Defining new mechanistic roles for αII spectrin in cardiac function

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Spectrins are cytoskeletal proteins essential for membrane biogenesis and regulation and serve critical roles in protein targeting and cellular signaling. αII spectrin (SPTAN1) is one of two α spectrin genes and αII spectrin dysfunction is linked to alterations in axon initial segment formation, cortical lamination, and neuronal excitability. Furthermore, human αII spectrin loss-of-function variants cause neurologic disease. As global αII spectrin knockout mice are embryonic lethal, the in vivo roles of αII spectrin in adult heart are unknown and untested. Here, based on pronounced alterations in αII spectrin regulation in human heart failure we tested the in vivo roles of αII spectrin in the vertebrate heart. We created a mouse model of cardiomyocyte-selective αII spectrin-deficiency (cKO) and used this model to define the roles of αII spectrin in cardiac function. αII spectrin cKO mice displayed significant structural, cellular, and electrical phenotypes that resulted in accelerated structural remodeling, fibrosis, arrhythmia, and mortality in response to stress. At the molecular level, we demonstrate that αII spectrin plays a nodal role for global cardiac spectrin regulation, as αII spectrin cKO hearts exhibited remodeling of αI spectrin and altered β-spectrin expression and localization. At the cellular level, αII spectrin deficiency resulted in altered expression, targeting, and regulation of cardiac ion channels NaV1.5 and Kv4.3. In summary, our findings define critical and unexpected roles for the multifunctional αII spectrin protein in the heart. Furthermore, our work provides a new in vivo animal model to study the roles of αII spectrin in the cardiomyocyte.

Cardiovascular disease is the number one cause of mortality in the United States, accounting for ~31% of all deaths (1). Although significant initiatives in both prevention and therapeutics have reduced cardiovascular mortality, the field lacks effective solutions for a host of cardiovascular phenotypes. Furthermore, the literature is just beginning to address the striking complexity of organ, cell, and molecular pathways linked with human cardiovascular disorders. Thus, there remains a clear need to understand the fundamental cellular and molecular pathways associated with normal cardiac function as well as dysfunction of critical pathways in cardiovascular disease.

Cardiac function requires finely tuned integration of myocyte mechanical and electrical pathways. Membrane-associated ion channels, transporters, receptors, signaling proteins, and cell adhesion molecules are important for cardiac function. The organization of these proteins within the vertebrate myocyte is essential for normal excitation-contraction coupling. In fact, decades of research have illustrated the role of the submembrane cytoskeleton and cytoskeletal-associated proteins in excitable cell biology (2–7). Moreover, in the heart alone, defects in myocyte cytoskeletal proteins have been linked with a host of structural and electrical phenotypes in human cardiovascular disease as well as in animal disease models (4).

Spectrins are cytoskeletal proteins, initially identified through their roles in erythrocyte structure and flexibility (8–10). In diverse tissues, spectrins act as an essential link between the actin-based cytoskeleton and membrane, serving to maintain cellular structure and polarity while providing flexibility and strength. In excitable cells and nonexcitable cells, spectrins form complexes with ankyrines and membrane-associated proteins, playing essential roles in the localization and regulation of critical ion channels and transporters (11–14). In humans, there are five β but only two α spectrin genes (15). SPTAN1 encodes nonerythroidic αII spectrin, which forms functional heterotetramers with β spectrins (16). αII spectrin has been extensively studied in neurons due in part to its link to human neurologic disease. Variants in SPTAN1 are linked to
early infantile epileptic encephalopathy, type 5 (EIEE5) or West Syndrome, characterized by refractory seizures, intellectual arrest/regression, agenesis of the corpus callosum, and hypomyelination, carrying a poor prognosis (11, 17, 18). Beyond the brain, recent work has defined roles for β-spectrins in cardiac structure, excitability, and signaling and linked dysregulation in β spectrin pathways with both acquired and congenital forms of human cardiovascular disease (19, 20). In contrast, the number of studies on αI spectrin in heart are limited (21–26), and the in vivo roles of cardiac α spectrins are essentially unstudied.

Here, we report dysregulation of αI spectrin in human heart failure. Moreover, using a newly engineered model of cardiomyocyte-specific deletion of αI spectrin, we illustrate the role of αI spectrin in normal cardiac physiology. αI spectrin cKO mice display both structural and electrical phenotypes at baseline that are intensified by physiological and pathological stress. Furthermore, we illustrate striking post-transcriptional roles for αI spectrin for global spectrin family protein regulation and unanticipated roles for αI spectrin in ion channel expression, targeting, and regulation. Together, our findings define critical in vivo roles for αI spectrin in the stabilization of the cardiomyocyte spectrin network, ion channel regulation, cardiac electrophysiology, structure, function, and stress response.

Results

**all spectrin regulates cardiac function**

**Creation and validation of in vivo model of cardiomyocyte αI spectrin deficiency**

Based on the critical role of spectrins in excitable cells and dysregulation in heart failure (Fig. 1), we investigated the role of αI spectrin for in vivo cardiac function. To date, due to the embryonic lethality of global αI spectrin knockout mice (29), the role of αI spectrin in the adult heart is unknown and unstudied. Thus, we generated a cardiomyocyte-specific αI spectrin mouse knockout model (αI spectrin cKO) using the Cre-lox system in which exon 8 of *Sptan1* is flanked by loxP sites (30) and the expression of Cre recombinase is driven under the α-myosin heavy chain promoter (Fig. 2A) (31).

αI spectrin cKO mice are viable, showed no gross visible phenotypes, and reproduced normally. As expected, cardiomyocyte preparations from αI spectrin cKO mouse myocytes displayed near-complete loss of αI spectrin protein expression (Fig. 2, B–D). This loss was selective for cardiomyocytes as αI spectrin protein levels were similar between control and αI spectrin cKO cerebellum (Fig. 2, E–G) and skeletal muscle. In the control mouse heart, αI spectrin is highly expressed and localizes to the Z-disc, lateral membrane, and intercalated disc (Fig. 2, H and I). Notably, this intracellular distribution does not completely overlap with αI spectrin (localized to the Z-disc and lateral membrane, absent from the intercalated disc (25)) suggesting potential unique roles for αI spectrin versus αI spectrin. Consistent with immunoblot data, αI spectrin cKO mice displayed complete loss of αI spectrin in the cardiomyocyte by immunofluorescence (Fig. 2). Thus, the newly generated knockout mouse line provides a viable model to study in vivo αI spectrin deficiency in cardiomyocytes.

**all spectrin cKO mice display cardiac dysfunction**

To test the in vivo role of αI spectrin in vertebrate heart, we performed detailed cardiac structural and functional phenotyp-
all spectrin regulates cardiac function

Figure 2. Generation and validation of all spectrin cKO mice. A, strategy for cardiomyocyte-specific knockout of all spectrin in mice. B–G, representative and quantification of immunoblots for all spectrin normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in isolated adult ventricular myocytes and cerebellum from all spectrin cKO and control mice. Results demonstrate (B–D) reduction of full-length (>250 kDa) and degraded (~150 kDa) all spectrin from isolated adult ventricular myocytes from all spectrin cKO mice compared with control mice (n = 8.5 for control and all spectrin cKO mice, respectively, p < 0.0001). E–G, no differences in all spectrin levels were observed in cerebellum. Values are represented as mean ± S.E. H–J, staining of all spectrin (red) and desmin (green) in adult cardiomyocytes isolated from (H) control and (J) all spectrin cKO mice. I, magnified image of control myocytes demonstrates localization of all spectrin at lateral membrane (white arrows), Z-lines (white arrowheads), and intercalated disc (yellow arrowheads). Scale bars = 20 µm.

ing of all spectrin cKO mice and control littermates. In adult mice (20–24 weeks of age), we observed a significant decrease in stroke volume, ejection fraction, and fractional shortening, indicative of early stages of heart failure and/or cardiac remodeling (Fig. 3, A–F). Furthermore, all spectrin cKO mice displayed cardiomyocyte hypertrophy, as assessed by histologic analysis of cross-sectional cardiomyocyte size (Fig. 3L, Fig. S2). However, this did not translate into gross increases in heart weight, even when normalized to body weight or to tibia length (Fig. S3). Histologically, we observed increased cardiac fibrosis in all spectrin cKO mice, as assessed by automated quantification of Masson’s Trichrome-stained heart samples (Fig. 3, I–K). Fibrosis was not accompanied by an increase in apoptosis, as assayed by TUNEL staining (Fig. S4). Notably, this dysfunction develops with age, as all spectrin hearts from younger mice (12–16 weeks of age) displayed no significant changes in cardiac structure or function and no fibrosis, necrosis, or hypertrophic phenotypes (Fig. S5). Thus, all spectrin deficiency promotes an age-dependent decline in cardiac ejection fraction and increased cardiac fibrosis.
We next tested the impact of spectrin deficiency on cardiac excitability. We observed electrical dysfunction in spectrin cKO mice, regardless of age. In young 12–16-week-old mice, prior to structural dysfunction, we observed ECG phenotypes in spectrin cKO mice compared with control mice including minor, but significant increases in P wave and QRS duration by conscious recordings (Table S1; no change in QRS by DSI telemetry), indicating slowed conduction through the atria and ventricles. Notably, this slowed conduction is not associated with reduced levels or altered localization of connexin 43 (Fig. S6).

Treatment of conscious spectrin cKO, but not control animals with epinephrine revealed significant differences in heart rate as well as PR, QT, and rate corrected QT (QTc) intervals when compared with control littermates (Table S1). Furthermore, epinephrine-treated spectrin cKO mice (but not control littermates) displayed arrhythmia phenotypes including sustained runs of premature ventricular contractions (PVCs) (Fig. 4, A–C). In summary, 12–16-week-old spectrin cKO mice display defects in electrical activity that precede cardiac structural and functional phenotypes. Furthermore, electrical phenotypes are increased by catecholamine treatment.

**all spectrin regulates cardiac function**

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**all spectrin cKO mice display in vivo electrical dysfunction**

We next tested the impact of all spectrin deficiency on cardiac excitability. We observed electrical dysfunction in all spectrin cKO mice, regardless of age. In young 12–16–week-old mice, prior to structural dysfunction, we observed ECG phenotypes in all spectrin cKO mice compared with control mice including minor, but significant increases in P wave and QRS duration by conscious recordings (Table S1; no change in QRS by DSI telemetry), indicating slowed conduction through the atria and ventricles. Notably, this slowed conduction is not associated with reduced levels or altered localization of connexin 43 (Fig. S6).

Treatment of conscious all spectrin cKO, but not control animals with epinephrine revealed significant differences in heart rate as well as PR, QT, and rate corrected QT (QTc) intervals when compared with control littermates (Table S1). Furthermore, epinephrine-treated all spectrin cKO mice (but not control littermates) displayed arrhythmia phenotypes including sustained runs of premature ventricular contractions (PVCs) (Fig. 4, A–C). In summary, 12–16–week-old all spectrin cKO mice display defects in electrical activity that precede cardiac structural and functional phenotypes. Furthermore, electrical phenotypes are increased by catecholamine treatment.

**all spectrin is required for normal myocyte excitability**

Based on in vivo all spectrin cKO mouse phenotypes, we investigated electrical properties at the individual myocyte level. Compared with control myocytes, we observed statistically significant changes in action potential duration (APD) at 50, 75, and 90% repolarization (APD<sub>50</sub>, APD<sub>75</sub>, and APD<sub>90</sub>, respectively) or in maximum upstroke velocity (dV/dT) in all spectrin cKO myocytes (Fig. S7, A–C; 0.5 Hz). Furthermore, we observed a statistically significant increase in action potential amplitude (APA) in all spectrin cKO myocytes compared with control cells (Fig. S7, D). For example, unlike control myocytes that initiated normal action potentials at frequencies up to 10 Hz, all spectrin cKO myocytes began failing to excite at just 2 Hz, indicating a
all spectrin regulates cardiac function

Figure 4. Cardiac all spectrin loss results in arrhythmia. A, all spectrin cKO mice show an increase in premature ventricular contractions (PVCs) following epinephrine injection compared with control littermates at 12–16 weeks of age, before structural changes. B and C, sample ECG traces from control (B) or all spectrin (C) cKO mice. Red arrows indicate PVCs (n = 4 and 4 for all spectrin cKO and controls, respectively). Values are represented as mean ± S.E. * indicates a significant difference from controls.

key role of all spectrin in myocyte excitability (Fig. S7G). In summary, all spectrin cKO myocytes display multiple electrical phenotypes.

Defining all spectrin pathways in heart: all spectrin is required for cardiac spectrin family regulation

Our findings support significant in vivo and cellular defects in the absence of cardiac αI spectrin. We therefore directly tested the impact of αI spectrin deficiency on spectrin family regulation in heart. Consistent with our hypothesis, β spectrin proteins (obligate α spectrin-binding partners) were significantly reduced in all spectrin cKO cardiomyocytes. We observed a 77.9% reduction in βI spectrin protein levels with βII spectrin protein levels reduced 93.2% (Fig. 5, A–D). Remaining βII spectrin was normally localized (Fig. 5H). Normal mRNA expression of βI (Sptbh) and βII (Sptbn1) spectrin (Fig. 5G) support that altered protein levels are due to post-transcriptional regulation of β spectrins. αI spectrin, a protein unique to mammals, is structurally similar to αII spectrin, having likely arisen from a gene duplication of αII spectrin (32). Notably, in cardiomyocytes lacking αI spectrin, αI spectrin is strongly up-regulated at both transcript (Fig. 5G) and protein levels (Fig. 5, E and F).

In control cardiomyocytes, αI spectrin localizes to the Z-disc and lateral membrane, and is absent from the intercalated disc (Fig. 5, I and J). However, in αII spectrin cKO cardiomyocytes, αI spectrin is present at the intercalated disc (Fig. 5, I and J), supporting a putative compensatory role of αI spectrin in the αII spectrin cKO model. In summary, our data support a key role of αI spectrin expression for the normal regulation of both βI spectrin and βII spectrin expression in cardiomyocytes. Moreover, our findings support that αI spectrin levels are significantly increased in αII spectrin cKO heart as a potential compensatory mechanism to preserve cardiac function. However, our data suggest that this compensatory pathway is insufficient to restore normal function to αII spectrin cKO mice, potentially due to the inability of αI spectrin to associate with cardiac αII spectrin-binding partners.

all spectrin is required for cardiac Na\textsubscript{v}1.5 expression and function

Spectrin dysfunction is linked with altered voltage-gated Na\textsubscript{v} channel regulation in the heart and brain (2, 3, 6, 33–35). We therefore tested the impact of αII spectrin-deficiency on cardiac Na\textsubscript{v}1.5 regulation. Na\textsubscript{v}1.5 expression was significantly reduced in αII spectrin cKO hearts compared with hearts from control littermates (Fig. 6, A and B; reduced 59.5% when normalized for total protein expression). This decrease was likely due to post-translational dysregulation, as mRNA levels of Scn5a (encoding Na\textsubscript{v}1.5) were unchanged between control and αII spectrin hearts (Fig. 6C). In the control heart, Na\textsubscript{v}1.5, whereas present at multiple membrane domains, is preferentially expressed at the intercalated disc membrane (Fig. 6D). In αII spectrin cKO hearts, we observed modestly decreased intercalated disc staining relative to lateral membrane expression of Na\textsubscript{v}1.5 (Fig. 6, D and E). However, neither immunoblot nor immunofluorescent microscopy are quantitative measures of functional Na\textsubscript{v}1.5. To determine the function of the remaining Na\textsubscript{v}1.5, analysis by single-cell electrophysiology was conducted, which revealed nearly a 2-fold decrease in peak \(I_{\text{Na}}\) in αII spectrin cKO myocytes compared with littermate controls (Fig. 7, A and B). However, we observed no difference in voltage-dependent inactivation or time-dependent recovery from inactivation between myocytes from control and αII spectrin cKO hearts (Fig. 7, C and D). In summary, αI spectrin is required for normal Na\textsubscript{v}1.5 expression, localization, and function in mouse heart.

Identification of putative αII spectrin-dependent cardiac pathways

Reduced Na\textsubscript{v}1.5 (\(I_{\text{Na}}\)) in αII spectrin cKO hearts (Figs. 6 and 7) supports the observed decrease in αII spectrin cKO myocyte excitability (Fig. S7, E–G), but is striking in the context of a normal dv/dt\textsubscript{max}. As assessed by an unbiased partial least-squares regression analysis using the Hund–Rudy action potential model (36–39), Na\textsubscript{v}1.5-dependent dysregulation alone is unlikely to produce observed experimental data from control and αII spectrin cKO action potential measurements (e.g. APD, APA, and dv/dt\textsubscript{max}). Our computational analyses strongly predicted secondary current alterations in αII spectrin cKO myocytes, most specifically associated with several repolarizing potassium currents.

To support the computational model, we performed transcriptional analysis of mRNA expression of ion channel subunits in 12–16-week-old αII spectrin cKO versus control littermate hearts. In support of the modeling predictions, we observed significant changes in ion channel subunits involved
in cardiac repolarization, particularly potassium channel α- and β-subunits (Fig. S9). Analysis of mRNA expression of Kcnb2 (encoding Kv4.2) and Kcnb3 (encoding Kv4.3), major contributors to $I_{K_{peak}}$, $I_{TO,fast}$, revealed normal expression of Kcnb3, but a 0.57-fold decrease in Kcnb2 expression in αII spectrin cKO hearts (Fig. S9). Additionally, we observed a down-regulation of Kv1.7 (Kcnb7), a potassium channel subunit that modulates both $I_{K_{ur}}$ and $I_{TO}$ in αII spectrin cKO hearts. Although we did not observe a difference in the resting membrane potential of isolated αII spectrin cKO cardiomyocytes (Fig. S8), there was a significant down-regulation of Kv2.1 (Kcnj3) expression was decreased, whereas GIRK4 (Kcnj5) expression was unchanged in αII spectrin cKO hearts. Kcne1, linked with long QT syndrome type 5 (LQT5) showed increased expression, whereas Kcnq1, which is linked with long QT syndrome type 1, is unchanged in αII spectrin cKO hearts. Finally, Kcnip2 (Kchip2), Kenb1 (contributing to $I_{K_{SS}}$), Kcnb5 (contributing to $I_{K_{ur}}$), Kcnj8 (Kv6.1), Kcnj11 (Kv6.2), Kcnj12 (Kv2.2), and Kcnj14 (Kv2.4) were all similarly expressed between control and αII spectrin cKO hearts (Fig. S9). Thus, whereas we observed dysregulated expression of many potassium channels, many remained unchanged, demonstrating a selective disruption of potassium channel subunit expression in αII spectrin cKO hearts.

Beyond potassium channel subunits, we observed alterations in other ion channel subunits. Although the mRNA expression

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**Figure 5. Loss of cardiac αII spectrin results in broad spectrin dysregulation.** A, C, and E, representative, and B, D, and F, quantification, of immunoblots of cardiomyocytes for β, βII, and αII spectrin (n = 7 and 5 for control and αII spectrin cKO, respectively, p value $<0.0001$, $<0.0001$, = 0.043 for β, βII, and αII spectrin, respectively). G, mRNA expression of Sptb, Sptbn1, and Spta1 in adult cardiac tissue (n = 8 and 8, p = 0.57). H–J, immunofluorescence staining of αII spectrin (yellow arrowhead) in all spectrin cKO tissue compared with controls. Scale bar = 20 μm. J, magnified images of intercalated discs. Scale bar = 5 μm.
of the α-subunit of Na\(_{\text{v}}\)1.5, Scn5a, was unchanged, the expression of the β1-subunit, Scn1b, was up-regulated ~2-fold in αII spectrin cKO hearts (Fig. S9). Furthermore, we noted an increase in the expression of two α subunits of the L-type calcium channel (Cacna1s and Cacna1c) accompanied by an up-regulation of the β1 (Cacnb1) and β3 (Cacnb3) subunits in αII spectrin cKO hearts (Fig. S9). In summary, consistent with computational modeling, transcriptional analysis of αII spectrin cKO hearts illustrates alterations in multiple unexpected cardiac ion channels, with a notable association with cardiac \( K^+ \) channels.

**αII spectrin cKO myocytes display altered repolarizing potassium currents**

Based on modeling and transcript analyses, we hypothesized that select potassium currents may be altered in αII spectrin cKO hearts. In line with our data showing no difference in resting membrane voltage (Fig. S8), we observed no change in \( I_{\text{K1}} \) between genotypes (Fig. 8, A–C). We did, however, observe significant increases in \( I_{\text{TO}} \) at both peak \( (I_{\text{TO,peak}}) \) and steady-state potassium currents \( (I_{\text{TO,ss}}) \) in αII spectrin cKO mice compared with littermate controls (Fig. 8, D–F). In further support of a role for αII spectrin in potassium channel regulation, we observed a significant increase in protein expression of K\(_{\text{v}}\)4.3 (Fig. 9, A and B). Of note, the increased expression of K\(_{\text{v}}\)4.3 in αII spectrin cKO mice was accompanied by more diffuse localization of K\(_{\text{v}}\)4.3 when compared with control mice (Fig. 9, C and D), suggesting a potential role for αII spectrin in K\(_{\text{v}}\)4.3 trafficking and/or localization. In summary, these data support an unexpected role for αII spectrin in regulation of cardiomyocyte potassium currents. Future experiments will be critical to define the mechanistic relationship for this regulation (direct versus compensatory due to \( I_{\text{Na}} \) loss).

**αII spectrin associates with α\(_{\text{v}}\)1.5 and K\(_{\text{v}}\)4.3**

To investigate the relationship of αII spectrin with ion channel subunits underlying \( I_{\text{Na}} \) and \( I_{\text{TO}} \), we examined potential association between αII spectrin and Na\(_{\text{v}}\)1.5 and K\(_{\text{v}}\)4.3. We observed association of αII spectrin with both Na\(_{\text{v}}\)1.5 and K\(_{\text{v}}\)4.3 in co-immunoprecipitation experiments from detergent-soluble lysates of adult mouse heart (Fig. S10, A and B). In contrast, we observed no association of the structurally similar αI spectrin with Na\(_{\text{v}}\)1.5 or K\(_{\text{v}}\)4.3 in parallel co-immunoprecipitation experiments (Fig. S10, C and D). Thus, in addition to supporting association of αII spectrin with multiple ion channel subunits, these new data suggest selectivity of α spectrin polypeptides for myocyte membrane targets. Furthermore, these data suggest that αI spectrin may not completely compensate for loss of αII spectrin in the αII spectrin cKO mouse model. Future experiments will be important to define the
mechanisms (direct versus indirect) and structural requirements for αII spectrin association with Naᵥ1.5 and Kᵥ4.3.

**Key cardiac regulatory pathways are altered in αII spectrin hearts**

As heart failure and arrhythmia are complex pathways involving the interplay between structural, membrane, signaling, and transcriptional pathways, we investigated αII spectrin-dependent transcriptional pathways in heart in 12–16–week-old mice, prior to cardiac electrical or structural remodeling using RNAseq (Fig. S11). Using GO Pathway analysis, we identified significantly altered regulation of many pathways, including several that may be relevant to the observed phenotypes of αII spectrin KO mice, such as extracellular matrix organization, cell adhesion, voltage-gated channel activity, and collagen metabolic process. We focused on the dysregulation in two major transcript classes. First, several cardiac intracellular proteases including calpains and caspasas, known to regulate neuronal and cardiac spectrins (24, 27, 40, 41), were significantly altered (Fig. S12A). Second, we observed significant alterations in transcripts associated with extracellular matrix (ECM) organization and remodeling (Fig. S12B). For example, we observed significant transcriptional up-regulation of TIMP1 (over 12-fold) that is essential for membrane metalloprotease regulation. Additionally, increased RNA expression of many collagens (Fig. S12C) is consistent with increased fibrosis and with increased ECM turnover. Thus, consistent with a critical role of αII spectrin for cardiac remodeling, αII spectrin KO mice display significant alterations in key pathways that promote cardiac protein turnover and ECM remodeling, thus promoting heart failure phenotypes. Furthermore, established markers of cardiac hypertrophy were significantly altered, foreshadowing the increased cardiomyocyte size observed in older (20–24–week-old) mice (Fig. S13). We hypothesize that these changes serve to augment and/or accelerate phenotypes in αII spectrin KO mice.

**αII spectrin mice display mortality and severe cardiac phenotypes in response to stress**

αII spectrin levels are dysregulated in human HF. Moreover, αII spectrin KO mice display significant structural and electrical phenotypes at baseline and in response to catecholaminergic stress and aging. Furthermore, in addition to observing alterations in critical global spectrin pathways in all spectrin mice, we also observed alterations in pathways that would favor structural and electrical remodeling. We used a well-validated model for afterload-induced heart failure to test the hypothesis that αII spectrin KO mice would display accelerated disease phenotypes in response to stress. When using our standard protocol that produces heart failure in 6–8 weeks with no early mortality (5), ~75% of all spectrin KO mice died within 2 weeks of transverse aortic constriction (TAC).

Based on the high mortality of αII spectrin KO mice following standard TAC, a less severe model of pressure overload (using a 25-gauge constriction) was performed. Consistent with a critical role for αII spectrin in normal cardiac function, αII spectrin KO mice displayed an accelerated heart failure phenotype when compared with control mice following TAC (Fig. 10). Notably, αII spectrin cKO TAC mice exhibit accelerated and decreased contractility (decreased ejection fraction, Fig. 10A) and cardiac dilation (increased left ventricular internal diameter (LVID) (Fig. 10B)), without compensatory hypertro-
phy (decreased left ventricular posterior wall (LVPW) thickness (Fig. 10C)) compared with control TAC littermates. Furthermore, compared with control TAC littermates, αII spectrin cKO TAC mice displayed more pronounced ECG changes, including QT and QTc prolongation, and T-wave depression (Fig. 10, F and G, Table S2). Additionally, histologic analysis with automated quantification of fibrosis in hearts of αII spectrin cKO and control mice indicated increased fibrosis in αII spectrin cKO hearts following TAC (Fig. S14, A–C). TUNEL staining revealed a large, but statistically insignificant (p = 0.0689) increase in apoptosis in αII spectrin cKO mice (Figs. S14, G–I, and S15), suggesting that the observed fibrosis may be replacement fibrosis. Finally, increased vacuolization of cardiac tissue was observed in αII spectrin cKO mice but not control mice (Fig. S14, B, C, E, and F). Notably, these changes occur in the absence of generalized disruption of the cardiomyocyte sarcomere or intercalated disc structure (Fig. S16). In summary, αII spectrin is required for normal physiologic cardiac remodeling, as loss of αII spectrin results in accelerated heart failure phenotypes in response to standard experimental models of heart failure and hypertrophy.

Discussion

Spectrins are broadly expressed, forming a submembrane network with actin essential for membrane organization, flexibility, and stability (8–10). Furthermore, recent work has demonstrated roles for spectrins in the trafficking, regulation, and stabilization of membrane-associated receptors and channels in diverse tissue types (12, 13, 20). Here, we define new roles of αII spectrin in normal cardiac function, in vivo. Based on obser-


vations of striking alterations in αII spectrin regulation in human heart failure and the lack of knowledge about the role of cardiac αII spectrin, we tested the impact of αII spectrin deficiency on the vertebrate heart. We establish that αII spectrin is required for normal cardiac structure and function, as adult αII spectrin cKO mice display reduced contractility. Histologically, αII spectrin cKO mice display simultaneous increases in cellular hypertrophy and fibrosis. Furthermore, unlike control mice, αII spectrin cKO mice displayed striking mortality in response to catecholamines. At the molecular level, we illustrate a central role of αII spectrin in the regulation of α and β spectrin expression and localization in vivo. Furthermore, we illustrate a critical role of αII spectrin in the expression and targeting of Na\textsubscript{v}1.5 and K\textsubscript{v}4.3, resulting in reductions of \( I_{Na} \) and \( I_{TO} \) in the heart. Finally, we define the impact of αII spectrin expression on upstream spectrin regulatory pathways. These findings provide new insight of the central nodal role of αII spectrin in the formation and regulation of key structural, electrical, and signaling pathways in heart.

**αII spectrin regulates global cardiac spectrin pathways**

αII spectrin is abundantly expressed in the heart where it forms heterotetramers with βI, βII, and βIV spectrin. Importantly, the role of cardiac αII spectrin, specifically, is relatively unexplored. Given the essential role of αII spectrin in excitable cells (21–26, 30, 42), in vivo roles of αII spectrin were anticipated. However, the severity of in vivo and in vitro αII spectrin cKO phenotypes were unexpected, particularly the impact of αII spectrin loss on both α and β spectrin expression. For most functions, the spectrin heterotetramer is the functional unit, with the presence of both α and β spectrin being required for spectrin stability (29). Thus, work on the in vivo role of β spectrins provides insight into the function of α spectrins. Cardiomyocyte-specific βII spectrin-deficient (βII spectrin cKO) mice experience a variety of electrophysiological abnormalities, including increased heart rate variability, atrioventricular block, prolonged QT intervals, and widened QRS complexes at baseline, along with pronounced ventricular arrhythmias and death following catecholaminergic stress (19). Similar to αII spectrin cKO mice, βII spectrin cKO mice displayed accelerated heart failure phenotypes following transverse aortic constriction. The near absence of βII spectrin in the αII spectrin model (that did not display such striking electrical phenotypes) supports that remodeling of other cardiac pathways is sufficient to mitigate more severe phenotypes.

**αII spectrin relationship with αI spectrin**

Cardiac αII spectrin localizes to the Z-disc, lateral membrane, and intercalated disc. Alternately, αI spectrin is localized only to the Z-disc and lateral membrane, leaving αI spectrin as the only α spectrin at the intercalated disc. Although we hypothesized that this would lead to deficits in intercalated disc organization and function in αII spectrin cKO mice, we were
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Figure 10. all spectrin cKO mice exhibit accelerated heart failure phenotypes following TAC. A–D, following TAC, all spectrin cKO mice have decreased cardiac function, as evaluated by echocardiography. A, ejection fraction decreased precipitously in all spectrin cKO mice, whereas, B, LVID at systole, increases compared with control mice. C, LVPW at systole failed to increase in all spectrin cKO mice. D and E, representative M-mode traces from control (D) and all spectrin (E) cKO mice at 12 weeks following TAC (n = 5 and 6 for control and all spectrin cKO mice, respectively). F and G, representative ECG traces from control (F) and all spectrin (G) cKO mice demonstrating T-wave depression (n = 5 and 6 for control and all spectrin cKO mice, respectively). H and I, representative images of cardiac structure in control (H) and all spectrin (I) cKO mice. Scale bar indicates 2 mm (n = 3 and 4 for control and all spectrin cKO mice, respectively). All values are represented as mean ± S.E. * indicates a statistical differences from controls, p < 0.05.

The opposite is observed here, with loss of all spectrin causing an increase in αI spectrin and a decrease in β spectrins. Based on these observations and on the nature of α-β spectrin tetramer formation, we hypothesize the dysregulation occurs due to increased degradation of unbound spectrin monomers, which are unable to form stable α-β tetromers due to perturbed ratios of α and β spectrins.

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The relatively normal action potential duration in all spectrin cKO mice supports the impressive pathways likely evolved in cardiac tissue to maintain excitability. The association of all spectrin with Na₈,1.5 was not unexpected (3, 5). However, our new findings illustrate altered Kᵥ₄.3 and Iᵥ₄.3 in all spectrin cKO myocytes. Although co-immunoprecipitation experiments demonstrate association of all spectrin with Kᵥ₄.3 (and Na₈,1.5), the relationship of these proteins may be indirect and instead related to secondary cytoskeletal interactions or functional interactions between channel subunits. Prior work from Remme and colleagues (44) has elegantly demonstrated regulation of Iᵥ₄.3 by Kᵥ₄.3, beyond electrophysiologic interference. Furthermore, work by Deschénes et al. (45) has demonstrated

surprised to observe translocation of αI spectrin to the intercalated disc in the absence of cardiac all spectrin (Fig. 5). This change in localization, along with a somewhat mild phenotype at baseline suggests functional redundancy among cardiac α spectrins, and a partial compensation by αI spectrin for αII spectrin loss. This is, perhaps not surprising, as only mammals have two α spectrin genes, αI having arisen from a gene duplication in terrestrial vertebrates (43). However, it is apparent from our characterization of αII spectrin cKO mice that this compensation is not complete. Although young, unchallenged mice are able to maintain normal cardiac function and conduction, age, physiologic (catecholamines) and pathologic (TAC) stress resulted in dysfunction. Although compensatory dysregulation of the spectrin cytoskeleton and opposing reductions of currents in the heart are able to preserve cardiac function at baseline, even somewhat subtle disruptions (age, mild pressure overload) resulted in a failure to maintain cardiac function, resulting in reduced contractility and arrhythmia. This is likely due to the relative inability of αI spectrin to associate with αII spectrin targets.

When cardiac βII spectrin is knocked out, there is an up-regulation of other β spectrins, whereas α spectrins are decreased.

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physical association of subunits responsible for $I_{Na}$ and $I_{Ko}$ in neonatal rat ventricular myocytes. Given the dependence of both currents on all spectrin for stability at the membrane, it is also possible that there is a co-trafficking mechanism contributing to their concurrent reduction, as has been observed between Na$_r$, K$_r$, and $I_{Kf}$ $(46)$. Whether $I_{Ko}$ (K$_r$, A.3) remodeling is a direct impact of all spectrin loss, or a secondary compensatory factor to preserve action potential dynamics will be an important future area for research $(43)$.

Although our study notes extensive transcriptional dysregulation in hearts lacking all spectrin, similar to the dysregulation observed following the loss of binding partner, ankyrin G $(5)$, the mechanism of this dysregulation is not known. Although all spectrin does localize (albeit at low levels) to the nucleus and is known to play a role in DNA repair $(47, 48)$, a role for all spectrin in direct transcriptional regulation in heart has not been described to our knowledge. It is likely that the transcriptional changes observed are a cellular response to the described disruptions of the cytoskeleton network, however, the mechanism of this regulation remains unexplored.

There are important aspects of the all spectrin cKO mouse phenotype that are not yet fully elucidated. First, the normal dV/dt in the setting of reduced $I_{Na}$ is surprising, and is incompletely explained by the simultaneous reduction in $I_{Ko}$. Furthermore, fibrosis and hypertrophy were observed to occur simultaneously, preventing an investigation of any potential causal relationship. These limitations elucidate important areas for future study.

It is important to note that although experiments were done using isolated cardiomyocytes when possible (electrophysiology, immunoblotting), experiments conducted on whole heart tissue (RNAseq, histology) are confounded by the presence of noncardiomyocytes in heart tissue. Although efforts were made to obtain the most cardiomyocyte-rich sample possible, the contribution of blood, fibroblasts, adipocytes, and immune cells cannot be eliminated.

Finally, heart failure results in dysregulated calcium handling, which can contribute to increased calcium-activated cain pain activity. We have shown increased calpain-mediated degradation of all spectrin is associated with heart failure. Consistent with our new findings, work from Jain et al. $(24)$ have previously demonstrated all spectrin breakdown products in the serum of neonates with congenital heart disease. However, future studies investigating the localization of all spectrin and other spectrins in human heart failure, in addition to the potential effect of all and BII spectrin degradation in heart failure are essential to further our understanding of the role of spectrin dysregulation in both congenital and acquired heart failure.

**Experimental procedures**

**Human heart tissue**

Ischemic and nonischemic failing left ventricular tissue samples from explanted hearts of patients undergoing heart transplantation were obtained through The Cooperative Human Tissue Network: Midwestern Division at The Ohio State University. Nonfailing hearts were obtained through the Lifeline of Ohio Project. The Ohio State University Institutional Review Board approved the use of human subject tissue. This investigation conforms to the principles outlined in the Declaration of Helsinki.

**Animal studies**

Cardiomyocyte-specific all spectrin knockout (all spectrin cKO) mice were produced using the Cre-flox system. Mice with exon 8 of the Sptan1 gene flanked by LoxP sites (Sptan1$^{f/f}$ mice) $(30)$ were backcrossed onto a C57BL/6j background for greater than five generations. Mice with Cre recombinase expression driven by the α-myosin heavy chain promoter were acquired from Jackson Laboratories (B6.N.FVB-(B6)-Tg(Myh6-cre)2182Mds/J, stock number 018972). Sptan1$^{f/f}$ breeders were established by maintaining one cre-positive parent per breeding pair. The genotype was confirmed with PCR (primers: Cre forward, ATGACAGACAGATCCCCCTCTATCC; Cre reverse, CTCTACATC-TGTAGATCATCTGGAC; Cre internal control forward, CAA-ATGTGTGTTGTCTGG; Cre internal control reverse, GTCACTCGAGTGCACAGTTT; Sptan1Box forward, AACAGTCACACCCTCTGAGTGCCA; Sptan1Box reverse, ATTCAGTGGAAAGCTGAGAGCAG). Male and female mice between 16 and 24 weeks of age were used for experiments, unless otherwise noted. Littermate Sptan1$^{WT/WT}$, Cre$^+$, or Sptan1$^{f/f}$ Cre$^-$ mice were used as controls for Sptan1$^{f/f}$, Cre$^+$, and all spectrin cKO mice. Young mice were between 12 and 16 weeks of age. Adult mice were 20–24 weeks of age. The Ohio State University IACUC approved all animal studies. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the NIH.

**Echocardiography**

Echocardiographic analysis was performed on mice lightly anesthetized with isoflurane (1.75% in 1 liter/min oxygen). Mice were immobilized on a heated imaging stage during image acquisition. HR was monitored throughout imaging and recordings that obtained heart rates ≤400 bpm were excluded. Long and short axis analyses were conducted using the GE LOGIQ E, whereas Doppler analysis was conducted on a VEVO 2100. Analysis was conducted following acquisition using at least three nonadjacent contractions. Researchers blinded to genotype performed the image collection and analysis.

**Electrocardiogram**

Surface electrocardiogram analysis was conducted on mice anesthetized with isoflurane (2% in 1 liter/min oxygen). Mice were immobilized on a heated imaging stage during acquisition. Lead II ECGs were collected using PowerLab equipment (ADInstruments). Conscious ECGs were collected using: 1) the ECGenie system with eMouse analysis software (Mouse-Specific Inc.) or 2) implanted radiotelemetry using an ETA-F10 miniature telemeter (DSI) and Ponemah acquisition software. All conscious ECGs were analyzed using LabChart software. Researchers blinded to genotype performed the collection and analysis.

**Transverse aortic constriction**

Mice were anesthetized with 2% isoflurane and intubated for artificial ventilation at 120–160 breaths per minute, tidal vol-
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volume of 0.2–0.35 ml. Heating pads were used to keep body temperatures at 37 °C throughout the procedure. The transverse aorta was accessed via a left lateral thoracotomy and 6-0 suture is used to ligate the aorta overlying a blunted 25- or 27-gauge needle. The needle was removed immediately following ligation leaving a discrete region of stenosis of the aorta. Successful constriction was confirmed by measuring the velocity of blood flow at the aortic root and after the construction site using echocardiography. The surgeon was blinded to genotype.

**Cardiomyocyte isolation**

Adult cardiomyocytes were isolated using aortic cannulation and retroperfusion of enzymes (for action potential and sodium current recordings, protease and collagenase type II (Worthington biochemical) or for potassium current recordings, Liberase TH (Roche Applied Science)) into the coronary circulation. The hearts were excised and flash frozen or fixed in 4% paraformaldehyde for 24 h. Masson’s trichrome staining was performed on 5-μm sections at room temperature. The bath solution contained (in mM): NaCl (136), KCl (4), CaCl2 (1.8), CdCl2, 0.1; HEPES (10), glucose (5.5); MgCl2 (2), HEPES (15), EGTA (1), adjusted to pH 7.4 with NaOH. The transient outward K⁺ current (Ito) was blocked with fish gelatin buffer (3% fish gelatin, 0.1% DMSO, 0.075% Triton X-100) and incubated with primary antibody in fish gelatin buffer overnight at 4 °C. Primary antibodies targeted αI spectrin (BioLegend; 803101), αII spectrin (BioLegend; 803201), β1 spectrin (ThermoFisher; MA3-062), βII spectrin (Custom from Covance), Kv4.3 (Neuromab; clone K75/41), Na+,1.5 (Covance (3)), Mena (a generous gift from Dr. Benz), and Vasp (ImmunoGlobe; 0012-02). Secondary antibodies used were donkey anti-rabbit or donkey anti-mouse (Jackson ImmunoResearch Laboratories, Bio-Rad). Densitometric analysis was performed using ImageJ.

**Immunofluorescence**

Isolated cardiomyocytes or cryopreserved cardiac tissue cryosliced at 5 μm were blocked with fish gelatin buffer (3% fish gelatin, 0.1% DMSO, 0.075% Triton X-100) and incubated with primary antibody in fish gelatin buffer overnight at 4 °C. Primary antibodies targeted αI spectrin (BioLegend; 803101), αII spectrin (BioLegend; 803201), β1 spectrin (ThermoFisher; MA3-062), βII spectrin (Covance), Kv4.3 (Neuromab; clone K75/41), Na+,1.5 (Covance (3)), Mena (a generous gift from Dr. Benz), Vasp (ImmunoGlobe; 0012-02), NCAD (ThermoFisher, 33-3900), Desmin (Sigma), or α-actinin (Sigma). Following incubation with secondary antibodies (donkey anti-rabbit 568 and donkey anti-mouse 488) in fish gelatin buffer for 4 h at room temperature, samples were washed repeatedly with fish gelatin buffer, mounted with Vectashield (Vector Laboratories, Bio-Rad). Densitometric analysis and analysis. Levels of fibrosis were quantified using an add-on to MATLAB (Mathworks). The add-on converts images from the RGB color space into CIE Lab color space, and segments the images using the k-means algorithm. Finally, the fibrosis (blue) segment is filtered through a color mask to remove noise, and the ratio of fibrosis to other tissues was calculated. TUNEL-stained images were analyzed using ImageJ “Color Threshold” and “Analyze Particles” functions. Only particles between 200 and 5000 pixels were counted as nuclei. Numbers of TUNEL-positive nuclei were normalized to total nuclei (by 4′,6-diamidino-2-phenylindole).

**Immunoblotting**

Cardiomyocytes or ventricular tissue samples were homogenized using a Cryolys-cooled Precellys 24-bead homogenizer (Bertin Corp.) using a combination of 1.4- and 2.8-mm ceramic beads at 6000 rpm for three bouts of 15 s in homogenization buffer (0.025 M Tris-HCl, 0.15 M NaCl, 0.001 M EDTA, 1% (v/v) Nonidet P-40, 5% (v/v) glycerol, pH 7.4). Homogenates were then centrifuged for 30 min at 21,130 × g at 4 °C. Following quantification by a BCA assay (Pierce), lysates were separated on 4–15% precast ProteanTGX gels (Bio-Rad) and transferred quantification by a BCA assay (Pierce), lysates were separated on 5-μm sections at room temperature. The bath solution contained (in mM): NaCl (136), KCl (4), CaCl2 (1), MgCl2, 1; CaCl2, 1.8; CdCl2, 0.1; HEPES, 20; CsCl, 127.5; glucose, 11. The pipette solution contained (in mM): NaCl, 5; CsF, 135; EGTA, 10; MgATP, 5; HEPES, 5. To characterize the voltage dependence of the peak Ina single cells were held at −120 mV, and 200 ms voltage steps were applied from −100 to +10 mV in 5 mV increments. The interval between voltage steps was 3 s. Action potentials were elicited using square wave pulses (1–2 nA amplitude, 2–3 ms duration) generated by a DS8000 digital stimulator (World Precision Instruments, Sarasota, FL) and recorded at room temperature with a pipette solution containing (in mM): MgCl2 (1), EGTA (1), KCl (150), HEPES (5), phosphocreatine (5), K₂ATP (4.46); β-hydroxybutyric acid (2), adjusted to pH 7.2 with KOH; and extracellular solution containing (in mM): NaCl (148), Na₂HPO₄ (0.4), MgCl₂ (1), glucose (5.5), KCl (5.4), CaCl₂ (1), HEPES (15), EGTA (1), adjusted to pH 7.2 with NaOH. The transient outward K⁺ current (Ito) recordings were conducted at room temperature. The bath solution contained (in mM): NaCl (136), KCl (4), CaCl₂ (1.8), MgCl₂ (2), HEPES (10), tetrodotoxin (0.03), nifedipine (0.005), pH adjusted at 7.4 with NaOH. Recording pipettes contained (in mM): KCl (135), MgCl₂ (1), EGTA (10), HEPES (10), glucose...
(5), adjusted to pH 7.2 with KOH. \(I_{\mathrm{TO}}\) was recorded using a step protocol with a holding potential of −70 mV and stepping from −40 to +60 mV in 10-mV increments of 5 s at each potential, every 20 s. Peak \(I_{\mathrm{TO}}\) was measured as the steady-state current at the end of the 5-s pulse.

**RNA sequencing**

RNA-Seq of RNA isolated from male and female cKO and littermate control mice was conducted in collaboration with Ocean Ridge BioSciences (Deerfield Beach, FL). Mice were sacrificed using an isoflurane overdose, and the heart were immediately excised. The aorta was then cannulated and the heart was retroperfused with ice-cold Hanks’ balanced salt solution to remove blood contamination. Ventricles were snap-frozen in liquid nitrogen. Total RNA was isolated from the tissue using the TRI Reagent\textsuperscript{®} (Molecular Research Center, part number TR118). Total RNA was quantified and assessed for quality on a 1% agarose, 2% formaldehyde gel. The RNA was then treated with RNase-free DnaseI (Epicerin; part number D9905K) and re-purified using Agencourt RNAclean XP beads (Beckman Coulter; part number A63987). Final RNA samples were then quantified by spectrophotometry. cDNA libraries were prepared from 250 ng of DNA-free total RNA using the TruSeq Stranded mRNA Library Prep (96 samples) (Illumina Inc.; part number 20020595). The quality and size distribution of the amplified libraries were determined by CHIP-based capillary electrophoresis (Bioanalyzer 2100, Agilent Technologies). Libraries were Bioanalyzed (Bioanalyzer 2100, Agilent Technologies) and quantified using the KAPA Library Quantification Kit (Kapa Biosystems, Boston, MA). The libraries were loaded onto an Illumina HiSeq 4000 flowcell and bridge amplified to create sequence clusters and sequenced with 150 nucleotide paired-end reads plus dual index reads. Quality-filtered and base-trimmed reads were used for alignment. Sequence alignment was performed using HISAT2 version 2.0.5. The read summarization program feature Counts2 version 1.5.1 was used for exon- and gene-level counting. Normalized RPKM values were calculated from the raw feature Counts read, and were then filtered to retain a list of genes with a minimum of ~50 mapped reads in 25% or more samples. The threshold of 50 mapped reads is considered the Reliable Quantification Threshold. GO Pathway Analysis was conducted to guide analysis and interpretation.

**Co-immunoprecipitation**

Co-immunoprecipitation experiments were conducted as previously described\textsuperscript{[53]}. WT mouse heart samples were homogenized in buffer (containing 0.025 M Tris–HCl, 0.15 M NaCl, 0.001 M EDTA, 1% (v/v) Nonidet P-40, 5% (v/v) glycerol, pH 7.4) using a Dounce homogenizer. Lysates were centrifuged for 30 min at 13,000 rpm at 4 °C on using a benchtop centrifuge.

The supernatant was removed from the beads using a magnetic stand, and the beads were washed 3 times with PBS. Bound protein was eluted with 2X Laemmli sample buffer and β-mercaptoethanol and heated to 95 °C for 10 min before immunoblotting with α-actinin spectrin (BioLegend; 803101) or αII spectrin (BioLegend; 803201) antibodies. 60 µg of lysate was used as an input loading control for each experiment.

**Statistical analysis**

All continuous variables are represented as mean ± S.E. A multivariate one-way analysis of variance with Tukey’s Honestly Significant Difference post hoc was used to identify differences among groups when data were normally distributed (passed Shapiro-Wilk normality test) in experiments with greater than two experimental groups. For experiments with just two experimental groups, an unpaired two-tailed Student’s \(t\) test was performed, provided data were normally distributed based on a Shapiro-Wilk normality test. When data failed to pass a normality test, a Mann-Whitney test was performed. For experiments where the same mice were followed over time (TAC mice), a repeated measured two-way (genotype by time) analysis of variance was conducted. When examining differences in a categorical variable (failure to capture) between genotypes, a Fisher’s exact test was used. Differences were considered significant at \(p < 0.05\). Statistical analysis was performed using SPSS 25.0 (IBM SPSS Statistics) and GraphPad Prism (version 7.01 for Windows, GraphPad Software).

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