Fibrosis is a pronounced feature of heart disease and the result of dysregulated activation of resident cardiac fibroblasts (CFs). Recent work identified stress-induced degradation of the cytoskeletal protein βIV-spectrin as an important step in CF activation and cardiac fibrosis. Furthermore, loss of βIV-spectrin was found to depend on Ca²⁺/calmodulin-dependent kinase II (CaMKII). Therefore, we sought to determine the mechanism for CaMKII-dependent regulation of βIV-spectrin and CF activity. Computational screening and MS revealed a critical serine residue (S2250 in mouse and S2254 in human) in βIV-spectrin phosphorylated by CaMKII. Disruption of βIV-spectrin/CaMKII interaction or alanine substitution of βIV-spectrin Ser2250 (βIV-S2254A) prevented CaMKII-induced degradation, whereas a phosphomimetic construct (βIV-spectrin with glutamic acid substitution at serine 2254 [βIV-S2254E]) showed accelerated degradation in the absence of CaMKII. To assess the physiological significance of this phosphorylation event, we expressed exogenous βIV-S2254A and βIV-S2254E constructs in βIV-spectrin-deficient CFs, which have increased proliferation and fibrotic gene expression compared with WT CFs. βIV-S2254A but not βIV-S2254E normalized CF proliferation, gene expression, and contractility. Pathophysiological targeting of βIV-spectrin phosphorylation and subsequent degradation was identified in CFs activated with the profibrotic ligand angiotensin II, resulting in increased proliferation and signal transducer and activation of transcription 3 nuclear accumulation. While therapeutic delivery of exogenous WT βIV-spectrin partially reversed these trends, βIV-S2254A completely negated increased CF proliferation and signal transducer and activation of transcription 3 translocation. Moreover, we observed βIV-spectrin phosphorylation and associated loss in total protein within human heart tissue following heart failure. Together, these data illustrate a considerable role for the βIV-spectrin/CaMKII interaction in activating profibrotic signaling.

Increased fibrosis, characterized by excessive accumulation of extracellular matrix (ECM) protein, is a critical component of the repair and remodeling response to chronic neurohumoral or biomechanical stress in tissues throughout the body, including heart, lung, kidney, liver, skeletal muscle, and skin (1). In heart, fibrosis is not only important for replacement of damaged/dead myocardium in response to ischemic injury but also promotes electrical and mechanical dysfunction in a wide range of cardiac diseases (2–4). Cardiac fibroblasts (CFs) are the underlying cell types responsible for fibrotic remodeling. Under baseline (nondiseased) conditions, CFs play an essential supportive role in maintaining ECM homeostasis. Following injury, however, CFs undergo a transition from their basal state to an activated phenotype, characterized by enhanced proliferation, migration, and ECM deposition leading to increased fibrosis (5). Importantly, a diverse array of signaling events, including neurohumoral, biomechanical, and paracrine factors, has been identified in the CF activation process (3), providing opportunities for therapeutic strategies aimed at convergent molecular targets shared among these numerous signaling cascades.

Spectrins are a family of proteins initially identified for their role in providing structural support to cellular membranes (6, 7). The family includes two α- and five β-spectrin gene products where dimers of α- and β-spectrin interact with one another to form heterotetramers to facilitate interaction of membrane proteins with the actin cytoskeleton (8). Since their discovery, our understanding of spectrin function has grown to include critical spatiotemporal regulation of cell signaling events (9). Importantly, novel roles for the βIV-spectrin isoform have been discovered in organizing local signaling domains important for stress-induced cardiac remodeling, including arrhythmia and fibrosis (10–13). Specifically, βIV-spectrin targets a subpopulation of the multifunctional Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) to the
cardiac myocyte intercalated disc membrane for regulation of voltage-gated Na⁺ channels. More recently, a broader role for βIV-spectrin/CaMKII has been identified in regulating gene programs in both cardiac myocytes and fibroblasts through interaction with the signal transducer and activation of transcription 3 (STAT3) (12, 13). Importantly, βIV-spectrin sequesters STAT3 near the membrane and out of the nucleus under basal (unstimulated) conditions. CaMKII activation in response to long-term pacing in vitro or chronic pressure overload in vivo promotes loss of βIV-spectrin and redistribution of STAT3 with nuclear accumulation, ultimately leading to changes in gene expression.

Here, we tested the hypothesis that βIV-spectrin is a novel target for direct CaMKII phosphorylation with effects on stability/expression of βIV-spectrin. Moreover, given the identified role for CaMKII in regulating cardiac fibrosis, we sought to explore involvement of the βIV-spectrin/CaMKII/STAT3 regulatory nexus in modulating CF gene expression and phenotypes. Using MS guided by a computational screen for consensus motifs, a putative CaMKII phosphorylation site was identified in the βIV-spectrin C terminus (Ser2250 in mouse and Ser2254 in human). Heterologous expression of human βIV-spectrin constructs lacking the putative site (βIV-spectrin with alanine substitution at serine 2254 [βIV-S2254A]) or mimicking constitutive phosphorylation (βIV-spectrin with glutamic acid substitution at serine 2254 [βIV-S2254E]) demonstrated a significance for this site in modulating the rate of βIV-spectrin degradation in vitro. Adenoviral expression of WT and mutant βIV-spectrin constructs in isolated mouse CFs further illustrated the importance for βIV-spectrin phosphorylation in regulating spectrin stability. STAT3 localization, gene expression, cell proliferation, and contractility in genetic models of spectrin deficiency and in response to angiotensin II (AngII). Finally, using a novel custom phospho-specific βIV-spectrin antibody, we identified increased βIV-spectrin phosphorylation at Ser2254 in human failing hearts, supporting an important role for this molecular pathway in human disease. These data advance our understanding of the dynamic range of βIV-spectrin function, including orchestration of its own degradation in response to chronic stress with coordinate changes in gene expression and cell function.

Results

Previous results revealed that chronic pressure overload induced prominent loss of βIV-spectrin in WT mice. However, mutant mice expressing truncated βIV-spectrin lacking an identified CaMKII interaction motif (mutant βIV-spectrin allele C-terminal region containing CaMKII interaction motif [qβ] allele) resulted in maintained βIV-spectrin protein expression (12). Furthermore, in vitro studies showed that rapid pacing of WT myocytes under hyperphosphorylating conditions recapitulated loss of βIV-spectrin observed in vivo, which was prevented by the CaMKII inhibitor autacantide-2-related inhibitory peptide as well as when using qβ myocytes (12). Based on these data, we hypothesized that CaMKII directly phosphorylates βIV-spectrin to induce degradation in response to stress. Initial bioinformatics screening of the fragment absent in the qββ-spectrin allele revealed several putative CaMKII phosphorylation motifs found in both mouse and human, including serine residues 2250, 2268, 2301, 2435, and 2557 (2254, 2272, 2305, 2438, and 2560 in human) (Fig. 1A). Notably, Ser2250/2254 received the highest score as a predicted phosphorylation target (residue highlighted in red, Fig. 1A). We next performed MS analysis on immunoprecipitated βIV-spectrin derived from COS-7 cell lysates transfected with hemagglutinin (HA)-tagged versions of mouse or human βIV-spectrin encoding spectrin repeats 10 through the C terminus. These constructs were coexpressed with or without constitutively active CaMKII. Phosphorylated and non-phosphorylated tryptic peptide fragments were identified corresponding to βIV-spectrin residues 2247 to 2259 in mouse (mβIV,2247–2259) and residues 2251 to 2263 in human (hβIV,2251–2263) (associated tandem MS/MS [MS/MS] spectra are shown in Fig. 1B, Table S1). The percentage of phosphorylated mβIV,2247–2259 (out of total detected mβIV,2247–2259) increased from 0% in the absence of CaMKII to 36.2% when coexpressed with CaMKII. A similar effect was observed for the human sequence with an increase in phosphorylated hβIV,2251–2263 from 15.9% to 48.3% in the absence and presence of CaMKII, respectively (Fig. 1D). Notably, human βIV-spectrin achieved higher percentages of phosphorylated peptide at both baseline and in response to constitutively active CaMKII coexpression, suggesting that differences in steady-state levels of phosphorylation may exist between mouse and human βIV-spectrin. Importantly, the identified fragment and corresponding phosphorylation site (Ser2250 in the mouse and Ser2254 in human) are highly conserved across species (Fig. 1E), supporting the physiological significance. MS/MS spectra were unable to detect peptide fragments for the other remaining predicted CaMKII target motifs, preventing assessment of potential phosphorylation of additional sites.

Based on our MS results, we generated a rabbit affinity-purified polyclonal antibody for detection of phosphorylated βIV-spectrin Ser2250/2254. The antibody was tested by immunoblot on lysates generated from COS-7 cells transfected with human βIV-spectrin encoding repeats 10 through the C terminus (WT βIV-spectrin) alone, WT βIV-spectrin with constitutively active CaMKII, or a phosphoablated βIV-spectrin derived from the WT βIV-spectrin construct utilizing a serine to alanine mutation at residue 2254 (βIV-S2254A) and coexpressed with constitutively active CaMKII. Increased immunoreactive signal was observed with CaMKII coexpressed with WT βIV-spectrin but not βIV-S2254A (Fig. 2, A and B). In order to more thoroughly evaluate antibody specificity, preadsorption with the antigenic peptide used in antibody generation and affinity purification was performed. As expected, this resulted in the complete loss of signal when treated with the phosphorylated peptide (Fig. S1A). Further antibody characterization was performed by pretreatment of the membrane with alkaline phosphatase to eliminate immunoreactive signal corresponding to phosphorylation of βIV-spectrin. This resulted in signal loss for both WT βIV-spectrin alone (identified to have basal phosphorylation status in MS data; Fig. 1A)
Figure 1. Identification of CaMKII phosphorylation site in βIV-spectrin. A, schematic of βIV-spectrin protein domains. βIV-spectrin comprises an N-terminal actin-binding domain, 17 tandem repeat domains (TCD) and C-terminal domain (CTD) and specific domain (SD). A computational screen identified five consensus phosphorylation motifs in the SD/CTD region of βIV-spectrin from both mouse and human using Group-Based Prediction System (GPS 3.0); serine residues 2250/2254 (highlighted in red), 2268/2272, 2301/2305, 2435/2438, and 2557/2560 for mouse and human, respectively. The qv3J allele is a mutation resulting in a premature stop codon producing a truncated βIV-spectrin lacking the CaMKII binding motif as well as the putative phosphorylation sites. The qv4J allele is a mutation resulting in a premature stop codon producing an extreme truncation ablating the STAT3-binding residue. B, the MS/MS spectra for mouse and human βIV-spectrin phosphopeptides, 2248 (QePSVDQPEETAR) (2259) [mouse] and 2252 (QePSVDQSEEAAR) (2263) [human] are shown. COS-7 cells expressing mouse and human HA-tagged βIV-spectrin ± constitutively active CaMKII (T287D) were used. The mouse doubly charged peptide has an observed m/z of 714.7884 Da. These spectra contain H3PO4 loss from the precursor, which is consistent with the presence of a phosphorylated serine residue, whereas the mass difference between the y10 and y9 ions is consistent with phosphorylation at S2254. The human doubly charged peptide has an observed m/z of 734.8034 Da. The mass difference between the y10 and y9 ions is consistent with phosphorylation at S2254. C, chromatograms were plotted for the unmodified and phosphorylated forms of the mouse and human peptides for both the minus and plus CaMKII samples. The degree of pS2250/2254 increases in the presence of CaMKII. D, integrated areas from chromatograms showing increase in phosphorylated peptide in the presence of CaMKII. Data reflect percentages from a single replicate for each group. E, alignment of βIV-spectrin protein sequence showing conservation across species of the putative CaMKII target sequence (underlined) and the phosphorylated serine (green). CaMKII, Ca2+/calmodulin-dependent kinase II; CTC, C-terminal construct; HA, hemagglutinin; MS/MS, tandem MS/MS; qv3J, mutant βIV-spectrin allele lacking repeats 10 through C terminus; STAT3, signal transducer and activation of transcription 3.

and WT βIV-spectrin with CaMKII but marginal loss for βIV-S2254A with CaMKII (Fig. S1, B and C). It is important to note that all validation experiments indicate some degree of crossreactivity with unphosphorylated epitopes. Treatment with the unmodified antigenic peptide also resulted in partial reduction of signal for all evaluated conditions, suggesting partial reactivity with unphosphorylated βIV-spectrin protein. Also, residual immuno reactive signal was apparent in cells expressing βIV-S2254A + CaMKII and in samples pretreated with alkaline phosphatase. Together, these data support the importance of including appropriate controls (i.e., normalization to total βIV-spectrin immunoreactive signal) when using the antibody. That said, these data support the ability of the antibody to detect phosphorylation of βIV-spectrin at S2254.

We next tested the hypothesis that CaMKII-dependent phosphorylation of βIV-spectrin modulates spectrin stability/ expression. We cotransfected COS-7 cells with constitutively active CaMKII and the WT and βIV-S2254A constructs. We also included a qv4J construct lacking the C-terminal region containing CaMKII binding and phosphorylation site (starting at spectrin repeat 10 as the case for WT and βIV-S2254A). Cells were treated with cycloheximide (CHX) 48 h post-transfection to stop new protein synthesis, allowing for assessment of potential differences in protein degradation. Lysates were collected at baseline, as well as 1 and 2 h post-CHX treatment to evaluate the rate of βIV-spectrin loss. WT βIV-spectrin levels (expressed as percentage of baseline expression) were significantly lower 2 h post-CHX treatment compared with qv4J or βIV-S2254A (Fig. 2, C and D), without dramatic changes at longer time points (up to 24 h, data not shown). Notably, there was no difference in relative βIV-S2254A and qv4J expression at 2 h post-CHX. Given that MS results were unable to detect coverage of the other predicted phosphorylation sites (S2272, S2305, S2438, and S2560), a series of phosphoalated mutants were generated to similarly test their functional significance in regulating βIV-spectrin loss. Coexpression of these additional mutants with constitutively active CaMKII led to no change in the rate of βIV-spectrin loss compared with WT (Fig. S2), reinforcing a central role for Ser2254. To subsequently determine whether phosphorylation of Ser2254 was sufficient to induce spectrin degradation, COS-7 cells were transfected with phoshomimetic βIV-spectrin (βIV-S2254E) or WT constructs in the absence of constitutively active CaMKII (GFP cotransfected with βIV-spectrin instead as the cotransfected control). Following treatment with CHX, we again assessed levels of βIV-spectrin expression, which identified a significant loss of spectrin with the βIV-S2254E but not WT construct, even in the absence of CaMKII coexpression (Fig. 3, A and B). Together, these data support an important role for Ser2254 phosphorylation in modulating stability/expression of βIV-spectrin.
CaMKII phosphorylates βIV-spectrin in heart

Figure 2. CaMKII-dependent phosphorylation of βIV-spectrin at S2254 promotes degradation. A, representative immunoblots and B, densitometric measurements for phospho-βIV-spectrin (S2254) and total βIV-spectrin from COS-7 cells transfected for 48 h with WT βIV-spectrin ± CaMKII T287D or phosphoablated βIV-spectrin (S2254A) with CaMKII T287D. *p < 0.05 and **p < 0.01 by one-way ANOVA followed by Tukey HSD test (F = 42.67; p < 0.0001); n = 7. C, representative immunoblots (bands at expected size for expressed constructs comprising repeat 10 through the C terminus) and D, densitometric measurements from COS-7 cells transfected with WT, qv4J, or S2254A βIV-spectrin coexpressed with constitutively active CaMKII T287D. Cells were cultured for 48 h after which degradation assays were performed by treating COS-7 cells with CHX (20 μM) to stop new protein synthesis for 1 and 2 h to measure the rate of βIV-spectrin loss. βIV-spectrin expression was normalized against cotransfected CaMKII to account for transfection control. *p < 0.05 versus WT and #p < 0.05 versus qv4J by one-way ANOVA followed by Tukey HSD test (F = 14.68, p = 0.0006, 1 h; F = 15.33, p = 0.0005, 2 h); n = 5. Summary data are presented as mean ± SEM. CaMKII, Ca2+/calmodulin-dependent kinase II; CHX, cycloheximide; HSD, honest significant difference; qv4J, mutant βIV-spectrin allele C-terminal region containing CaMKII interaction motif.

CFs isolated from βIV-spectrin–deficient mice (mutant βIV-spectrin allele lacking repeats 10 through C terminus [qv4J]) mice expressing truncated βIV-spectrin lacking repeats 10 through the C terminus) were previously shown to have enhanced expression of profibrotic genes, increased proliferation, increased contractility, and associated in vivo fibrosis compared with WT animals (13). Therefore, as a first step in assessing the potential physiological significance of βIV-spectrin phosphorylation at S2254, we evaluated CF phenotype in qv4J CFs, as a model for βIV-spectrin deficiency. qv4J CFs were subjected to adenosivral expression of human phosphoablated (Ad.βIV-S2254A) or phosphomimetic (Ad.βIV-S2254E) βIV-spectrin or control (Ad.GFP) (Fig. 4A). Mouse and human βIV-spectrin share 96% sequence homology, supporting use of human constructs in mouse CFs. Importantly, qv4J CFs expressing Ad.βIV-S2254A showed a significant reduction in proliferation at both 48 and 72 h post-transduction compared with Ad.βIV-S2254E or control (Fig. 4B). Furthermore, delivery of Ad.βIV-S2254A significantly reduced CF contractility in relation to Ad.βIV-S2254E or Ad.GFP treated cells, as assessed by collagen gel volume compaction (Fig. 4, D and E) as another functional readout for profibrotic activity. At the same time, a significant reduction in select profibrotic genes was observed in Ad.βIV-S2254A expressing CFs compared with Ad.βIV-S2254E or control (Fig. 4C). Interestingly, while CFs expressing Ad.βIV-S2254E showed a similar proliferation rate compared with control, they had significant enhancement of profibrotic gene expression. This change in fibrotic gene expression was specific to the recognized βIV-spectrin/STAT3 pathway, as evaluation of additional fibrosis-associated genes (pdgfra, postn, and vim) previously shown to be unaffected by βIV-spectrin/STAT3 (13) remained unregulated between groups (Fig. S3).

To ensure that observed phenotypic changes with expression of βIV-spectrin constructs were not an artifact of the qv4J model (expression of truncated βIV-spectrin), we repeated a subset of experiments using our fibroblast-specific βIV-spectrin model utilizing a periostinfl/fl, CreERT2 crossed with βIV-spectrin–floxed mice (inducible fibroblast-specific βIV-spectrin knockout mouse [βIVifKO]) (Fig. S4A) (13). Periostin expression is highly specific to activated CFs and can be induced with AngII. Therefore, βIVifKO and control (βIVif, Cre–) mice

Figure 3. Constitutive phosphorylation of βIV-spectrin at S2254 induces degradation. A, representative immunoblots and B, associated summary data from COS-7 cells transfected with WT βIV-spectrin or phosphomimetic βIV-spectrin (S2254E) and coexpressed with GFP. Degradation assays were performed by treating COS-7 cells with CHX (20 μM) to stop new protein synthesis for 1- and 2-h time points to measure the rate of βIV-spectrin loss. βIV-spectrin expression was normalized against cotransfected GFP to account for transfection control. Resulting values normalized to baseline. *p < 0.05 versus WT by Student’s two-tailed t test; n = 3. Summary data are presented as mean ± SEM. CHX, cycloheximide.
were treated for 7 days with tamoxifen/AgII for activation of the MerCreMer peristin promoter to induce $\beta_{IV}$-spectrin KO in CFs (Fig. S4, A and B), as previously described (13). CFs isolated from $\beta_{IV}$-ifKO mice were then transduced with Ad.$\beta_{IV}$-S2254A, Ad.$\beta_{IV}$-S2254E, or Ad.GFP. Consistent with the phenotype observed in the $q^{q4}$-derived CFs, Ad.$\beta_{IV}$-S2254A treatment reduced contractility (as assessed by collagen gel compaction) compared with Ad.$\beta_{IV}$-S2254E and Ad.GFP treated cells. Collectively, these data support a functional role for the phosphorylation of $\beta_{IV}$-spectrin at S2254 in its ability to regulate CF gene expression and profibrotic activity.

$\beta_{IV}$-spectrin alters CF gene expression by controlling subcellular localization of the transcription factor STAT3 through physical interaction and sequestration at the membrane (12, 13). Notably, conditions of $\beta_{IV}$-spectrin loss through genetic or acquired means untethers STAT3, resulting in greater nuclear changes in $\beta_{IV}$-spectrin stability, altered STAT3 localization, $\beta_{IV}$-spectrin KO

Figure 4. CaMKII-dependent phosphorylation of $\beta_{IV}$-spectrin alters cardiac fibroblast (CF) gene expression and function. A, representative immunoblots of CF lysates from $q^{q4}$ mice expressing truncated $\beta_{IV}$-spectrin lacking interaction with the transcription factor STAT3. CFs were transduced with adenovirus-encoding GFP (control, Ad.GFP), human phosphoablated S2254A $\beta_{IV}$ (Ad.$\beta_{IV}$-S2254A), or human phosphomimetic S2254E $\beta_{IV}$ (Ad.$\beta_{IV}$-S2254E), validating functional and comparable level of expression from adenovirus delivery. B, summary data of manual cell counts from $q^{q4}$ CFs expressing Ad.GFP (control), Ad.$\beta_{IV}$-S2254A, and Ad.$\beta_{IV}$-S2254E. Cell counts were taken at 24, 48, and 72 h post-transduction. Data are normalized to 24 h control condition. $**p < 0.01$ by correlated one-way ANOVA followed by Tukey HSD test ($F = 15.51, p = 0.0018, 48$ h; $F = 29.25, p = 0.0002, 72$ h); $n = 5$. C, summary data for expression of select profibrotic genes (relative to Rpl7) evaluated by quantitative PCR from $q^{q4}$ CFs 72 h after transduction with Ad.GFP, Ad.$\beta_{IV}$-S2254A, or Ad.$\beta_{IV}$-S2254E; $**p < 0.05$ and $*p < 0.01$ by correlated one-way ANOVA followed by Tukey HSD test ($F = 22.76, p = 0.0002, col1a1; F = 49.22, p < 0.0001, col2a1; F = 34.03, p = 0.0001, serpin3g; F = 23.43, p = 0.0002, mmp19; and F = 55.23, p < 0.0001, serpin3g); $n = 6$. D, representative images of collagen gels seeded with $q^{q4}$ CFs expressing Ad.GFP, Ad.$\beta_{IV}$-S2254A, or Ad.$\beta_{IV}$-S2254E just after collagen gel formation and release (baseline) and 16 h after incubation. E, summary data of the change in collagen gel volume over the course of 16 h in Ad.GFP, Ad.$\beta_{IV}$-S2254A, or Ad.$\beta_{IV}$-S2254E conditions. $**p < 0.05$ by one-way ANOVA followed by Tukey HSD test ($F = 6.51; p = 0.0314$); $n = 3$. F, representative confocal microscopy images (60x magnification) of permeabilized $q^{q4}$ CFs 48 h post-transduction with Ad.GFP, Ad.$\beta_{IV}$-S2254A, or Ad.$\beta_{IV}$-S2254E immunostained for $\beta_{IV}$-spectrin (red), STAT3 (green), phallolidin (gray in merged image), and DAPI (blue in merged image). The scale bar represents 50 $\mu$m. G, summary data of STAT3 nuclear localization in Ad.GFP, Ad.$\beta_{IV}$-S2254A, or Ad.$\beta_{IV}$-S2254E-treated $q^{q4}$ CFs. Data were analyzed from at least five different fields per preparation. $**p < 0.01$ by one-way ANOVA followed by Tukey HSD test ($F = 29.76; p < 0.0001$); $n = 4$ experiments from three different biologic preparations. Summary data are presented as mean ± SEM. CaMKII, Ca$^{2+}$/calmodulin-dependent kinase II; DAPI, 4',6-diamidino-2-phenylindole; HSD, honest significant difference; STAT3, signal transducer and activator of transcription 3; $q^{q4}$, mutant $\beta_{IV}$-spectrin allele lacking repeats 10 through C terminus.
prominent nuclear localization of STAT3 because of the absence of STAT3/βIV-spectrin binding in the qv4J allele (Fig. 4, F and G). Expression of Ad.βIV-S2254A but not Ad.βIV-S2254E in qv4J CFs significantly reduced the relative amount of STAT3 in the nucleus compared with control, consistent with proliferation, gene expression, and compaction assay results. Interestingly, Ad.βIV-S2254A was colocalized with STAT3 in a diffuse and perinuclear pattern, whereas Ad.βIV-S2254E showed a distinct punctate pattern (Fig. 4F).

Beyond genetic models of βIV-spectrin deficiency (qv4J and βIVifKO), we sought to test whether βIV-spectrin phosphorylation at Ser2250/2254 in fluenced CF phenotype under pathophysiological stress conditions involving AngII treatment, which alters CF phenotype (including increased proliferation) in part through activation of CaMKII (14–18). CFs treated with AngII (1 μM) showed an increase in phosphorylated βIV-spectrin (expressed as fraction of total) with an overall decrease in total βIV-spectrin expression (Fig. 5, A–C). To...
CaMKII phosphorylates \( \beta_{IV} \)-spectrin in heart

Determine whether phosphorylation of \( \beta_{IV} \)-spectrin regulated the CF functional response to AngII. WT CFs were transduced with Ad.\( \beta_{IV} \)-WT, Ad.\( \beta_{IV} \)-S2254A, or Ad.GFP prior to treatment with AngII. Consistent with immunoblot data, AngII treatment led to a loss in \( \beta_{IV} \)-spectrin signal in Ad.GFP-expressing CFs (Fig. 5D), together with a significant increase in STAT3 nuclear signal and increased proliferation compared with Ad.GFP-expressing CFs (Fig. 5, D–G). Expression of Ad.\( \beta_{IV} \)-WT significantly reduced AngII-induced translocation of STAT3 into the nucleus and reduced the proliferation rate compared with Ad.GFP-expressing CFs. Interestingly, Ad.\( \beta_{IV} \)-S2254A-expressing CFs were resistant to AngII-induced STAT3 nuclear accumulation with levels comparable to untreated Ad.GFP-expressing CFs (Fig. 5, D and F). Similarly, Ad.\( \beta_{IV} \)-S2254A-expressing CFs treated with AngII showed a similar proliferation rate to untreated Ad.GFP CFs. Together, these studies establish a link between \( \beta_{IV} \)-spectrin phosphorylation, STAT3 localization, and CF pro-fibrotic phenotype.

Finally, as a first step in determining whether our findings in mouse translate to human, levels of phosphorylated \( \beta_{IV} \)-spectrin were evaluated in ventricular lysates from normal (nonfailing) and failing human hearts. A significant increase in phosphorylated \( \beta_{IV} \)-spectrin (as a fraction of total \( \beta_{IV} \)-spectrin) was observed together with a decrease in overall \( \beta_{IV} \)-spectrin levels in heart failure–compared control (Fig. 6, A–C), suggesting a potential role for this regulatory site in human disease.

Discussion

Here, we sought to identify the molecular mechanism for CaMKII-dependent regulation of \( \beta_{IV} \)-spectrin expression and CF activity. Using a computational screen, MS analysis, and in vitro functional assays, we identified a putative CaMKII phosphorylation site in the \( \beta_{IV} \)-spectrin C terminus conserved across species, including mouse and human (S2250/S2254). We observed that ablation of the CaMKII phosphorylation site in \( \beta_{IV} \)-spectrin (\( \beta_{IV} \)-S2254A) conferred resistance to CaMKII-induced degradation in vitro, whereas a phosphomimetic construct (\( \beta_{IV} \)-S2254E) demonstrated accelerated loss even in the absence of CaMKII. Using a custom antibody, we observed that AngII treatment of isolated mouse CFs induced phosphorylation of \( \beta_{IV} \)-spectrin together with increased nuclear localization of STAT3, increased proliferation, and contractility. Adenoviral expression of WT \( \beta_{IV} \)-spectrin was able to partially prevent AngII-induced changes in STAT3 localization and proliferation in CFs. Moreover, CFs expressing Ad.\( \beta_{IV} \)-S2254A treated with AngII were almost indistinguishable from untreated control with respect to STAT3 localization and CF phenotype. Finally, we reported an increase in phosphorylated \( \beta_{IV} \)-spectrin in samples from human failing hearts compared with nonfailing controls, suggesting a significant role for \( \beta_{IV} \)-spectrin phosphorylation and subsequent protein loss in pathologic cardiac remodeling. Our findings expand the dynamic nature of \( \beta_{IV} \)-spectrin to include coordination of its own disassembly in the presence of chronic stress. Furthermore, the system is imbued with a mechanism for reporting its fate back to the cell through STAT3-dependent changes in gene expression.

Recent studies have identified a role for \( \beta_{IV} \)-spectrin in mediating the fibrotic response in heart. Both cardiomyocyte- and CF-specific deletion of \( \beta_{IV} \)-spectrin led to a prominent increase in fibrosis, arrhythmia, and contractile dysfunction (12, 13). Conversely, disruption of \( \beta_{IV} \)-spectrin/CaMKII interaction (\( \gamma^{37} \) allele) preserved \( \beta_{IV} \)-spectrin expression, abrogated fibrosis, and improved cardiac function in response to pressure overload. Together, these studies intimately tie \( \beta_{IV} \)-spectrin expression with the onset of pathologic remodeling through both genetic and acquired disease pathways. Our new findings reveal the mechanistic relationship between \( \beta_{IV} \)-spectrin stability and CaMKII via direct phosphorylation of a conserved residue in the \( \beta_{IV} \)-spectrin C terminus. Although the phosphorylation site identified here is distinct from and proximal to (44 amino acids upstream) the previously identified CaMKII-binding site in \( \beta_{IV} \)-spectrin (give residues), these studies cannot rule out the possibility that CaMKII phosphorylation alters CaMKII binding to \( \beta_{IV} \)-spectrin itself with potential disruptions in associated signaling (10). While studies here concentrate on implications of \( \beta_{IV} \)-spectrin phosphorylation/expression related to STAT3 signaling, it will be interesting in the future to assess consequences for CaMKII signaling.

\( \beta_{IV} \)-spectrin expression is closely linked to that of \( \alpha_{II} \)-spectrin, which associate together in heterotetrameric

![Figure 6. Increased \( \beta_{IV} \)-spectrin phosphorylation in human failing hearts.](image)

A, representative immunoblots for phospho-\( \beta_{IV} \)-spectrin (S2254) and total \( \beta_{IV} \)-spectrin in human left ventricular nonfailing (NF) and heart failure (HF) tissue samples. B, associated summary data for phospho-\( \beta_{IV} \)-spectrin normalized to total \( \beta_{IV} \)-spectrin and C, total \( \beta_{IV} \)-spectrin normalized to GAPDH. HF samples comprise both ischemic and nonischemic samples (groups combined). *\( p < 0.05 \) and **\( p < 0.01 \) by Student's two-tailed t test; NF: \( n = 7 \), HF: \( n = 10 \). Summary data are presented as mean ± SEM.
increased mortality (27–29), spontaneous hypertrophy, decreased cardiac function, and ischemic disease models (30–32), reflecting states of remodeling consistent with $\beta_{IV}$-spectrin loss. Despite this, it is not clear what role $\alpha_{II}$-spectrin might play in the CaMKII-dependent degradation of $\beta_{IV}$-spectrin and subsequent fibrotic remodeling. Is $\alpha_{II}$-spectrin stability simply dependent on signaling events targeting $\beta_{IV}$-spectrin, or might there exist independent regulation of $\alpha_{II}$-spectrin that could dictate outcomes for $\beta_{IV}$-spectrin expression and fibrosis? Overall, this codependent regulation may even suggest that multiple disease pathways are able to converge on this spectrin complex to facilitate activation of common underlying remodeling events.

The identification of $\beta_{IV}$-spectrin as a novel target for CaMKII adds to a growing list of CaMKII substrates involved in disease remodeling (21–24). Importantly, CaMKII has long been distinguished as a critical factor in mediating pathologic electrical remodeling, hypertrophic signaling, and fibrosis (22, 25, 26). Genetic models of CaMKII overexpression result in spontaneous hypertrophy, decreased cardiac function, and increased mortality (27–29). In contrast, genetic and pharmacologic inhibition of CaMKII provides protection from chronic stress, limiting the development of cardiomyopathy and maintaining cardiac output in both ischemic and non-ischemic disease models (30–33). Functional effects of CaMKII activity are mediated through post-translational modifications to a range of ion-handling proteins such as phospholamban (34), ryanodine receptor (35), and the voltage-gated channels Ca$_{1.2}$ (36) and Na$_{1.5}$ (10, 37), as well as more direct hypertrophic drivers like the class 2 histone deacetylases 4 and 5 (38). Notably, our previous investigations found that the $\beta_{IV}$-spectrin qv$^{3}$ mouse with disruption of CaMKII/$\beta_{IV}$-spectrin interaction led to reduced fibrosis but maintained a hypertrophic response to chronic pressure overload (12), delineating the multiple etiologies of CaMKII-directed remodeling and the pivotal importance of $\beta_{IV}$-spectrin stability with regard to fibrosis. Indeed, direct inhibition of CaMKII in CFs has been shown to reduce CF activation and fibrosis (17, 18), further reinforcing the role for a CaMKII/$\beta_{IV}$-spectrin axis of regulation.

CaMKII resides at the hub of a vast signaling network and is well equipped to integrate a host of pathophysiological signaling inputs. Neurohumoral signaling, ischemic stress, inflammation, reactive oxygen species, and diabetes are all associated with activation of CaMKII resulting in cardiomyopathy and fibrosis (30, 39, 40), whereas specific profibrotic cytokines and neurohumoral factors such as AngII (41), interleukin-6 (42), tumor necrosis factor beta (17), and bone morphogenetic protein 4 (43) also provide CaMKII activation. Based on our findings that CaMKII-dependent phosphorylation of $\beta_{IV}$-spectrin modulates gene expression and activity of CFs at baseline and in response to AngII, it is interesting to consider the possibility that the CaMKII/$\beta_{IV}$-spectrin axis exists as a central common pathway for fibrotic remodeling response to stress.

Our data support that the CaMKII/$\beta_{IV}$-spectrin signaling node resides upstream of the transcription factor STAT3, whereas loss of $\beta_{IV}$-spectrin has been shown to drive fibrotic remodeling by promoting redistribution of STAT3 into the nucleus and subsequent changes in profibrotic genes (12, 13). These data add to the emerging status of STAT3 as a master controller of the fibrotic response (44). Pharmacologic inhibition of STAT3 directly (12, 45, 46) or genetic ablation of upstream STAT3 activators, such as interleukin-6 (42), have successfully attenuated cardiac fibrosis in both the atria and/or ventricles following ischemia and pressure overload, reducing adverse electrical remodeling and improving heart function. Moreover, STAT3 activation and nuclear translocation has been shown to be upstream of CaMKII activity and can be impaired by direct CaMKII inhibition (42). Importantly, the investigation here is not only supportive of these data but identifies $\beta_{IV}$-spectrin as the bridge between these signaling events, showing that stable expression of phosphoablated $\beta_{IV}$-spectrin is able to reduce nuclear STAT3 and CF proliferation even in the presence of CaMKII activation.

Characteristically, CaMKII activity often leads to altered function for its target substrate; however, we describe here the unexpected consequence of induced protein degradation. While there is precedence for phosphorylation-dependent degradation of target substrates for other protein kinases (47–49), such a mechanistic role for CaMKII is less established. Up to now, just one such target has been identified in the protein Liprin$\alpha_1$, a regulator for neuronal dendrite maturation (50). Interestingly, CaMKII phosphorylation of Liprin$\alpha_1$ activates a PEST sequence in the target protein, characterized by the enrichment of proline, glutamate, serine, and threonine residues. Such sequences facilitate basal regulation of protein half-lives as well as induced degradation following post-translational modification, recruiting both calpain and proteosome-dependent degradation pathways. It will be interesting to further analyze the $\beta_{IV}$-spectrin C terminus for putative PEST sequences to determine whether a similar mechanism may be involved in the behavior observed here. Importantly, other clues to the mechanisms responsible for CaMKII-dependent spectrin loss may be found in the confocal imaging studies of $\beta_{IV}$-spectrin localization. Specifically, phosphomimetic and WT $\beta_{IV}$-spectrin stimulated with AngII showed significant punctate patterns of localization (Figs. 4F and 5D), consistent with those for both proteosomal complexes and/or lysosomal bodies (51, 52). Notably, these puncta were absent when expressing $\beta_{IV}$-spectrin under nondegrading conditions such as phosphoablated $\beta_{IV}$-spectrin with AngII (Fig. 5D), highlighting a clear association with Ser2254 phosphorylation status, $\beta_{IV}$-spectrin stability, and nuclear STAT3 accumulation.

Overall, this study identifies $\beta_{IV}$-spectrin as a novel target of CaMKII and reveals CaMKII/$\beta_{IV}$-spectrin signaling to play an important role in modulating the response of CFs to neurohumoral stress. Given the paramount role that CaMKII activation plays in multiple cardiac disease pathways and the
established significance for βIV-spectrin in regulating cardiac fibrosis, the βIV-spectrin/CaMKII axis represents a comprehensive event and attractive therapeutic candidate in fibrotic remodeling.

**Experimental procedures**

**Mouse models**

Adult (2–4 months) C57BL/6 male and female WT and truncated βIV-spectrin (qv^θ) littermate mice were used. The qv^θ mice were obtained from Jackson Laboratory and express a Sptnb4 allele with a spontaneous insertion point mutation at C4234T (Q1358 → Stop) resulting in a premature stop codon proximal to the STAT3-binding region in βIV-spectrin (53, 54). PeriostinMerCreMer mice (55) were crossbred with βIV-spectrin-floxed mice (12) to obtain tamoxifen-inducible βIV-ifKO mice. Adult male and female βIV-ifKO or control (βIV-spectrin floxed, Cre−) mice were treated with tamoxifen (MilliporeSigma) dissolved in corn oil (75 mg/kg i.p. injection daily) and AngII (2.16 mg/kg i.p. injection daily) for 1 week to induce MerCreCre expression under regulation of the periostin promoter (36, 57). Animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH) following protocols that were reviewed and approved by the Institutional Animal Care and Use Committee at The Ohio State University.

**Bioinformatics screening of putative CaMKII phosphorylation target sites in βIV-spectrin**

A C-terminal fragment of the βIV-spectrin protein was evaluated for potential CaMKII target phosphorylation motifs using the Group-Based Prediction System (GPS 3.0) (58). Five potential sites of interest were identified that were conserved between mouse and human βIV-spectrin sequences (residues refer to position in human sequence): Ser2254, Ser2272, Ser2305, Ser2438, and Ser2560.

**MS**

A fragment of βIV-spectrin from spectrin repeat 10 through the C terminus (representing a natively expressed truncated version of βIV-spectrin) was cloned from mouse and human complementary DNA (cDNA) libraries into pcDNA3.1 with an HA tag for heterologous expression, as previously described (12). HA-tagged βIV-spectrin was expressed in COS-7 cells (American Type Culture Collection catalog no. CRL-1651, RRID: CVCL 0224) and purified using Anti-HA antibody (3724; Cell Signaling) loaded onto TrueBlot agarose beads (00-8800-25; Rockland). A subset of cells were coexpressed with constitutively active CaMKII (phosphomimetic mutation of Thr287 to Asp[T287D]), generated as previously described (12). About 300 ng of βIV-spectrin plasmid was transfected alone or codeposited with 300 ng CaMKII T287D using 2 μl Lipofectamine 2000 (11668030; Thermo Fisher Scientific), according to the manufacturer’s instructions. MS and subsequent analysis was performed by the Proteomics and Metabolomics Laboratory, Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, OH, USA. The protein samples were fractionated on an SDS-PAGE and submitted for analysis, and a small area around the band was cut from the gel. The gel pieces were washed with water and dehydrated in acetonitrile. The bands were then reduced with DTT and alkylated with iodoacetamide prior to the in-gel digestion. All bands were digested in gel by adding 5 μl of 10 ng/μl trypsin in 50 mM ammonium bicarbonate and incubating overnight at room temperature. The peptides that were formed were extracted from the polyacrylamide in two aliquots of 30 μl 50% acetonitrile with 5% formic acid. These extracts were combined and evaporated to <10 μl in Speedvac and then resuspended in 1% acetic acid to make up a final volume of ~30 μl for LC–MS analysis. The LC–MS system was a Dionex Ultimate 3000 nano-flow HPLC interfacing with a ThermoScientific Fusion Lumos mass spectrometer system. The HPLC system used an Acclaim PepMap 100 precolumn (75 μm × 2 cm, C18, 3 μm, 100 A) followed by an Acclaim PepMap RSLC analytical column (75 μm × 15 cm, C18, 2 μm, 100 A). About 5 μl volumes of the extract were injected, and the peptides eluted from the column by an acetonitrile/0.1% formic acid gradient at a flow rate of 0.3 μl/min were introduced into the source of the mass spectrometer online. The microelectrospray ion source is operated at 2.5 kV. The digest was analyzed using the data-dependent multitask capability of the instrument acquiring full scan mass spectra to determine peptide molecular weights and product ion spectra to determine amino acid sequence in successive instrument scans. The data were analyzed by using all collision-induced dissociation spectra collected in the experiment to search the Chlorocebus sabaeus reference sequence protein database and more specifically against the sequence of mouse and human βIV-spectrin as expressed in the pcDNA3.1 construct. In order to more accurately measure the relative abundance of the phosphopeptides across these samples, a second LC–MS/MS experiment was performed. Tandem mass spectra were extracted using the Spectrum Selector node bundled into Proteome Discoverer 2.4 (ThermoFisher). Charge state deconvolution and deisotoping were not performed. The data were analyzed using Proteome Discoverer, version 2.4, with the search engine Sequest. The databases used to search the MS/MS spectra included the Reference Sequence C. sabaeus database containing 61,803 entries along with the sequences for mouse Spectrin beta chain (Q62261) and human Spectrin beta chain (Q9H254). An automatically generated decoy database (reversed sequences) was generated for false discovery rate (FDR) calculation. The search was performed looking for fully tryptic peptides with a maximum of three missed cleavages. Oxidation of methionine, acetylation of protein N terminus, and phosphorylation at S, T, and Y were set as dynamic modifications. Carbamidomethylated cysteine was set as a static modification. The precursor mass tolerance for these searches was set to 10 ppm, and the fragment ion mass tolerance was set to 0.6 Da. Peptide and protein identifications were accepted if they could be established at greater than 5.0% probability to achieve an FDR less than 0.1%. Protein identifications were accepted if they could be established at greater than 5.0% probability to achieve an
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FDR less than 1.0% and contained at least three identified peptides. The positively identified phosphorylated peptides were further subjected to manual inspection with the requirements of a mass measurement less than 5 ppm, and a majority of the ions present were consistent with the identified phosphopeptide. The targeted experiments involve the analysis of specific βIV-spectrin peptides, including the phosphorylated and unmodified forms. The chromatograms for these peptides were plotted based on known fragmentation patterns, and the peak areas of these chromatograms were used to determine the extent of phosphorylation.

Heterologous expression and degradation assays

In order to assess the role of CaMKII activity and βIV-spectrin phosphorylation on the stability of βIV-spectrin, COS-7 cells (American Type Culture Collection CRL-1651) were transfected with 300 ng of the HA-βIV-spectrin pcDNA3.1 construct and 100 ng of constitutively active CaMKII (T287D) or enhanced GFP as previously described (12). Transfections were performed using 2 μl Lipofectamine 2000, according to the manufacturer’s protocol. HA-βIV-spectrin pcDNA3.1 was also mutared at serine residue 2254 (human βIV-spectrin) as well as additional serine residues 2272, 2305, 2438, and 2560 using the Agilent site-directed mutagenesis kit with primers listed in the following table.

| Serine residue | Primer | Sequence |
|----------------|--------|----------|
| S2254A         | Forward | 5’-CCTGAGGGGCAAGGGCAGCTGATGCTAACATCCG-3’ |
|                | Reverse | 5’-TTCCTGAGGATTGATCGACCTC |
| S2254E         | Forward | 5’-GGCTCTGATGCTGACCTCCTCTCTTTGGCAGGTCTAGGCGC-3’ |
|                | Reverse | 5’-GGCTCTGATGCTGACCTCCTCTCTTTGGCAGGTCTAGGCGC |
| S2272A         | Forward | 5’-GAAGCCCCGAGGAGCAGGCGAGCAG-3’ |
|                | Reverse | 5’-CTCGTGTCCCGGTCCTGATCCCCCAGCTGC-3’ |
| S2305A         | Forward | 5’-AGGCCGGACAGGAGCAGGCGAGCAGC-3’ |
|                | Reverse | 5’-CCACAGAGGGTGTCGCCGACATTGGGTTAG-3’ |
| S2438A         | Forward | 5’-CTAAACCCGACATTGGGCAACCCGGTCTGG-3’ |
|                | Reverse | 5’-CTTCTGCCGCGCGCAGCTCGATCT-3’ |

Underlining indicates mutant cells. Cells were incubated 36 h after transfection, at which time a baseline sample was collected. Remaining wells were then treated with 20 μg/ml CHX in incomplete Dulbecco’s modified Eagle’s medium (DMEM) to inhibit new protein synthesis. The drug was kept on the cells for 1 and 2 h, at which time cells were collected and lysates prepared for the evaluation of βIV-spectrin expression. About 20 μg of protein was loaded into a 10% polyacrylamide gel. An HA-antibody (catalog no. 3724; Cell Signaling) was used to assess levels of βIV-spectrin and normalized to the cotransfected CaMKII or GFP to account for variations in transfection efficiency.

Immunoprecipitation

COS-7 cell pellets were suspended in PhosphoSafe Extraction Buffer (71296; Millipore) supplemented with Protease Inhibitor Cocktail (P8340; Millipore). Whole cell lysates (175 μg) were incubated with HA monoclonal antibody (3724; Cell Signaling) overnight. The antibody-bound proteins were precipitated with TrueBlot Anti-Rabbit IgG agarose beads (00-8800-25; Rockland), washed with PBS supplemented with protease inhibitor and Halt Phosphatase Inhibitor Cocktail (78420; Thermo Fisher Scientific), and boiled with loading buffer containing 2% β-mercaptoethanol.

Isolation of primary mouse ventricular CFs

Mouse CFs were isolated from left and right ventricles under sterile conditions, as described (59). Briefly, mouse hearts were minced in 2 mg/ml collagenase II (Worthington) dissolved in 1× Ham’s F-10 buffer (Corning). After digestion, the extract was filtered and centrifuged. The supernatant was discarded, and cells were resuspended in DMEM; 1×, supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin/streptomycin. Cells were allowed to adhere to culture plates for approximately 4 to 5 h prior to media removal containing nonadherent cells (e.g., endothelial, myocytes). Fresh feeding media were replenished, and cells were grown for 5 to 7 days to 80% to 100% confluency at 37 °C in 5% CO2. All cell experiments were conducted at passage 1.

Adenoviral generation

Adenoviral βIV-spectrin constructs were generated by cloning the HA-tagged βIV-spectrin from the pcDNA3.1 construct into the adenovirus construct Ad.CIG (cynomolgus virus promoter combined with internal ribosome entry site expression of enhanced GFP in the second position and βIV-spectrin in the first). The resulting adenoviral plasmid was transfected along with the psi5 vector into CRE8 cells for the production and amplification of packaged viral constructs as described here (60).

CF proliferation assay

CFs were seeded into 12-well culture-treated plates, as described (13). Briefly, cells were adhered for 24 h with serum starvation. At the same time, cells were transduced with adenoviruses (Ad.) Ad.GFP (control), Ad.WT-βIV-spectrin, Ad.βIV-S2254A, or Ad.βIV-S2254E between WT and qψII isolated CFs. The next day medium was replaced with 10% FBS/DMEM/penicillin/streptomycin for qψII CFs or 2% FBS/DMEM/penicillin/streptomycin for WT cells. For qψII CFs, cells were trypsinized at 24, 48, and 72 h postplating. WT CFs were treated 100 nM AngII (1158; R&D Systems) and trypsinized at 24 and 72 h post-AngII stimulation. Cell pellets were resuspended in a fixed volume and manually counted using a hemacytometer to calculate total cell numbers. Accuracy and reproducibility of manual counting was previously confirmed through BrdU proliferation assay (Cell Signaling) (13).

Collagen gel formation and macroscopic gel contraction measurements

Type I rat-collagen gels (1 mg/ml) were prepared by mixing 10× PBS, sterile water, acidic rat tail collagen, and 1 M NaOH. Cells were added (100,000 cells/ml) and mixed before gelation.
Cell-collagen mixtures were cast into 24-well culture plates and incubated at 37 °C in 5% CO₂ for 45 min. After incubation, 1 ml of culture feeding media was added, and the gels were released from wells. Collagen gels were photographed immediately after release and again the next day (16 h). Photographs were analyzed using NIH ImageJ software (61).

Specifically, the diameter of each gel was measured in perpendicular directions and then averaged. As previously described, isotropic compaction was assumed to measure the volume ratio of gels before and after compaction (62).

Immunoblotting and immunostaining

Primary ventricular CF lysates were prepared by washing cells in PBS and scraping in PhosphoSafe Extraction Buffer (71296; Millipore) supplemented with Protease Inhibitor Cocktail and analyzed using SDS-PAGE and immunoblotting, as described (10, 12, 54). Briefly, equal protein loading was achieved using standard bicinechonic acid protein assay protocols and verified by Ponceau staining of immunoblots. The following antibodies were used for immunoblotting: HA antibody (for heterologous βIV-spectrin) (1:1000 dilution; 3724; Cell Signaling), CaMKII (1:2000 dilution; #A010-56AP; Badrilla), phospho-βIV-spectrin enzyme (Antarctic Phosphatase; NEB #M0289) at 150 units/ml in reaction buffer 10 mM Tris–HCl (pH 8.0 at 37 °C), 5 mM MgCl₂, 100 mM KCl, 0.02% Triton X-100, and 0.1 mg/ml bovine serum albumin, 3% goat serum in 1× PBS (Invitrogen) overnight at 4 °C. Primary antibodies (βIV-spectrin [1:100; Millipore; N393/76], total STAT3 [1:200; Cell Signaling; 4904]) were prepared in blocking buffer and added for overnight incubation at 4 °C. After washing, secondary Alexa Flour 568 antimouse (1:500; Invitrogen; A11004), 488 anti-rabbit (1:500; Invitrogen; A11008), and 633 phalloidin (1:200; Invitrogen; A22284) antibodies were prepared in blocking buffer and incubated for 2 h at room temperature. After washing, 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories) was applied, and dishes were stored at 4 °C in the dark until they were imaged using confocal microscopy. Images were acquired on a Nikon Ti2 Microscope in a blinded fashion. Multiple random fields were selected from preparation for analysis. STAT3 localization analysis was conducted by identifying nuclear area through DAPI staining and cell body areas through phalloidin staining. Background noise from acquired images was removed using a 3 × 3 median filter in MATLAB (MathWorks). Threshold values were established from these measurements, and binary images were generated. ImageJ software was then used to identify percent signal within DAPI-defined boundaries, relative to total signal within phalloidin-defined boundaries.

Quantitative real-time PCR

Total RNA from mouse CFs was extracted with TRIzol Reagent following manufacturer’s instructions. Multiscribe reverse transcriptase (Invitrogen) was used for the first-strand cDNA synthesis (20 ng/μl) using random primers. Quantitative real-time PCRs (qPCRs) were performed in triplicate on cDNA samples in 96-well optical plates using PowerUp SYBR Green Universal PCR Master Mix (Invitrogen) and a QuantStudio 3 Real-Time PCR System (Applied Biosystems). qPCR data were analyzed using relative standard curve method, and two delta Ct was used to calculate fold changes in relative gene expression. qPCR products were confirmed by melt-curve analysis, amplicon length, and DNA sequencing. Rpl-7 levels were used as a normalization control. Experiments were conducted in technical triplicates. Primers for gene targets are listed herewith.

| Gene name | Primer Sequence | Primers used for the first-strand cDNA synthesis (20 ng/μl) using random primers. Quantitative real-time PCRs (qPCRs) were performed in triplicate on cDNA samples in 96-well optical plates using PowerUp SYBR Green Universal PCR Master Mix (Invitrogen) and a QuantStudio 3 Real-Time PCR System (Applied Biosystems). qPCR data were analyzed using relative standard curve method, and two delta Ct was used to calculate fold changes in relative gene expression. qPCR products were confirmed by melt-curve analysis, amplicon length, and DNA sequencing. Rpl-7 levels were used as a normalization control. Experiments were conducted in technical triplicates. Primers for gene targets are listed herewith. |
|------------|----------------|
| Col1a1     | Forward TCAGCCTTTTGTGGACCTTCCG |
| Fmod       | Forward AGAAGATCCCTCCTGTCGAACAC |
| Serpina3n  | Forward GGACATTGATGGTGCTGGTGA |
| Col28a1    | Forward CCAGAACCAAAAAGCTTGGC |
| Mmp19      | Forward TCTTCTACCAAGCTGTCGAC |
| Serpina3g  | Forward TCAGCTTGCGGGTTCTCCTCC |
| Pdgfra     | Forward ATCCACCATACCAGGCCACAC |
| Postn      | Forward TGGACCACCAAGAAACCCGAGG |
| Von         | Forward CGCTGCGGTTGCTACATCA |
| Rpl7       | Forward CGGAAGGAGAAAGCCAGAAGG |

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### Human tissue

Left ventricular failing heart tissue was obtained from explanted hearts from patients undergoing heart transplantation at The Ohio State University by the Cardiac Transplant Team. Left ventricular tissue from nonfailing donor hearts (without a history of cardiac dysfunction) not suitable for transplantation was obtained through the Lifeline of Ohio Organ Procurement Organization. All samples were flushed with ice-cold cardioplegic solution and flash frozen within 45 min of removal and kept frozen at −80 °C. Frozen samples were ground before treatment with PhosphoSafe Extraction Reagent (71296; Millipore) supplemented with protease inhibitor (P8340; Sigma) and followed up by sonication to facilitate homogenization. The local institutional review board approved the use of human subject tissue. This investigation conforms to the principles outlined in the Declaration of Helsinki. Human hearts used in this study were deidentified and labeled with six digit random codes for reference to clinical descriptions.

### Statistics

SigmaPlot 14.0 (SYSTAT) was used for statistical analysis. A 2-tailed t-test was used to determine p values for single comparisons. For multiple comparisons, a one-way ANOVA with Tukey honest significant difference post hoc test was used (data presented as mean ± SEM). The null hypothesis was rejected for p value <0.05.

### Data availability

The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (63) partner repository with the dataset identifier PXD025776 and 10.6019/PXD025776. All remaining data are contained within the article.

### Supporting information—This article contains supporting information.

### Author contributions—D. M. N., N. J. P., S. D. U., J. Y., P. J. M., and T. J. H. conceptualization; D. M. N., N. J. P., J. Y., P. J. M., and T. J. H. resources; D. M. N., N. J. P., S. D. U., R. S., and J. Y. data curation; D. M. N., R. S., J. Y., and T. J. H. formal analysis; D. M. N., J. Y., P. J. M., and T. J. H. supervision; D. M. N., J. Y., and P. J. M. funding acquisition; D. M. N., N. J. P., J. Y., P. J. M., and T. J. H. validation; D. M. N., N. J. P., S. D. U., R. S., J. Y., P. J. M., and T. J. H. investigation; D. M. N. visualization; D. M. N., N. J. P., R. S., J. Y., P. J. M., and T. J. H. methodology; D. M. N., P. J. M., and T. J. H. writing—original draft; D. M. N., P. J. M., and T. J. H. project administration; D. M. N., J. Y., P. J. M., and T. J. H. writing—review and editing.

### Funding and additional information—This work was supported by the National Institutes of Health grants HL135096 and HL156652 (T. J. H.), HL134824 (T. J. H. and P. J. M.), HL135754 (P. J. M.), the Fondation Leducq (TNE 19CVD03), the American Heart Association Post-Doctoral Fellowship (18POST34030245, D. M. N.), and funding from the Bob and Corrine Frick Center for Heart Failure and Arrhythmia and the Dorothy M. Davis Heart and Lung Research Institute. The MS Fusion Lumos instrument was purchased via an NIH shared instrument grant, 1S10OD023436-01 (Cleveland Clinic). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

### Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

### Abbreviations—The abbreviations used are: β IVifKO, inducible fibroblast-specific β IV-spectrin knockout mouse; β IV-S2254E, β IV-spectrin with glutamic acid substitution at serine 2254 (phosphomimetic); AngII, angiotensin II; CaMKII, Ca²⁺/calmodulin-dependent kinase II; cDNA, complementary DNA; CF, cardiac fibroblast; CHX, cycloheximide; DAPI, 4',6-diamidino-2-phenylindole; DMEM, Dulbecco’s modified Eagle’s medium; ECM, extracellular matrix; FBS, fetal bovine serum; FDR, false discovery rate; HA, hemagglutinin; MS/MS, tandem MS/MS; NIH, National Institutes of Health; qPCR, quantitative real-time PCR; qv⁴⁹, mutant β IV-spectrin allele C-terminal region containing CaMKII interaction motif; qv⁶⁴, mutant β IV-spectrin allele lacking repeats 10 through C terminus; STAT3, signal transducer and activation of transcription 3.

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