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β_{IV}-Spectrin regulates STAT3 targeting to tune cardiac response to pressure overload

Sathya D. Unudurthi,1,2 Drew Nassal,1,3 Amara Greer-Short,1,2 Nehal Patel,1,2 Taylor Howard,1,2 Xianyao Xu,1 Birce Onal,1,2 Tony Satroplus,1,2 Deborah Hong,1,2 Cemanthla Lane,1,2 Alyssa Dalic,1,2 Sara N. Koenig,1,3 Adam C. Lehnig,1,3 Lisa A. Baer,1,3 Hassan Musa,1 Kristin I. Stanford,1,3 Sakima Smith,1,4 Peter J. Mohler,1,3,4 and Thomas J. Hund1,2,4

The Dorothy M. Davis Heart and Lung Research Institute, The Ohio State University Wexner Medical Center, Columbus, Ohio, USA. 2Department of Biomedical Engineering, College of Engineering, The Ohio State University, Columbus, Ohio, USA. 3Department of Physiology and Cell Biology, and 4Department of Internal Medicine, The Ohio State University College of Medicine, Columbus, Ohio, USA.

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Introduction

Heart failure (HF) represents a major burden on the US health care system, with 870,000 new cases annually and a total cost of $30.7 billion. By 2030, the incidence of HF is expected to affect more than 8 million Americans at a cost of almost $70 billion (1). Maladaptive cardiac remodeling is an important step in the progression of HF and is driven by a constellation of cell- and tissue-level factors including hypertrophic growth, inflammation, fibrosis, and genetic reprogramming (2). Growing evidence supports Ca^{2+}/calmodulin-dependent kinase II (CaMKII) as a master controller of the maladaptive cardiac remodeling process in failing hearts. Namely, it is well accepted that pathological conditions (e.g., excess Ca^{2+}, β-adrenergic stimulation, oxidative stress) promote the constitutive activation of CaMKII, which in turn leads to dysregulation of a host of intracellular proteins important for cardiac cell excitability, contractility, metabolism, and transcription (3). Recent studies show that genetic deletion of cardiac CaMKII isoforms abolges systolic dysfunction in response to chronic pressure overload due, in part, to direct effects on gene transcription pathways (4–6). Furthermore, it has become clear that CaMKII is an important node in a larger network of stress-induced kinases and phosphatases linked to pressure overload–induced changes in gene transcription and hypertrophy and HF (2). While great strides have been made in identifying the components of these integrated signaling networks, the field lacks a larger understanding of how the cell tunes the response of an extensive but highly pleiotropic signaling web to a specific perturbation in the cardiac neurohumoral state and/or biomechanical load. At the same time, there has been growing appreciation in the field for the importance of well-defined local signaling domains to maintain spatial and temporal control over pleiotropic networks involved in hypertrophy and HF (7).

Spectrins are important for membrane integrity and ultrastructure (8). Recent studies have demonstrated novel roles for β_{IV}-spectrin in regulating heart function through the organization of local signaling domains (9–11). Specifically, β_{IV}-spectrin targets CaMKII to membrane substrates at the intercalated disc, a specialized cardiac “synapse-like” structure important for intercellular mechanical and electrical communication. Importantly, our group and others have identified significant alterations in spectrins and spectrin-based pathways in human HF and animal models of HF, although the functional consequences are unknown and untested (9–16). On the basis of previous work showing an important role for CaMKII in modulating hypertrophy and HF in response to chronic stress (4–6, 12, 17), we hypothesized that β_{IV}-spectrin may serve as a novel therapeutic target for abrogating adverse cardiac remodeling and HF.
Here, we tested the role of the βiv-spectrin–CaMKII complex in modifying the cardiac response to chronic pressure overload. We found that chronic stress promotes CaMKII-dependent degradation of βiv-spectrin and that targeted disruption of spectrin-CaMKII interaction confers protection from pressure overload–induced maladaptive remodeling and cardiac dysfunction without blocking hypertrophy. Unexpectedly, we identified a role for the βiv-spectrin–CaMKII complex in controlling the subcellular localization of STAT3, a ubiquitous stress-activated transcription factor that regulates gene programs important for hypertrophy, fibrosis, and inflammation (18–20). STAT3 signaling is characterized by a high degree of pleiotropy, in which a single transcription factor has been linked to a wide variety of input stimuli and downstream regulatory events. Yet the mechanism underlying the pleiotropic actions of STAT3 remain unclear. Here, we define a direct interaction between βiv-spectrin and STAT3 that is essential for balanced STAT3 signaling and that may imbue the pathway with a degree of pleiotropy. Membrane-bound βiv-spectrin physically sequesters basal STAT3 nuclear transcriptional activity (analogous to cadherin sequestration of β-catenin in the Wnt signaling pathway [ref. 21]). Our data support the idea that dysfunction of the βiv-spectrin–CaMKII complex in disease alters STAT3 sequestration to favor profibrotic pathways and adverse remodeling. These data provide a potential link between spectrin dysfunction and remodeling in HF and establish a paradigm for cardiac βiv-spectrin as a critical regulatory node in a transcriptional highway for stress-induced cellular reprogramming in heart.

**Results**

**Targeted disruption of βiv-spectrin–CaMKII interaction abrogates maladaptive remodeling.** Previous work has identified a critical role for CaMKII in controlling the maladaptive response to chronic pressure overload (4–6, 17). Recent studies indicate that βiv-spectrin-associated CaMKII is an important mediator of CaMKII-induced pathological remodeling, although the exact mechanism is undefined (12, 14). Given these findings, we hypothesized that selective targeting of βiv-spectrin–CaMKII interaction would confer protection from pressure overload–induced pathological remodeling. To test this hypothesis, we performed transarticular constriction (TAC) on qv3J animals (carrying the spontaneous qv3J mutation in Spnb4) lacking spectrin-CaMKII interaction and on their WT littermates. The WT animals showed hypertrophy (increased myocyte area and left ventricular [LV] wall thickness) with HF symptoms, including LV dilation and decreased cardiac function following 6 weeks of TAC (Figure 1, A–D and Supplemental Figure 1; supplemental material available online with this article; doi: 10.1172/JCI99245DS1). While qv3J animals demonstrated a robust hypertrophic response with TAC similar to that seen in the WT animals (Figure 1, E and F), they maintained normal cardiac function with no evidence of LV dilation. In fact, the ejection fraction (EF) was no different in qv3J animals after 6 weeks of TAC compared with baseline EF values (Figure 1B). The qv3J animals showed an increased EF and less LV dilation, even up to 10 weeks after TAC compared with WT animals (Supplemental Figure 2). Mortality was low in both groups and not statistically different (12 of 13 WT animals survived out to 10 weeks compared with 8 of 9 qv3J animals; P = NS). To evaluate the possibility of systemic alterations in the qv3J animals contributing to their differential response to TAC, we measured the metabolic state and body composition in qv3J animals and their WT littermates at baseline. We found no baseline metabolic differences that could explain the observed phenotypic difference between WT and qv3J animals (Supplemental Figure 3 and Supplemental Table 1).

The βiv-spectrin–CaMKII complex mediates STAT3 dysregulation following TAC. Given the dramatically altered remodeling response to TAC observed in qv3J animals, we sought to use an unbiased approach to identify signaling pathways differentially regulated in qv3J TAC hearts, potentially downstream of aberrant spectrin-associated CaMKII. We performed microarray and pathway analysis on qv3J TAC and WT TAC hearts. Of the 201 genes
Figure 2. Differential regulation of STAT3-related genes in WT and qv3J TAC hearts. (A) Heatmap showing the expression profiles of select genes (>1.5-fold change, $P < 0.005$) between WT and qv3J TAC hearts analyzed by microarray. $n = 3$ from 3 separate hearts in triplicate per group. (B) Fold change of select STAT3-related genes differentially regulated in WT and qv3J TAC hearts. Genes with a reported change in human HF consistent with the observed change in WT TAC are indicated in red. (C) Selected STAT3-dependent genes (Nppb, Tnfrsf12a, Serpin3n, Tgfβ2, Timp1, Mmp2, Serpine1, Col14a1, Igfbp7, Hspa1a, and Vim) differentially regulated in HF were validated by qPCR. Camk2d was not identified in the microarray as being different between WT and qv3J hearts (3J) and was used as a control. *$P < 0.05$ versus baseline, by 1-way ANOVA with the Holm-Sidak post hoc test. $n = 3$ from 3 separate hearts in triplicate for WT baseline, qv3J baseline, and qv3J TAC; $n = 4$ from 4 separate hearts in triplicate for WT TAC. Summary data are expressed as the mean ± SEM.
phorylated STAT3, have also been identified (23–25). As a first step in determining whether STAT3 signaling was altered in qv 3J mice, we analyzed the expression of STAT3 in WT and qv 3j TAC hearts. Interestingly, under basal conditions, STAT3 was found to be concentrated at the intercalated disc membrane, where our previous studies have shown that βIV-spectrin colocalizes and associates with a subpopulation of CaMKII (Figure 3) (9, 11, 12). Furthermore, loss of βIV-spectrin and STAT3 from the intercalated disc membrane occurred in WT but not qv 3j hearts following TAC, without any change in total STAT3 levels (Figure 3 and Supplemental Figures 4 and 5). Together, these data indicate that TAC alters the subcellular distribution of STAT3, ostensibly as a result of stress-induced downregulation of βIV-spectrin (10, 26). Furthermore, our data show that targeted ablation of βIV-spectrin–CaMKII interaction in vivo (qv3J allele) prevents STAT3 dysregulation and preserves cardiac function in response to chronic pressure overload. Preservation of βIV-spectrin in qv 3J TAC hearts led us to ask whether βIV-spectrin was a target for CaMKII itself, with implications for its own stability. As a first step in determining the mechanism underlying stress-induced βIV-spectrin degradation, we subjected isolated WT and qv 3j ventricular myocytes to long-term stress conditions in vitro (Figure 4, A and B). Following 12 hours of pacing in the presence of isoproterenol and okadaic acid (hyperphosphorylating conditions to increase adrenergic tone), we detected a significant decrease in βIV-spectrin levels in WT but not qv 3j hearts (Figure 4, A and B). We performed quantitative PCR (qPCR), which confirmed that expression of several STAT3-dependent genes, including Nppb, Serpina3n, Timp1, Mmp2, Col14a1, and Vim, was elevated in WT but not qv 3j hearts compared with baseline expression levels (Figure 2C).

Under basal conditions, STAT3 resides in high-molecular-weight complexes, although the molecular constituency of these “statosomes” remains unclear (22). Canonical signaling involves the recruitment of STAT3 to activated JAK at the membrane, leading to phosphorylation at Tyr705, dimerization, and subsequent translocation to the nucleus. Noncanonical pathways involving, for example, the unique effects of unphosphorylated or hyperphosphorylated STAT3, have also been identified (23–25). As a first step in determining whether STAT3 signaling was altered in qv 3j mice, we analyzed the expression of STAT3 in WT and qv 3j TAC hearts. Interestingly, under basal conditions, STAT3 was found to be concentrated at the intercalated disc membrane, where our previous studies have shown that βIV-spectrin colocalizes and associates with a subpopulation of CaMKII (Figure 3) (9, 11, 12). Furthermore, loss of βIV-spectrin and STAT3 from the intercalated disc membrane occurred in WT but not qv 3j hearts following TAC, without any change in total STAT3 levels (Figure 3 and Supplemental Figures 4 and 5). Together, these data indicate that TAC alters the subcellular distribution of STAT3, ostensibly as a result of stress-induced downregulation of βIV-spectrin (10, 26). Furthermore, our data show that targeted ablation of βIV-spectrin–CaMKII interaction in vivo (qv3J allele) prevents STAT3 dysregulation and preserves cardiac function in response to chronic pressure overload.

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cytes. Furthermore, loss of βIV-spectrin was prevented in WT myocytes treated with the CaMKII inhibitor KN-93 and in myocytes from AC3-1 mice expressing a CaMKII-inhibitory peptide, similar to the protection afforded by the qv11 allele (Supplemental Figure 6). In vitro assays demonstrated that overexpression of active CaMKII increased phosphorylation and decreased the expression of βIV-spectrin (Figure 4, C–E), consistent with the hypothesis that local CaMKII activity regulates the stability of βIV-spectrin in response to long-term stress and provides a potential mechanism for resistance to maladaptive remodeling in qv11 hearts subjected to TAC.

Given our observation that STAT3 localizes to the myocyte intercalated disc membrane and that dysregulation of STAT3 localization and downstream signaling occur together with loss of βIV-spectrin following TAC, we hypothesized that βIV-spectrin defines a novel signaling complex with STAT3 (“statosome”) (ref. 22) to control gene transcription in response to chronic stress. A scan of the full-length βIV-spectrin sequence identified a proline-rich region in spectrin repeat 15, homologous to an identified STAT3-binding sequence in human IL-2γ (27) (Figure 5A and Supplemental Figure 7). We first tested for βIV-spectrin–STAT3 interaction using radiolabeled STAT3 and immobilized βIV-spectrin fragments with or without repeat 15 containing the putative STAT3-binding site. Consistent with a specific interaction, we found that STAT3 bound only to βIV-spectrin fragments containing repeat 15 (βIV-spec10-13, βIV-spec13-15) (Figure 5, B and C). In agreement with in vitro direct binding experiments, βIV-spectrin associated with endogenous STAT3 from heart lysates (Figure 5D). Furthermore, coimmunoprecipitation experiments showed that endogenous βIV-spectrin associated with endogenous STAT3 in heart lysates from WT mice but not from mice expressing truncated βIV-spectrin lacking the putative STAT3-binding motif (qv11 allele) with a premature stop codon in spectrin repeat 10; refs. 10, 28 and Figure 5E). Together, these data demonstrate for the first time to our knowledge that βIV-spectrin interacts directly with STAT3, possibly to coordinate downstream gene expression and a functional response to chronic stress in vivo.

**STAT3 dysregulation and cardiac dysfunction in cardiac-specific βIV-spectrin-KO mice.** To test the hypothesis that stress-induced loss of βIV-spectrin promotes STAT3 dysregulation, genetic reprogramming, and pathological remodeling, we generated a cardiac-specific βIV-spectrin–KO mouse (αMHC-CRE/Spnb4 fl/fl, referred to hereafter as βIV-cKO) (Figure 6, A and B). Use of a conditional KO allowed us to not only circumvent defects associated with global βIV-spectrin deficiency (28), but also to address the role of βIV-spectrin and STAT3 in myocytes versus other heart cells (fibroblasts, immune cells). βIV-Spectrin was not detectable by immunoblotting or immunostaining in βIV-cKO cardiomyocytes (Figure 6, C and D; Figure 7, A and B; and Supplemental Figure 8), similar to what was observed in WT TAC cardiomyocytes (Figure 3 and Figure 7C). We verified that βIV-spectrin levels were normal in pancreas, which also expresses the isofrom (Supplemental Figure 8). We found that expression of other spectrins (βII-spectrin, αΓ-spectrin) was not affected in βIV-cKO hearts (Supplemental Figure 8). Expression and localization of NaV1.5 were also normal in βIV-cKO compared with WT hearts (Figure 6D and Figure 7, A and B), consistent with previous results indicating βIV-spectrin–independent (but ankyrin-G–dependent) expression of NaV1.5 in heart (10, 11). CaMKII showed a small but significant decrease in βIV-cKO hearts (Figure 6, C and D). Interestingly, while STAT3 levels were normal, its localization was dramatically altered in βIV-cKO cardiomyocytes at baseline. Specifically, while STAT3 was highly localized to the intercalated disc in cells and tissue from WT baseline, qv11 base- line, and qv11 TAC hearts, cardiac-specific deletion of βIV-spectrin resulted in a loss of submembrane STAT3 comparable to that seen in WT TAC hearts (Figure 7, A, B, and D–F). qPCR demonstrated altered expression of STAT3-regulated genes in βIV-cKO hearts, which was consistent with our measurements in WT TAC hearts (Supplemental Figure 9). Together, these data support our hypothesis that βIV-spectrin organizes a “statosome” in heart and that loss of βIV-spectrin in TAC promotes dysregulation of STAT3 signaling.

On the basis of these data, we hypothesized that STAT3 dysregulation at baseline in βIV-cKO animals would lead to pathological remodeling and/or depressed cardiac function at baseline in the absence of stress, similar to what is observed WT TAC hearts. Consistent with our hypothesis, echocardiography revealed a decrease in cardiac function, with a small but significant degree
of LV dilation at baseline compared with WT cardiac function (Figure 8, A–D). In vivo telemetry recordings revealed a normal electrocardiogram with no arrhythmias at baseline in βIV-cKO animals, but a trend toward increased susceptibility to arrhythmic events with acute stress (exercise plus epinephrine; Supplementary Figure 10). While the functional significance of the small amount of LV dilation in βIV-cKO hearts was unclear, we also found increased fibrosis in βIV-cKO hearts, which may further compromise function compared with WT hearts (Figure 8, E and F). In fact, the degree of fibrosis in βIV-cKO hearts was comparable to levels measured in WT hearts following 6 weeks of TAC. Interestingly, fibrosis in qv3J TAC hearts was similar to that seen in WT hearts at baseline, consistent with preserved STAT3 localization and activity and cardiac function in the qv3J TAC animals.

Therapeutic benefit of STAT3 inhibition in the setting of βIV-spectrin deficiency. STAT3 signaling has been implicated in the inflammatory and fibrotic response to chronic stress (29–31). Furthermore, as discussed above, βIV-cKO hearts showed abnormal STAT3 localization and increased fibrosis compared with WT hearts at baseline (Figure 8, E and F). To determine the specific
role of STAT3 dysregulation in cardiac dysfunction in the setting of βIV-spectrin deficiency, we treated βIV-cKO and WT animals with the selective STAT3 inhibitor S3I-201 (32) (20 mg/kg i.p. daily). STAT3 inhibition significantly improved basal cardiac function at 1 and 2 weeks in βIV-cKO but not WT animals (Figure 9A). In fact, βIV-cKO animals showed a greater than 25% increase in EF with just 1 week of S3I-201 treatment, while WT animals showed no response to the drug. At the same time, STAT3 inhibition eliminated the differences in fibrosis observed in WT and βIV-cKO animals at baseline (compare Figure 9, B and C, with Figure 8, E and F). These data indicate that loss of βIV-spectrin in myocytes contributes to remodeling via a STAT3-dependent pathway. Furthermore, these studies indicate that dysfunction associated with βIV-spectrin deficiency may be ameliorated with STAT3 inhibition.

To test the hypothesis that βIV-spectrin/STAT3 dysfunction promotes remodeling in pressure overload conditions and to determine the therapeutic potential of STAT3 inhibition, we treated WT TAC animals with S3I-201 (20 mg/kg i.p. daily beginning 2 days after surgery until study termination) (Figure 9D). Six weeks after the TAC procedure, the S3I-201-treated animals showed a greater than 25% increase in EF with just 1 week of S3I-201 treatment, while WT animals showed no response to the drug. At the same time, STAT3 inhibition eliminated the differences in fibrosis observed in WT and βIV-cKO animals at baseline (compare Figure 9, B and C, with Figure 8, E and F). These data indicate that loss of βIV-spectrin in myocytes contributes to remodeling via a STAT3-dependent pathway. Furthermore, these studies indicate that dysfunction associated with βIV-spectrin deficiency may be ameliorated with STAT3 inhibition.

Consistent with our observation in qP/L TAC hearts, the drug did not have a significant effect on the hypertrophic ability of the heart (measured by LV anterior wall thickness), although it trended lower in the treated versus untreated hearts. In parallel with the preservation of cardiac function, STAT3 inhibition with S3I-201 abrogated the degree of fibrosis in WT TAC hearts (Figure 8E).

Finally, as an initial effort to determine the relevance of our findings to humans, we assessed nonfailing and failing human heart samples for expression of the STAT3-regulated genes NPPB, TNFRSF12A, and COL14A1, which were confirmed by qPCR to be differentially regulated in WT and qP/L TAC hearts and in βIV-cKO hearts. Consistent with our findings in WT TAC and βIV-cKO hearts, we detected an increase in mRNA levels for NPPB, TNFRSF12A, and COL14A1, but not CAMK2D, in failing human hearts compared with levels in nonfailing human hearts (Supplemental Figure 11). These findings, together with our previous reports of decreased βIV-spectrin levels in failing human hearts, indicate that the spectrin/STAT3 pathway investigated here may have implications for human disease (10).

Discussion

Despite decades of work, the field lacks a robust model with which to adequately explain the complex cardiac response to chronic pathophysiological stress. Important gaps remain in our understand
same time, we identify a new hypertrophic response to maladaptive remodeling and HF. At the same time, therapeutic approaches add to mounting evidence (6, 42) that it is possible to prevent (or at least delay) the transition from an adaptive stage characterized by adverse remodeling (fibrosis, ventricular chamber dilation, ventricular wall thinning) and ultimately HF. The conventional view of hypertrophy is that, at least in the short term, it serves an adaptive role, allowing the heart to normalize changes in wall stress induced by increased afterload (33). However, it has been almost 20 years since the seminal observation that LV mass serves as a prognostic indicator for cardiovascular event risk (34). These data and subsequent studies (35–38) have called into question the adaptive nature of hypertrophy, suggesting that perhaps an optimal therapy involves suppressing the hypertrophic response altogether (39). While hypertrophy remains a viable therapeutic target for HF (especially in the presence of preserved EF) (38, 40, 41), our studies using both genetic and pharmacological approaches add to mounting evidence (6, 42) that it is possible to prevent (or at least delay) the transition from an adaptive hypertrophic response to maladaptive remodeling and HF. At the same time, we identify a new βν-spectrin–based pathway that specifically regulates maladaptive remodeling, making it an attractive potential target for HF therapy. Specifically, we propose that chronic pressure overload induces the disintegration of a key spectrin-based “statosome” that promotes noncanonical and maladaptive STAT3 signaling (Figure 10).

An important unresolved issue to address relates to the complex web of pleiotropic signaling networks linked to hypertrophy and HF (2). CaMKII regulates multiple transcriptional pathways involved in hypertrophy and maladaptive remodeling (e.g., HDAC/MEF2, calcineurin/NFAT) (4, 6, 43). Our data identify βν-spectrin–STAT3 as a CaMKII-dependent network that specifically regulates the maladaptive response. The question remains: how does the cell decide which networks are activated or suppressed and when in the time course of the cardiac response? Furthermore, how is signaling via distinct but related pathways integrated to determine the phenotype at the cell and organ levels? Clearly, an important determinant is the specific biomechanical/neurohumoral challenge. For example, elegant experiments by the Rockman group showed that the duration of the stimulus has less to do with the specific cardiac response than the nature of the stimulus itself (e.g., physiological vs. pathological challenge) (44). An aspect of our study relevant to this discussion is the focus on local control of a subpopulation of CaMKII/STAT3 at the cardiomyocyte intercalated disc membrane. Interestingly, our findings in the qv3J mouse align with those of recent work showing hypertrophy with preserved systolic function in response to pressure overload in both CaMKIIδ-KO and CaMKIIδ/γ double-KO mice (5, 6). Although the qv3J allele directly affects only a subpopulation of CaMKII (9), the afforded protection is comparable to complete KO of the 2 cardiac CaMKII isoforms. We propose that this spectrin-based signaling node has evolved for the specific purpose of sensing local, abnormal stress and that βν-spectrin itself serves as an important target for pathological CaMKII signaling, although potential contributions from other targets (including Na,1,5, Ca2+ cycling proteins, and mitochondrial proteins) cannot be ruled out (3, 9, 45, 46). Together, these data suggest that the βν-spectrin C-terminal domain (CTD) serves as a “pathological” membrane dock for intracellular CaMKII (probably multiple isoforms) and that disruption of this CaMKII subpopulation is sufficient to abrogate maladaptive remodeling in response to chronic stress. While our initial data support a model whereby CaMKII-dependent phosphorylation of βν-spectrin ultimately leads to its degradation, release of STAT3, and large-scale changes in gene transcription (Figure 10), future studies are required to determine the precise mechanism by which spectrin-associated CaMKII destabilizes spectrin-STAT3 interaction and/or alters STAT3 activity.

**Figure 8.** Cardiac-restricted deletion of βν-spectrin induces maladaptive remodeling at baseline. (A) Representative echocardiograms and (B–D) summary data (mean ± SEM) from WT and cardiac-specific βν-cKO animals at baseline. *P < 0.05 versus WT, by 2-tailed t test. n = 18 for WT; n = 12 for βν-cKO. (E and F) Masson’s trichrome–stained heart sections (collagen is labeled in blue) and summary data (mean ± SEM) showing fibrosis as a percentage of the tissue area. *P < 0.05 versus WT baseline; *P < 0.05 versus WT TAC, by 1-way ANOVA with Holm-Sidak post hoc test. n = 3 for WT baseline, βν-cKO baseline, and qv3J TAC; n = 5 for WT TAC, n = 4 for WT TAC plus the STAT3 inhibitor S3I-201 (20 mg/kg i.p. daily). Scale bars: 200 μm.
While our findings show that disruption of βⅡ-spectrin–CaMKII interaction in vivo preserves the integrity of the βⅡ-spectrin–STAT3 complex and prevents maladaptive remodeling in response to chronic pressure overload, we cannot rule out the possibility of a contribution to the phenotype of STAT3-independent pathways. CaMKII targets a large number of intracellular substrates (ion channels, Ca<sup>2+</sup> cycling proteins, transcription factors other than STAT3, mediators of inflammatory response) that are potentially important in regulating the hypertrophic/HF response. It is likely that direct or indirect changes in activity of several of these targets play a role in the protection afforded by the q<sup>βⅡ</sup> allele. At the same time, it is likely that cardiac cells (e.g., fibroblasts and immune cells) other than myocytes are affected in the q<sup>βⅡ</sup> mouse (global truncation of βⅡ-spectrin) and may mediate the altered remodeling response with TAC. Likewise, we cannot rule out the possibility of a role for other signaling pathways independent of CaMKII. In this regard, a fascinating avenue for further study is related to signaling between the lipid bilayer and spectrin. Despite these limitations, our data support a central role for the spectrin-based cytoskeleton in the heart’s adaptation to chronic stress and identify targets to modify this response for therapeutic benefit.

Discovered as structural proteins in the erythrocyte membrane (8), spectrins are classically viewed as “static” adapter proteins responsible for maintaining membrane integrity. However, our findings in multiple excitable cell types support spectrins as critical signaling nodes (9, 16). Our new data expand the roles of spectrins (in any cell) as tunable sensors of the cellular stress response, serving to modulate key transcriptional pathways important for fibrotic response and cardiac function. Specifically, we propose that βⅡ-spectrin organizes a critical “statosome” in cardiac myocytes to preserve temporal and spatial control over STAT3 signaling (22) (Figure 10). Although previous work has identified a potential feedback mechanism between βⅡ-spectrin (encoded by Sptbn1 in the mouse) levels and STAT3 expression (49), we did not find evidence that βⅡ-spectrin controls the expression of STAT3 itself in cardiomyocytes; rather, we found that it controls STAT3 subcellular localization and activity, perhaps reflecting functional differences across βⅡ-spectrin isoforms (e.g., βⅡ-spectrin vs. βⅡ-spectrin). At the same time, earlier work has uncovered an association between spectrin and TGF-β/Smad signaling, with potential implications for remodeling responses to pressure overload (50). While we cannot rule out the possibility of a role for TGF-β/Smad independent of STAT3 in the βⅡ-spectrin-deficient phenotype, it is interesting to note that STAT3 controls the expression of a wide variety of genes including TGF-β and may serve as the key node linking spectrin to other signaling pathways.

Despite prior work showing dysregulation of STAT3 in animal models and human disease (18, 19, 51–53), the in vivo roles of STAT3 remain unclear and controversial. On one hand, studies using

**Figure 10. Schematic of proposed spectrin-based complex to regulate gene expression in response to chronic stress.** (A) Spectrin-based macromolecular complex at the cardiomyocyte intercalated disc membrane, which is composed of βⅡ-spectrin, CaMKII, and STAT3. Previous studies have identified the association with cardiac voltage-gated Na<sup>+</sup> channel Na<sub>1.5</sub> via the adapter protein ankyrin-G. (B and C) CaMKII activation during stress leads to phosphorylation of βⅡ-spectrin and eventual degradation, releasing STAT3 and allowing for its translocation to the nucleus, where it is able to induce profibrotic genes.
genetic mouse models support the notion that STAT3 is protective against acute and chronic stress in heart as a result of antiapoptotic and antioxidant effects (20, 54–56). On the other hand, continuous STAT3 activation is associated with worse outcomes in myocardial infarction, HF, and atrial fibrillation because of proinflammatory, profibrotic pressure (29, 30, 32). Confusion regarding the role of STAT3 stems in part from limitations of animal models and reagents that suffer from (a) a lack of fine control (STAT3 is either completely eliminated or constitutively active), and/or (b) an inability to study “local” versus “global” regulation of STAT3 signaling. We believe our data provide resolution to the apparent conflicting hypotheses regarding STAT3 function in heart, illustrating that, like β-catenin/ Wnt signaling (21), tightly balanced regulation of STAT3-localized activity is required for normal heart function, with loss of balance promoting maladaptive cardiac remodeling in chronic disease.

Methods

Animals. qβ<sup>−/−</sup> and qβ<sup>+/+</sup> mice were obtained from The Jackson Laboratory (9, 10, 28). WT littermate mice were used as controls. β<sub>β</sub>-spectrin conditional KO mice were generated (genOway) on a C57/B6 background using an Flp-mediated strategy to remove the neomycin selection cassette. Genotyping was performed by PCR using the following primers: 5'-GAGCTGCATAAGTTCTACGGATGC-3' (sense) and 5'-ACCCCCATCTCAAAGCTTCTG-3' (antisense), yielding bands of 341 and 440 bp for WT and floxed alleles, respectively. The resulting animals expressing the floxed spectrin allele were crossed with mice expressing Cre under the cardiac promoter α-mysin heavy chain (αMHC-Cre, purchased from The Jackson Laboratory), resulting in cardiac-specific deletion of β<sub>β</sub>-spectrin (αMHC-Spnb4<sup>β β</sup>). Experiments were performed in 2-month-old male mice. Animals were euthanized using CO₂ and cer-.

Microarray and qPCR. Total RNA from mouse heart tissues was extracted with TRizol Reagent plus RNeasy column purification (QIA-GEN) following the manufacturer’s instructions. RNA integrity was analyzed using the Agilent 2100 Bioanalyzer (Agilent Technologies). A 100-ng aliquot of total RNA was linearly amplified, and 5.5 μg cDNA was labeled and fragmented using the GeneChip WT PLUS Reagent Kit (Affymetrix) according to the manufacturer’s instructions. Labeled cDNA targets were hybridized to the Affymetrix GeneChip Mouse Transcriptome Array 1.0 for 16 hours at 45°C. The arrays were washed and stained using the Fluidics Station 450 and scanned using the GeneChip Scanner 3000. For gene expression analysis (>29,000 genes), the arrays were normalized using the RNA-SST algorithm in Expression Console, and comparisons were made with the Transcriptome Analysis Console (Affymetrix). Genes with a P value cutoff below 0.05 and a fold change of greater than 1.5 were used for pathway analysis (Ingenuity Pathway Analysis, QIAGEN Inc., www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis/) and for analysis of transcription factor binding using MatInspector (Genomatrix). Select genes (Nppb, Camk2d, Tnfrsf12a, Serpina3n, Tgfβ2, Tmip1, Mmp2, Serpinel1, Col1a4d, Igfbp7, Hspa1a, and Vim) were confirmed by quantitative real-time PCR (qPCR) analysis, as described previously (10). The primer sequences for mice and humans are provided in Supplemental Tables 4 and 5. Total RNA (500 ng), treated with DNase I, was used for first-strand complementary DNA synthesis with the SuperScript III Reverse Transcriptase VILO cDNA Synthesis Kit (Invitrogen, Thermo Fisher Scientific). qPCR reactions were performed in triplicate on cDNA samples in 96-well optical plates with TaqMan Gene Expression Assays (Life Technologies, Thermo Fisher Scientific) and TaqMan Universal PCR Master Mix (No AmpErase UNG, Thermo Fisher Scientific) to maximize PCR precision and uniformity. PCR was performed at 95°C for 3 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute on an Applied Biosystems 7900HT Fast Real-Time PCR System or a StepOnePlus Real-Time PCR System (Life Technologies, Thermo Fisher Scientific). PCR data were analyzed using the relative standard curve method, and the ΔΔCt method was used to calculate fold changes in relative gene expression. PCR products were confirmed by melt-curve analysis, amplicon length, and DNA sequencing. Rpl7 levels were used as a normalization control.

Human tissue. LV tissue was obtained from explanted hearts from patients undergoing heart transplantation at The Ohio State University. LV tissue from nonfailing donor hearts not suitable for transplantation was obtained through Lifeline of Ohio.

Statistics. SigmaPlot 13.0 (Systat Software) was used for statistical analysis. Data distribution for all comparisons was first tested for normality using the Shapiro–Wilk test. A 2-tailed t test or Mann-Whitney U rank-sum test was used to determine P values for single comparisons, and a P value of less than 0.05 was considered significant. Multiple comparisons were performed using either a 1-way ANOVA with the Holm-Sidak post hoc test (data are presented as the mean ± SEM) or 1-way ANOVA on ranks with Dunn’s multiple comparisons test for determination of significant P values (data are presented as the median with 25th and 75th percentiles [box] and 10th and 90th percentiles [whiskers]). A P value of less than 0.05 was considered significant.
Study approval. Animal studies were conducted in accordance with the NIH’s Guide for the Care and Use of Laboratory Animals (National Academies Press, 2011), following protocols that were reviewed and approved by the IACUC of The Ohio State University. Approval for the use of tissue from human subjects was obtained from the IRB of The Ohio State University. The study conformed with the principles outlined in the Declaration of Helsinki.

See Supplemental Methods for additional details on the methods.

Author contributions
SDU, KIS, SS, PJM, and TJH designed the research studies. SDU, AGS, NP, TH, XX, BO, TS, DH, CL, AD, SNK, DN, ACL, LAB, and HM conducted the experiments. SDU, KIS, and TJH analyzed the data. SDU and TJH wrote the manuscript. SDU, SS, PJM, and TJH edited the manuscript.

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Address correspondence to Thomas J. Hund, The Dorothy M. Davis Heart and Lung Research Institute, The Ohio State University Wexner Medical Center, 473 W. 12th Avenue, Columbus, Ohio 43210, USA. Phone: 614.292.0755; Email: Thomas.Hund@osumc.edu.

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