc protein N-cadherin (Figure 6C). In summary, these data define β II spectrin as required for the selective local organization of RyR₂ calcium release channels. Of note, defects in local RyR₂ organization have been linked with aberrant calcium-dependent release, arrhythmia, and heart failure phenotypes in humans and animal models.^{18–20}

β II spectrin is required for expression and targeting of ankyrin-B

In addition to RyR₂, β II spectrin cKO mice displayed loss of expression and localization of ankyrin-B in adult heart (Figure 6I-M). These findings were initially unanticipated, as prior work implicates ankyrin-B as critical for β II spectrin targeting and localization in post-natal day two neonatal cardiomyocytes.¹³ To define the functional relationship of ankyrin-B and β II spectrin in myocyte localization, we evaluated β II spectrin and ankyrin-B localization in wild-type neonatal cardiomyocytes. As shown in Supplemental Figure 8, striated expression of ankyrin-B precedes β II spectrin at post-natal day 1 in mice. However, both proteins are expressed and co-localized by post-natal day three (Supplemental Figure 8). In support of a role of ankyrin-B in the targeting of β II spectrin and in agreement with prior work¹³, ankyrin-B cKO neonatal cardiomyocytes lack β II spectrin expression or striation at post-natal day 1 or day 3 (Supplemental Figure 8). However, we were surprised to observe that later in myocyte maturation (post-natal day 7), ankyrin-B expression was not requisite for β II spectrin expression. In fact, β II spectrin expression in post-natal day myocytes is equivalent to expression/staining observed in wild-type cardiomyocytes (Supplemental Figure 8). This is further illustrated in adult myocytes where we observe no difference in β II spectrin expression or localization in ankyrin-B cKO hearts (Supplemental Figures 9-10). Finally, β II spectrin cKO cardiomyocytes demonstrate normal expression and localization of ankyrin-B at post-natal day 1 and 3, but loss of ankyrin-B expression and targeting at post-natal day 7 (Supplemental Figure 8). Together, these findings demonstrate a complex relationship between ankyrin and β II spectrin in heart. More specifically, our data show that ankyrin-B plays a dominant role for β II spectrin targeting in immature, developing myocytes whereas in maturing and mature myocytes, β II spectrin assumes the dominant targeting role for ankyrin-B. While we assume this transition likely represents a shift in the maturity of the membrane/cytoskeletal network (i.e. myocyte transverse-tubules begin to develop ~7 days in

culture²¹), future work will be important to better define the underlying mechanisms. Notably, while β II spectrin cKO mice displayed reduced ankyrin-B expression, we observed no change in the expression of ankyrin-R (*Ank1*) in β II spectrin cKO adult heart compared to control (Figure 6N-O). However, ankyrin-G (*Ank3*), a third ankyrin gene product expressed in heart²², showed elevated expression in β II spectrin cKO hearts (Figure 6N-O), likely as a compensatory mechanism in response to reduced ankyrin-B levels.

β II spectrin is required for organization of ankyrin-B-associated membrane proteins

Ankyrin-B targets $\text{Na}^+/\text{Ca}^{2+}$ exchanger and Na^+/K^+ ATPase and controls regulation of dyadic proteins, including RyR_2 .²³ Consistent with our above findings, we observed a significant decrease in I_{NCX} in β II spectrin cKO myocytes (Figure 7A-B). These changes were confirmed by both immunoblot and immunostaining where we observed decreased expression of Na/Ca exchanger as well as Na/K ATPase (Figure 7C-E). Notably, despite the major alterations in electrical activity in β II spectrin cKO myocytes, we did not observe differences in I_{Na} current (peak, activation/ inactivation/late current) and $\text{Na}_v1.5$ and connexin43 protein levels were unchanged in β II spectrin cKO hearts (Figure 7C and Supplemental Figure 11). Together with our data on RyR_2 , these findings identify β II spectrin as essential for the localization of multiple membrane proteins required for myocyte Ca^{2+} regulation.

β II spectrin regulates α II spectrin and the myocyte microtubule network

While Na/Ca exchanger and Na/K ATPase loss is likely related to loss of ankyrin-B targeting in β II spectrin cKO mice, the mechanism for RyR_2 dysfunction is less clear. We hypothesized that this defect reflects an important role for β II spectrin in organizing local cardiac cytoskeleton. In metazoans, α - and β -spectrin form a submembrane lattice through formation of hetero-tetramers via anti-parallel N- and C-terminal interactions. However, the requirement of β II spectrin expression for the targeting and formation of the hetero-tetramer in heart is unknown. We examined the abundance of α II spectrin, an *in vivo* partner for cardiac β II spectrin. Consistent with prior reports demonstrating degradation of α -spectrin in the absence of adequate erythroid β -spectrin^{24–26}, α II spectrin levels were reduced nearly 50% in β II spectrin cKO hearts (Figure 7F-G). In line with these data, we observed reduced abundance of α II spectrin in β II spectrin cKO myocytes by immunostaining (Figure 7H-K). Based on these data, we further investigated the integrity of the cytoskeleton in β II spectrin cKO myocytes. While actin was unchanged in expression or localization in β II spectrin cKO mice, we observed significant increases in levels of β I spectrin and α - and β -tubulin in β II spectrin cKO hearts by both immunoblot and/or immunostaining (Figure 7L-O and Supplemental Figure 12), likely as a compensatory response to β II spectrin deficiency, and consistent with prior findings of tubulin remodeling in failing hearts.²⁷ However, levels of desmin, an intermediate filament protein critical for myocyte cytoskeletal infrastructure, were unchanged between control and β II spectrin cKO hearts (Supplemental Figure 12). In summary, these data demonstrate that myocytes lacking β II spectrin display significant, yet selective cytoskeletal remodeling.

β II spectrin cKO mice display accelerated heart failure phenotypes

Heart failure is characterized by significant electrical and structural remodelling. Based on severe electrical and structural phenotypes present in β II spectrin cKO mice at baseline, we hypothesized that β II spectrin cKO mice would display accelerated and more pronounced cardiac damage following induction of heart failure through transverse aortic constriction (TAC). Notably, unlike control littermates or sham β II spectrin cKO mice, β II spectrin cKO mice displayed cardiac arrhythmia and death associated with major structural remodelling following six weeks of banding (Figure 8A-G). Examination of β II spectrin cKO mouse banded sections revealed high prevalence of widespread myocardial degeneration of the LV free wall and septum that was characterized by vacuolation, pallor, interstitial and ventricular myocyte necrosis (Figure 8A-D). Further, at six weeks post-banding β II spectrin cKO mice displayed severe AV block, ST segment depression, frequent PVCs, and junctional rhythms (Figure 8E-G). While we observed standard pre-heart failure phenotypes in control mice in response to the TAC protocol, we did not observe the extensive electrical or structural phenotypes found in β II spectrin cKO littermates.

Discussion

The spectrin superfamily is comprised of two α - and five β - spectrin genes. Most information regarding spectrin function comes from the erythrocyte, although a growing body of literature points to key roles of spectrin family members in cytoskeletal infrastructure in complex cells. In the red blood cell, α - and β -spectrin tetramerize and form the basis of the submembrane ultrastructure through interactions with membrane proteins (e.g. anion exchanger) and cytoskeleton (actin). Mutations in α - or β -spectrin in humans or animals result in loss of membrane integrity, spherocytosis, and hemolytic anemia.²⁸ In fact, spectrin mutations are the cause of the most common forms of hereditary spherocytosis in the Caucasian population. In complex cells, spectrins are critical for membrane assembly and maintenance and both α II and β II spectrin deficient mice are embryonic lethal.^{29, 30} β II spectrin is required for lateral membrane formation of columnar epithelial cells in the lung³¹ and β II and β IV spectrin are critical for development and maintenance of the axon initial segment and nodes of Ranvier in the central nervous system.³²⁻³⁴ Little is known on the role of β II spectrin in heart as mice homozygous for β II spectrin allele deficiency die *in utero*.³⁰ However, data from the β III spectrin literature offers insight into the likely mechanisms for β II spectrin function in heart. For example, work from Stankewich, Morrow, Ranum, and colleagues demonstrate that β III spectrin is essential for membrane protein targeting in the nervous system.^{35, 36} Similar to our findings in heart, targeted deletion of β III spectrin in brain results in impaired assembly of the post-synaptic membrane, endomembrane retention of multiple synaptic proteins, as well as ataxia and seizure phenotypes.³⁶ Moreover, human β III spectrin gene mutations found in the region where β III spectrin associates with the dynactin subunit Arp1³⁷ cause human spinocerebellar ataxia (SCA5) due in to defects in membrane protein (glutamate transporter EAAT4, metabotropic glutamate receptor 1 α) targeting.^{35, 38} Thus, based on β III spectrin data, as well as our new findings, we hypothesize that β II spectrin is a critical player in membrane protein sorting, versus simply a static membrane structural protein. In fact, the demonstrated links between spectrins and dynactin³⁷ provide a logical rationale for the cell and molecular phenotypes observed in β II

spectrin cKO animals. While this study focused on the relationship between β II spectrin and ankyrin-B, based on our new data (Figure 6N-O), it will be important to identify the relationship of β II spectrin with other cardiac ankyrin and spectrin gene products.

An important, but unexpected finding of this study is the essential role of β II spectrin for the local organization of RyR₂. The cardiac RyR₂ is a central player for myocyte calcium release and RyR₂ dysfunction has been linked with a broad spectrum of heart failure and arrhythmia phenotypes in human and animal models.^{39–42} Our data demonstrate a role of β II spectrin for both the expression and targeting of RyR₂. However, potentially more significant, remaining RyR₂ populations in β II spectrin cKO myocytes are disorganized, displaying a heterogeneous pattern. In parallel, loss of β II spectrin results in reorganization of the microtubule network that may underlie the heterogeneous RyR₂ distribution. Alternatively, microtubule reorganization may be a compensatory response of the cell to β II spectrin loss, as we also identified increased expression of β I spectrin (Supplemental Figure 12). Notably, abnormal organization of the calcium release structure was also identified in a mutant mouse model harboring a calsequestrin mutation linked with human catecholaminergic polymorphic ventricular tachycardia (CPVT).⁴² These mice, like the β II spectrin cKO mice, display unstable electrical events and significant cytosolic calcium management phenotypes. Beyond spectrin, junctophilin, a lipophilic molecule anchored to the junctional SR, has also been linked with the organization of the ryanodine receptor in heart. In fact, *JPH2*^{-/-} mice are embryonic lethal and display an enlarged dyadic cleft.⁴³ While organization of the RyR₂ is altered in β II spectrin cKO mice, we observed normal localization and expression of T-tubule L-type calcium channels (Ca_v1.2). Further, we observed no difference in T-tubule morphology between control and β II spectrin cKO mice through Di-8-ANEPPS imaging. Therefore, the β II spectrin pathway appears to regulate select populations of membrane proteins without altering membrane morphology. It will be critical for future experiments to define the specific pathways underlying β II spectrin-dependent regulation of RyR₂ clusters. Importantly, β II spectrin-dependent regulation of RyR₂ clusters is likely independent of ankyrin-B, as ankyrin-B^{+/-} mice display no defects in RyR₂ expression or localization.¹⁰ Finally, our work supports a relationship between β II spectrin and the microtubule system. As spectrins have previously been linked with microtubule-based proteins including kinesin II and the dynein complex^{44–46}, and spectrin-associated proteins including protein 4.1R have been linked with arrhythmia in animals⁴⁷, work to integrate the role of the β II spectrin pathway with intracellular targeting versus cytoskeletal organization will be an important future area of research. Likewise, as our data show accelerated heart failure phenotypes in β II spectrin cKO mice (Figure 8), it will be important to investigate the long-term function of β II spectrin in well-phenotyped human and animal disease models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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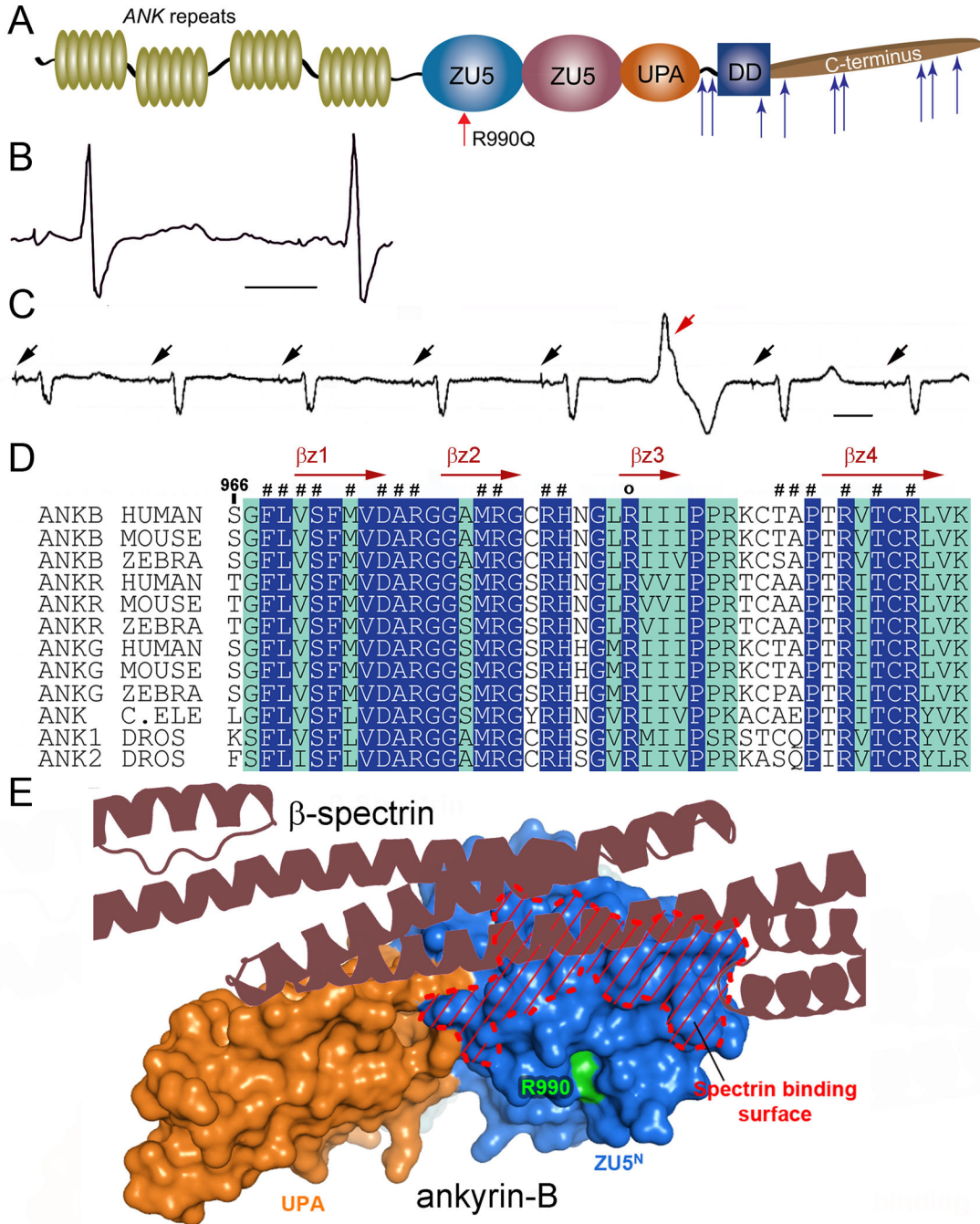


Figure 1. Ankyrin-B arrhythmia variant identified in conserved spectrin-binding domain. (A) Ankyrin-B includes ANK repeats, a spectrin-binding domain comprised of two ZU5 and one UPA domain, and a regulatory domain comprised of a death and C-terminus. Identified ANK2 loss-of-function mutations are noted by blue arrows and novel p.R990Q variant is indicated in red. (B) ANK2 p.R990Q proband displays QT-prolongation. (C) 10 second rhythm strip in p.R990Q proband demonstrating atrial demand pacing (black arrows) with premature ventricular contraction (red arrow). (D) Sequence alignment of ankyrin-B

spectrin-binding sequence. Residues that are absolutely conserved and highly conserved are in blue and green, respectively. Secondary structural elements are indicated above the alignment. p.R990Q is conserved across species and marked with “o”. (E) Structure of the ZU5^N-UPA tandem of ankyrin-B SBD reveals the spectrin-binding surface and location of p.R990 (*green*).

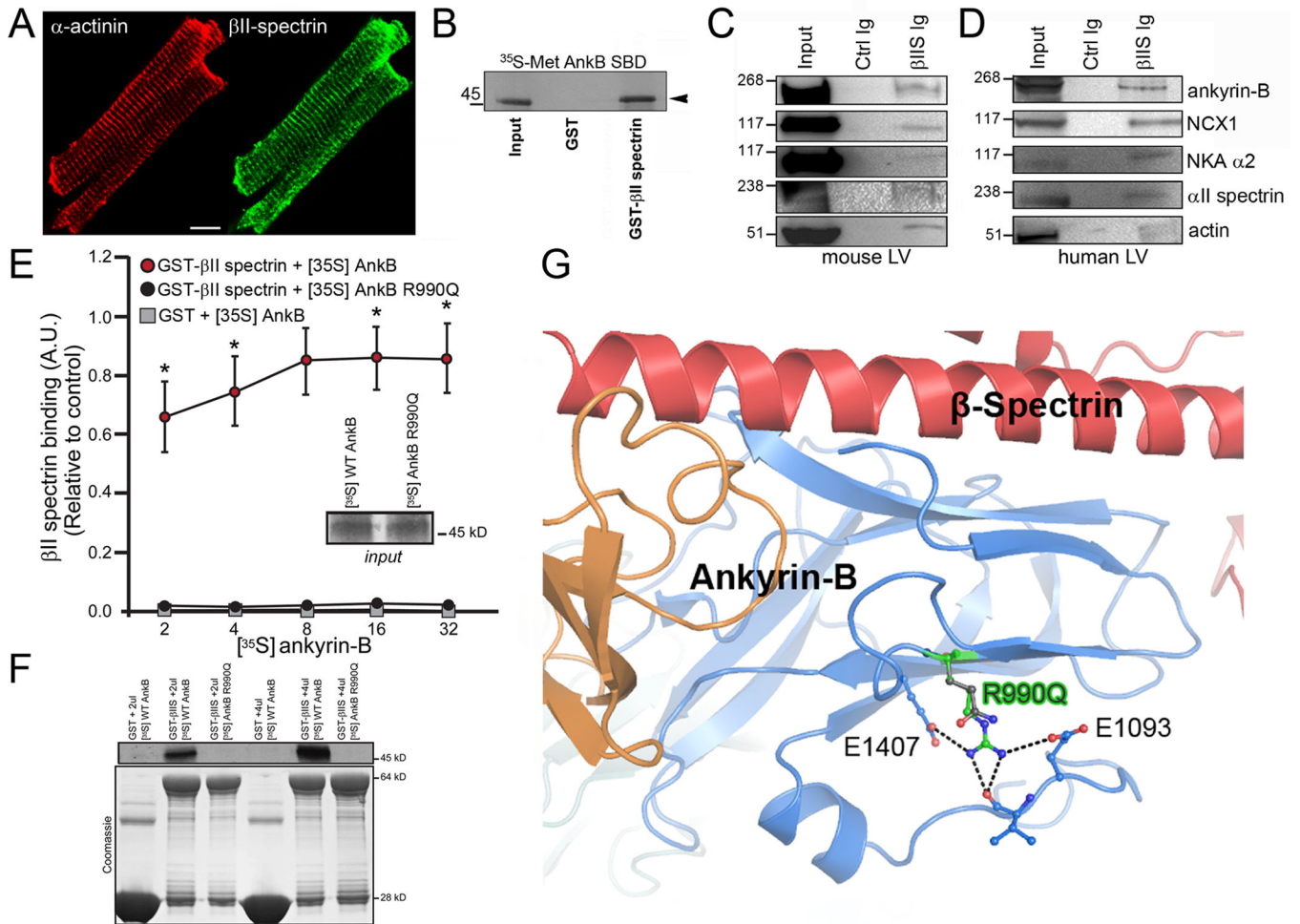


Figure 2.

Ankyrin-B/ β II spectrin complex is blocked by human disease mutation. (**A**) β II spectrin (green) is localized in a striated pattern in isolated mouse myocytes (co-labelled with α -actinin, red). Scale=10 μm . (**B**) Radiolabelled ankyrin-B (spectrin-binding domain) associates with GST β II spectrin fusion protein, but not GST. (**C-D**) β II spectrin (β IS) Ig co-immunoprecipitates ankyrin-B, NCX1, Na/K ATPase, α II spectrin, and actin from detergent soluble lysates of non-failing mouse and human LV. (**E**) Human ANK2 p.R990Q arrhythmia variant displays aberrant β II spectrin-binding. Data in inset represents equal inputs for experiments. Curves denote binding for GST- β II spectrin or GST alone with a concentration range of radiolabelled ankyrin-B or radiolabelled ankyrin-B R990Q (n=5/group; * represents p<0.05). Panel **F** represents primary binding data and Coomassie Blue-stained gels for fusion proteins in **E**. (**G**) Ribbon structure from co-crystal structure of ankyrin-B/ β II spectrin. Human p.R990Q variant shown in green.

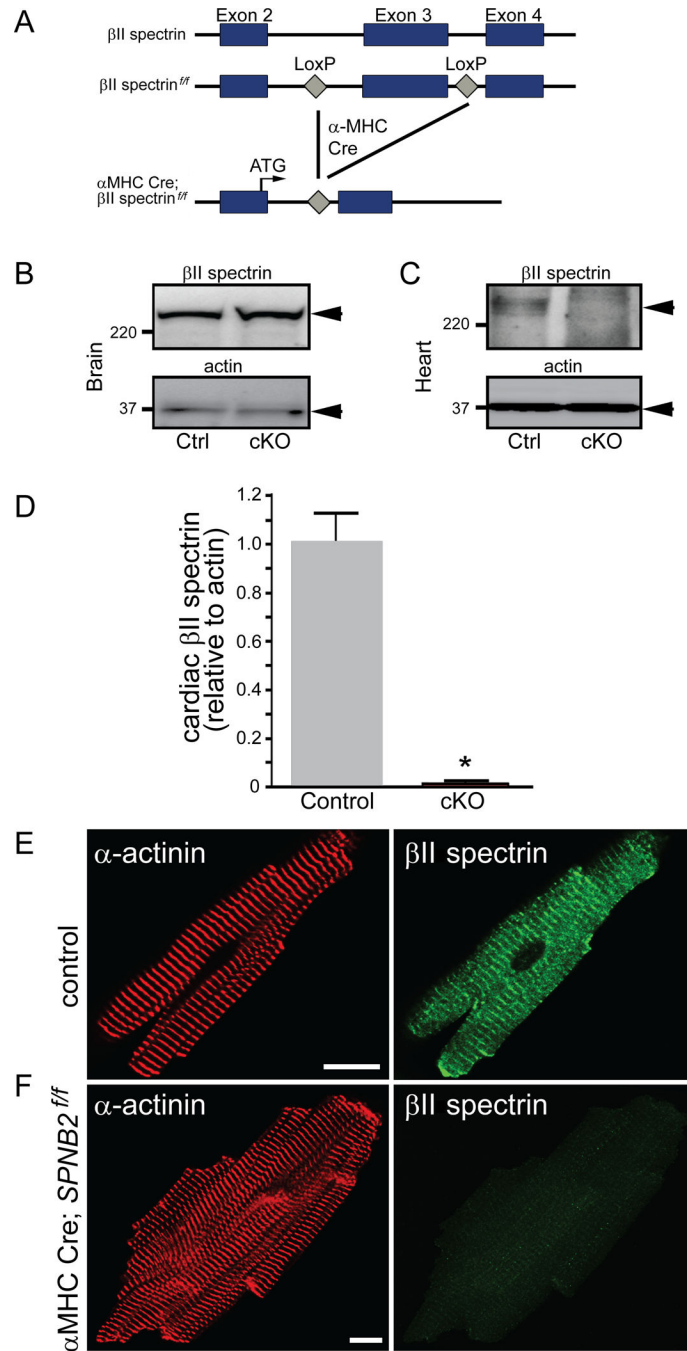


Figure 3. Generation and validation of mice lacking β II spectrin in cardiomyocytes. **(A)** Targeting strategy to generate Cre-dependent loss of cardiac β II spectrin. β II spectrin in brain **(B)** and heart **(C)** of control and β II spectrin cKO mice. **(D)** β II spectrin levels in control (n=5) versus β II spectrin cKO hearts (n=5; $p < 0.05$). **(E)** β II spectrin localization in control and β II spectrin cKO adult ventricular myocytes. Bar= 10 μ m.

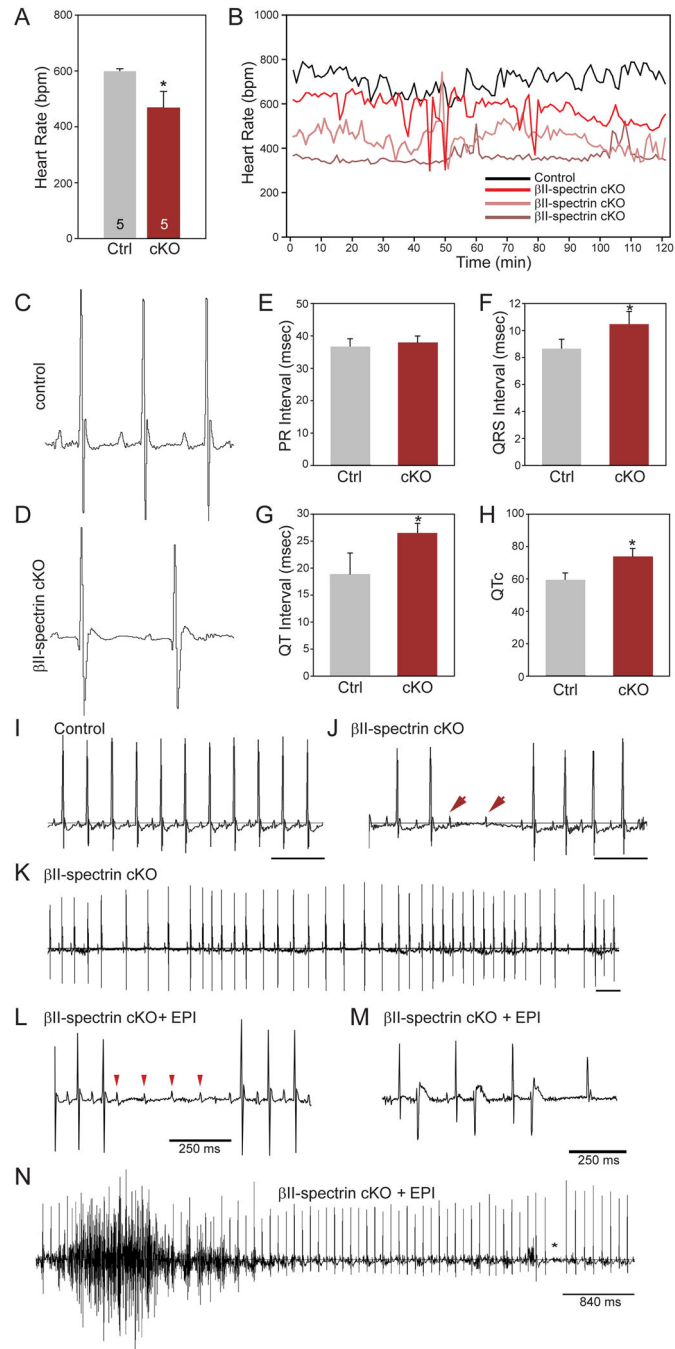


Figure 4.

Loss of β II spectrin causes bradycardia, rate variability, and arrhythmia. (**A-B**) β II spectrin cKO mice display reduced heart rate (n=5) as assessed by telemetry compared to control mice (n=5, $p < 0.05$). (**B**) Heart rates of control and three β II spectrin cKO mice that show bradycardia and rate variability. (**C-D**) ECGs from control and β II spectrin cKO mouse showing increased RR, QRS, and QT intervals. Mean data for parameters are shown in **E-H** (n=5 mice/genotype; $p < 0.05$). (**I-J**) Control ECG recording over 1-second demonstrating no R-R variability or heart block vs. ECG recording from β II spectrin cKO littermate

demonstrating type II heart block, confirmed by P-waves (arrow heads) without ventricular conduction. (**K**) 3-second ECG recording of β II spectrin cKO mouse demonstrating significant R-R variability with heart block. (**L-N**) β II spectrin cKO mice demonstrate severe arrhythmia phenotypes and death following injection of epinephrine. Examples include (**L**) four sinus P-waves (arrow heads) without ventricular conduction consistent with type II AV block, (**M**) bigeminy, and (**N**) polymorphic ventricular arrhythmia.

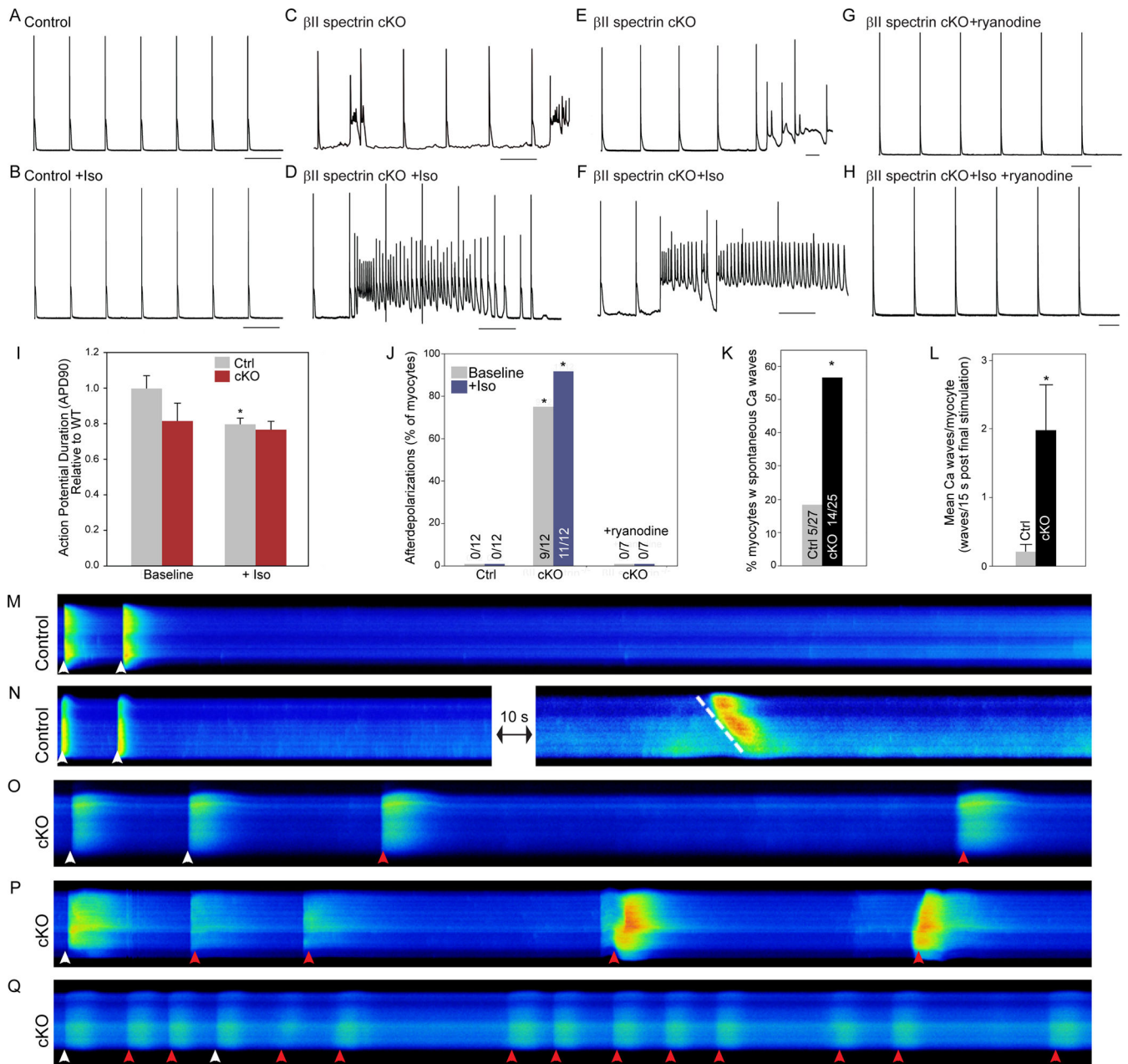


Figure 5. β II spectrin cKO myocytes display electrical instability, afterdepolarizations, and aberrant Ca waves. Action potential measurements of (A-B) control and (C-D) β II spectrin cKO myocytes measured at baseline at 0.5 Hz pacing protocol \pm 1μ M Iso. (E-F) Electrical instability was present in β II spectrin cKO myocytes independent of pacing frequency (shown at 1 Hz). (G-H) Ryanodine (100 nM) blocked abnormal electrical instability of β II spectrin cKO myocytes \pm Iso. (I) Mean APD₉₀ of control and β II spectrin cKO myocytes \pm Iso ($n > 10$ myocytes/genotype; $p < 0.05$ for control vs. control + Iso). (J) Prevalence of afterdepolarizations for control and β II spectrin cKO myocytes \pm Iso and in the presence of ryanodine ($n > 10$ myocytes/treatment; $p < 0.05$ for control versus cKO at baseline; $p < 0.05$ for

control +Iso versus cKO +Iso). **(K-L)** β II spectrin cKO myocytes were more likely to form spontaneous Ca^{2+} waves (n=27 control, n=25 β II spectrin cKO; $p < 0.05$). **(M-Q)** Linescan images of fluo-4-loaded myocytes field stimulated at 0.5 Hz. Following stimulation, myocytes were continuously monitored for spontaneous Ca^{2+} wave formation for 15 sec. **(M-N)** Control myocytes with no spontaneous wave activity. When waves formed they were slow moving (N, dashed white line). **(O-Q)** Spontaneous waves in β II spectrin cKO myocytes (red arrowheads). Stimulated transients are indicated by white arrowheads.

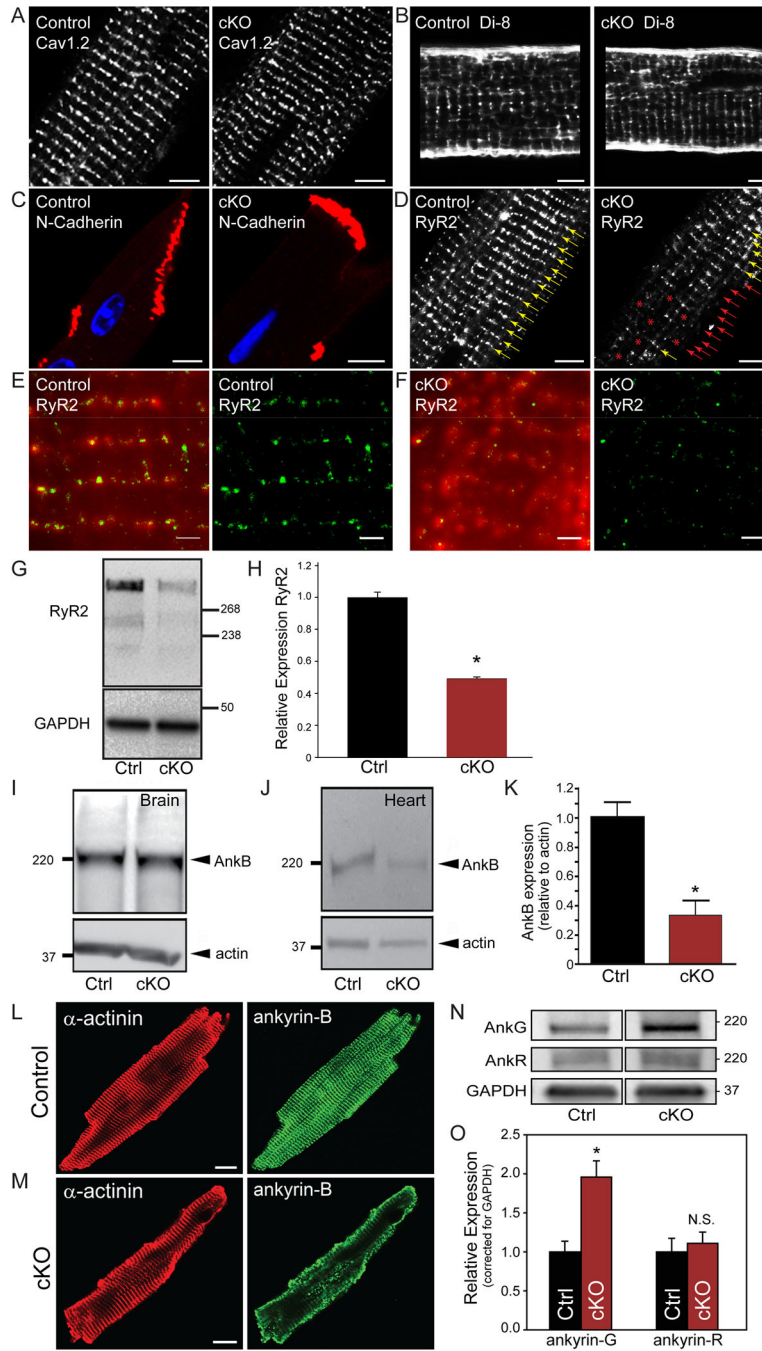


Figure 6. β II spectrin is required for organization of calcium release units and ankyrin-B. Like control mouse myocytes, β II spectrin cKO myocytes display normal localization of transverse-tubule $\text{Ca}_v1.2$ (A), transverse-tubule organization (B, visualized by Di-8-ANEPPs), and intercalated disc N-cadherin (C). In contrast, RyR_2 expression was reduced and heterogeneous in β II spectrin cKO myocytes (D, red *). (E-F) RyR_2 expression was further analyzed by total internal reflection fluorescence (TIRF) and super-resolution imaging. (E-F, left) Overlay of a TIRF image (red) and the corresponding super-resolution image (green)

of RyR₂ in control (**E**) and β II spectrin cKO (**F**) cardiomyocyte. (**E-F, right**) Super-resolution images of RyR₂ in control and β II spectrin cKO myocytes. Note that data in **F** was collected from area of RyR₂ heterogeneity (red * cKO panel **D, right**) that illustrates reduced RyR₂ cluster size and intensity. Scale bar for **E-F**: 1000 nm. (**G-H**) RyR₂ levels were significantly reduced in β II spectrin cKO myocytes (n=4 hearts/genotype, p<0.05). (**I-K**) Ankyrin-B levels are decreased in the heart but not brain of β II spectrin cKO mice (n=5 hearts/genotype; p<0.05). (**L-M**) Ankyrin-B shows reduced expression and abnormal targeting in β II spectrin cKO versus control myocytes (Bar=10 microns). (**N-O**) Ankyrin-G, but not ankyrin-R levels are decreased in the heart of β II spectrin cKO mice (n=5) versus control mice (n=6; p<0.05).

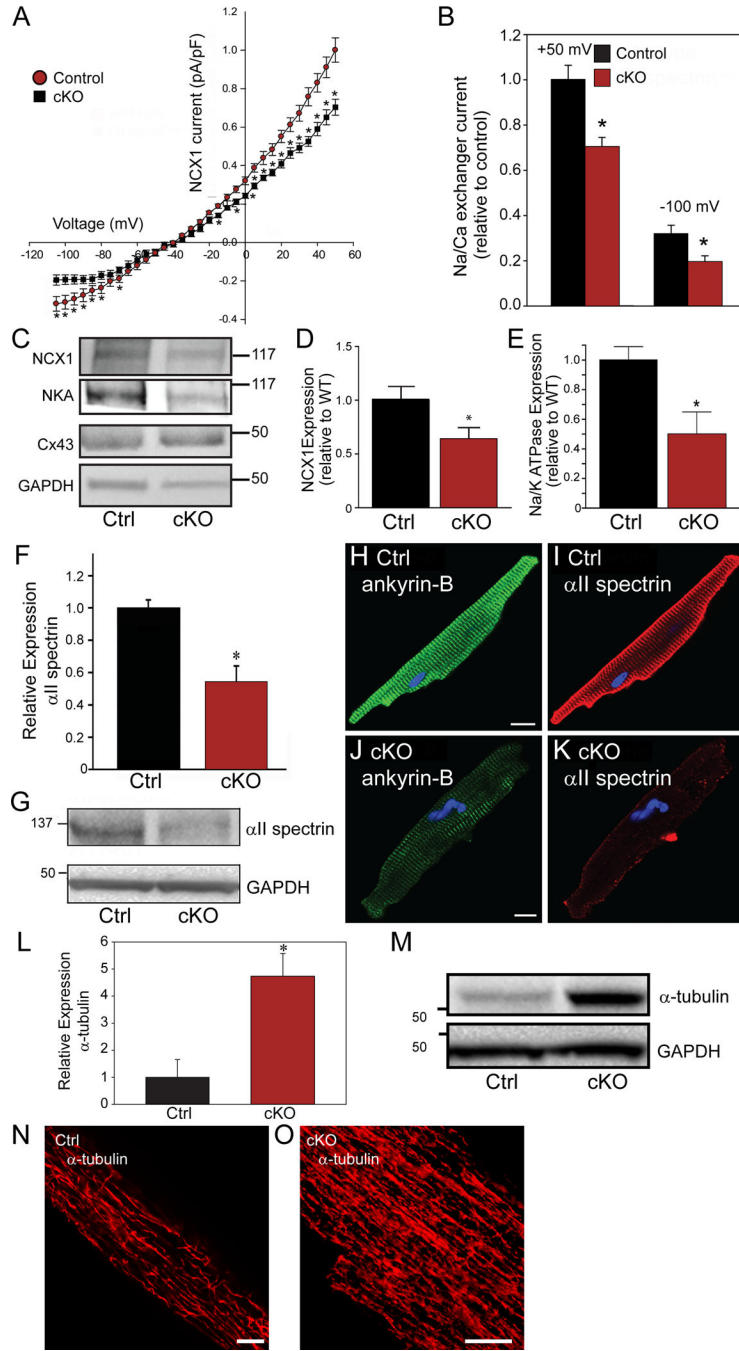


Figure 7.

β II spectrin deficiency results in reduced expression of ankyrin-B-associated membrane proteins and abnormal myocyte cytoskeletal organization. (A-B) β II spectrin cKO myocytes (n=12) display reduced I_{NCX} compared with control mouse myocytes (n=14; $p < 0.05$). (C-E) β II spectrin cKO hearts display reduced NCX and Na/K ATPase expression but normal expression of Cx43 compared with control hearts (n=4 hearts/genotype; $p < 0.05$). (F-K) β II spectrin cKO hearts (n=5) display reduced α II spectrin expression and localization compared with control hearts (n=5) by immunoblot and immunostaining ($p < 0.05$). (L-O) β II

spectrin cKO hearts display significant increase in α -tubulin expression by immunoblot and immunostaining compared to control hearts (n=5 hearts/genotype for panel L; p<0.05). Bar=10 μ M for panels N-O.

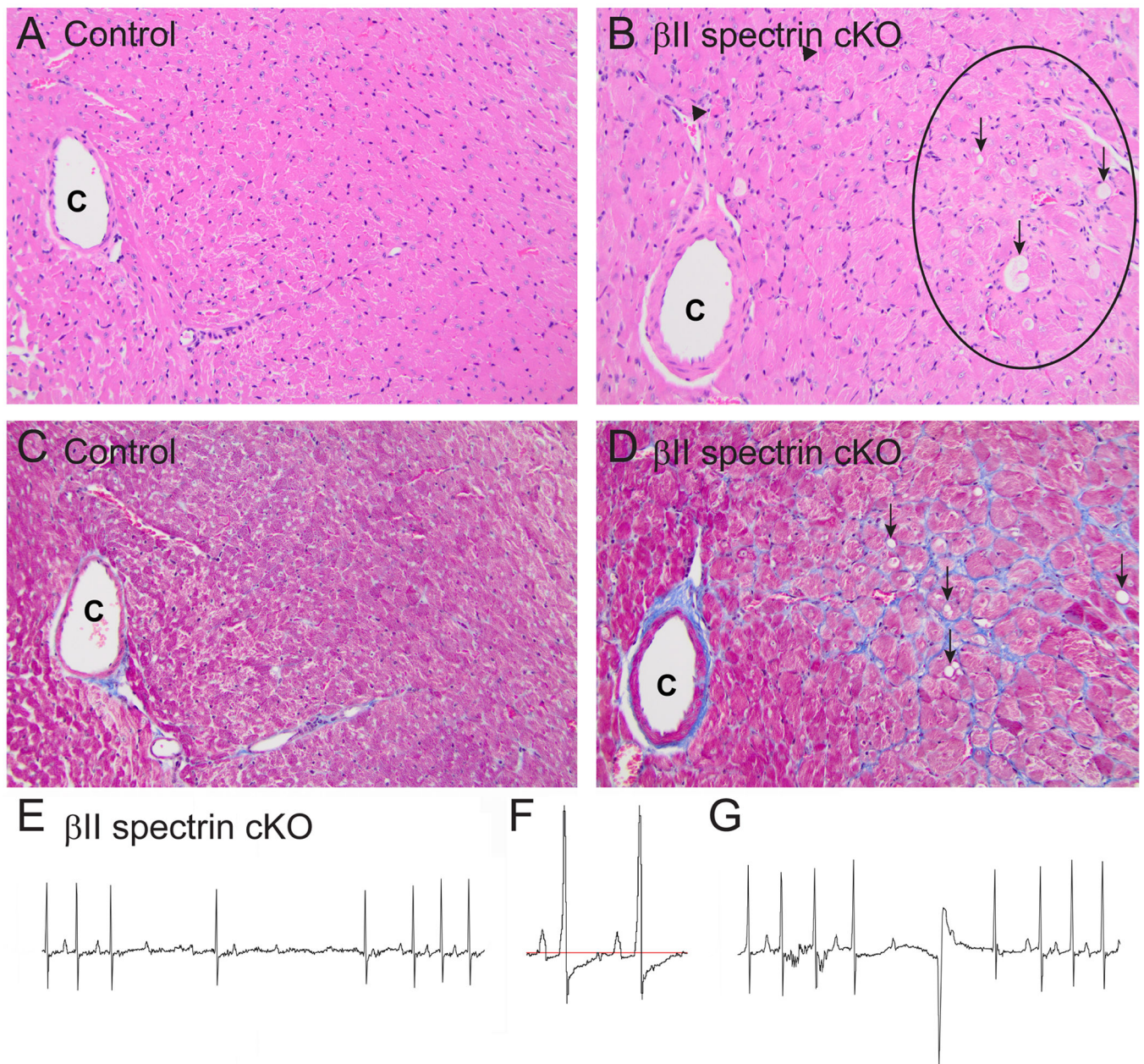


Figure 8.

β II spectrin cKO mice show severe damage and electrical phenotypes following aortic banding. (A-B) Unlike control TAC mice, β II spectrin cKO mice displayed widespread myocardial degeneration of the LV free wall and septum, characterized by vacuolation (arrows), pallor (inside circle), and necrosis of myocytes (C=coronary artery; H&E staining; 200x). (C-D) β II spectrin cKO hearts further displayed increased interstitial fibrosis (blue) of connective tissue compared with control sections (vacuolation noted by arrows; 200x). Electrical phenotypes observed in β II spectrin cKO and not control mice included (E) AV

conduction defects, (F) ST segment depression, and (G) intermittent PVCs and junctional beats.