Circulation Research

ORIGINAL RESEARCH

GSK-3β Localizes to the Cardiac Z-Disc to Maintain Length Dependent Activation

Marisa J. Stachowski-Doll, Maria Papadaki, Thomas G. Martin,Weikang Ma, Henry M. Gong, Stephanie Shao, Shi Shen, Nitha Aima Muntu, Mohit Kumar, Edith Perez, Jody L. Martin, Christine S. Moravec, Sakthivel Sadayappan, Stuart G. Campbell, Thomas Irving, Jonathan A. Kirk

BACKGROUND: Altered kinase localization is gaining appreciation as a mechanism of cardiovascular disease. Previous work suggests GSK-3β (glycogen synthase kinase 3β) localizes to and regulates contractile function of the myofilament. We aimed to discover GSK-3β's in vivo role in regulating myofilament function, the mechanisms involved, and the translational relevance.

METHODS: Inducible cardiomyocyte-specific GSK-3β knockout mice and left ventricular myocardium from nonfailing and failing human hearts were studied.

RESULTS: Skinned cardiomyocytes from knockout mice failed to exhibit calcium sensitization with stretch indicating a loss of length-dependent activation (LDA), the mechanism underlying the Frank-Starling Law. Titin acts as a length sensor for LDA, and knockout mice had decreased titin stiffness compared with control mice, explaining the lack of LDA. Knockout mice exhibited no changes in titin isoforms, titin phosphorylation, or other thin filament phosphorylation sites known to affect passive tension or LDA. Mass spectrometry identified several z-disc proteins as myofilament phospho-substrates of GSK-3β. Agreeing with the localization of its targets, GSK-3β that is phosphorylated at Y216 binds to the z-disc. We showed pY216 was necessary and sufficient for z-disc binding using adenoviruses for wild-type, Y216F, and Y216E GSK-3β in neonatal rat ventricular cardiomyocytes. One of GSK-3β's z-disc targets, abLIM-1 (actin-binding LIM protein 1), binds to the z-disc domains of titin that are important for maintaining passive tension. Genetic knockdown of abLIM-1 via siRNA in human engineered heart tissues resulted in enhancement of LDA, indicating abLIM-1 may act as a negative regulator that is modulated by GSK-3β. Last, GSK-3β myofilament localization was reduced in left ventricular myocardium from failing human hearts, which correlated with depressed LDA.

CONCLUSIONS: We identified a novel mechanism by which GSK-3β localizes to the myofilament to modulate LDA. Importantly, z-disc GSK-3β levels were reduced in patients with heart failure, indicating z-disc localized GSK-3β is a possible therapeutic target to restore the Frank-Starling mechanism in patients with heart failure.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: calcium ■ cardiac myocytes ■ connectin ■ mice ■ myofibrils

In This Issue, see p 809 | Meet the First Author, see p 811

A major mechanism driving the pathobiology of cardiovascular disease involves dysregulation of kinases and is frequently observed as either aberrant activation or deactivation.[1–3] There has been increasing appreciation of another layer of complexity in kinase regulation involving subcellular pools of the enzyme whose activities are independently controlled.[4,5] Furthermore, these separate pools can have different phospho-targets and thus behave like functionally distinct kinases.[4] While these kinase pools can complicate attempts to alter enzyme activity therapeutically, they also provide an opportunity to alter function

Correspondence to: Jonathan A. Kirk, PhD, Department of Cell and Molecular Physiology, Loyola University Chicago Stritch School of Medicine, Center for Translational Research, 2160 S First Ave, Maywood, IL 60153. Email kirk.jonathan@gmail.com

Supplemental Material is available at https://www.ahajournals.org/doi/suppl/10.1161/CIRCRESAHA.121.319491.
For Sources of Funding and Disclosures, see page 884.

Circulation Research is available at www.ahajournals.org/journal/res

Novelty and Significance

What Is Known?

- GSK-3β (Glycogen synthase kinase 3β) can target myofilament proteins and alter calcium sensitivity in vitro.
- Stretch increases myofilament calcium sensitivity through a process known as length-dependent activation that is not well understood but involves titin-based strain.
- Length-dependent activation is reduced in heart failure.

What New Information Does This Article Contribute?

- GSK-3β phosphorylates myofilament proteins at the z-disc and contributes to length-dependent activation in vivo.
- GSK-3β's effect on length-dependent activation occurs through interaction of its myofilament target, abLIM-1 (actin-binding LIM protein 1), with the z-disc region of titin.
- Loss of myofilament GSK-3β in human heart failure correlates with decreased length-dependent activation.

Length-dependent activation (LDA) refers to the process by which increased sarcomere length results in increased myofilament calcium sensitivity. While much work has been done to elucidate the mechanism by which LDA occurs, a clear picture remains elusive. Our in vivo work shows that the primarily cytosolic kinase GSK-3β localizes to the myofilament and alters LDA via titin-based strain. We provide evidence that GSK-3β's z-disc substrate, abLIM-1 is a negative regulator of LDA. Additionally, abLIM-1 is a novel binding partner to titin's z-disc bound Z1Z2 domains. In human heart failure myofilament GSK-3β is diminished in samples that also have reduced LDA. These findings suggest that LDA can be regulated by protein interactions at the z-disc, which could be beneficial for targeting the diminished LDA response that can occur in heart failure.

Nonstandard Abbreviations and Acronyms

abLIM-1	actin binding LIM protein 1
cMyBP-C	cardiac myosin binding protein C
EHT	engineered heart tissue
FHL	four and a half LIM domain protein
GSK-3β	glycogen synthase kinase 3β
LDA	length-dependent activation
LV	left ventricle
MLP	muscle LIM protein
SL	sarcomere length
TCAP	telethonin

more selectively and avoid off-target effects. Traditional experimental approaches, however, may not detect dysregulation of small, localized pools of a kinase.

GSK-3β (Glycogen synthase kinase 3β) is a prolific cytosolic serine/threonine kinase. In the heart, GSK-3β acts as a negative regulator of hypertrophic signaling, preventing translocation of proteins to the nucleus that activate hypertrophic gene expression.[6–9] There is a lack of consensus on whether GSK-3β is protective or harmful in heart failure (HF),[6,10–12] possibly because there exists a subcellular pool of GSK-3β with unexplored actions. Indeed, we previously found that GSK-3β activity was depressed in a dog model of HF concurrent with cardiac dyssynchrony,[13] which typically arises from conduction abnormalities and results in premature activation of one region of the ventricular wall.[13] The observed decrease in GSK-3β activity correlated with depressed myofilament calcium sensitivity, which could be rescued in vitro with exposure to exogenous GSK-3β. Furthermore, GSK-3β was uncoupled from Akt, its canonical upstream de-activator, suggesting an independently regulated pool of GSK-3β.[13] However, there is no direct evidence that GSK-3β localizes to the sarcomere or regulates sarcomere function in vivo.

Changes in myofilament calcium sensitivity are frequently observed in HF due to altered phosphorylation of thin filament proteins.[14] However, dependent on the specific HF cause,[15–17] comorbidities,[18] and treatments,[19] myofilament calcium desensitization and oversensitization are both observed. Unfortunately, both situations are detrimental, with desensitization resulting in hypocontractility and worsening of the weakened heart while oversensitization can cause arrhythmias and slowed relaxation.[15] In addition, calcium sensitivity is dynamically regulated in response to acute changes, such as stretch. Length-dependent activation (LDA) is the mechanism by which stretch increases calcium sensitivity in cardiomyocytes and underlies the organ level Frank-Starling Law[20,21] that allows the heart to respond to changes in blood volume on a beat-to-beat basis. Animal models of HF exhibit a depressed Frank-Starling response,[5,22] however, in humans this is less clear, as Frank-Starling/LDA has been found to be both diminished[23–25] and unaffected[26,27] by HF.

It is unknown whether GSK-3β regulates myofilament function in vivo, whether it does so through a localized pool at the myofilament, the mechanisms involved, and

whether this occurs in humans. In this study, we directly address these questions using inducible cardiomyocyte-specific GSK-3β knockout mice, engineered heart tissue (EHT), and human left ventricular (LV) tissue from non-failing rejected donor hearts and patients with HF. Confirming our hypothesis, ablation of GSK-3β in vivo results in reduction in LDA and interestingly, passive tension, a property of titin which has been established as a facilitator of LDA.[28] Exogenous treatment of skinned myocytes with recombinant GSK-3β can increase calcium sensitivity at long sarcomere lengths (SLs) and increase passive tension. We also found that GSK-3β's z-disc phosphorylation target, abLIM-1 (actin-binding LIM protein 1), binds to the z-disc region of titin (Z1Z2 domains) and modulates LDA. Importantly, in human HF GSK-3β mislocalizes away from the z-disc, which correlates with an absent LDA. GSK-3β localizes to the sarcomeric z-disc via its own phosphorylation at Y216, which reveals a novel mechanism for targeting this kinase to specific myofilament targets. Overall, this study uncovers a novel subcellular pool of GSK-3β that is critical for maintaining normal contractile function.

METHODS

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Study Approval

Human study protocols were approved by the Institutional Review Board at the Cleveland Clinic and Loyola University Chicago. All patients gave informed consent. Animal studies were approved by the Loyola University Chicago Health Sciences Division and University of Cincinnati Institutional Animal Care and Use Committees.

Mice

Cardiomyocyte-specific, conditional GSK-3β$^{flox/flox}$, α-MHC (α-myosin heavy chain) Mer Cre Mer + mice[6] (a kind gift from Dr Thomas Force and rederived at Jackson Laboratories) and littermate controls, GSK-3β$^{flox/flox}$ α-MHC Mer Cre Mer −, were generated from several crosses of GSK-3β$^{flox/flox}$ and α-MHC Mer Cre Mer strains, both on the C57BL/6J background. Mice were backcrossed with wild-type C57BL/6J mice every 10 to 12 generations. cMyBP-C (Cardiac myosin binding protein-C) null mice, cMyBP-C$^{t/t29,30}$ and cardiomyocyte-specific cMyBP-C$^{\Delta C0C1f}$, α-MHC mice[31] were generated as described and characterized previously. Approximately equal numbers of male and female mice, age 12 to 14 weeks, were used in all experiments.

Mass Spectrometry

LV tissue from control (n=4) and GSK-3β knockout (n=5) mice were enriched for the myofilament, digested in trypsin, desalted, and phospho-enriched using titanium oxide beads. Samples were analyzed by liquid chromatography mass spectrometry (LC-MS/MS) using Data-Independent Acquisition (Orbitrap Fusion Lumos Tribrid Mass Spectrometer). Data were processed using Data-Independent Acquisition-Umpire and then underwent Median-based normalization.

X-Ray Diffraction

X-ray diffraction patterns were collected from freshly skinned mouse muscle strips using the small-angle instrument at BioCAT beamline 18ID at the Advanced Photon Source, Argonne National Laboratory.[32] SL was adjusted by laser diffraction using a 4-mW HeNe laser. The data were analyzed using data reduction programs belonging to the MuscleX software package developed at BioCAT,[33] as described previously.[34]

Engineered Heart Tissues

EHTs were made by seeding induced pluripotent stem cell cardiomyocytes and human adult cardiac fibroblasts onto decellularized porcine ventricular scaffolds as previously reported.[35] Two weeks after seeding, EHTs were treated with scrambled or abLIM-1 siRNA for 4 hours. Seventy-two hours after treatment, passive and active mechanics of EHTs were measured as previously described.[36]

Statistics

Variables are expressed as mean±SE. Statistical significance was calculated in GraphPad Prism (version 9) by *t* test, 1-way ANOVA, or 2-way ANOVA followed by the Tukey or Mann-Whitney post hoc tests, as indicated. Normality (by Shapiro-Wilk test) and equal variance (by *F* test for *t* tests or Brown-Forsythe test for ANOVAs) were tested before all parametric tests (Table S1). Correlations between categorical variables were performed with linear regression. $P<0.05$ were considered statistically significant. A priori power analyses were performed based on data from published studies[13,37,38] and pilot experiments.

Further details are provided in the Supplemental Material.

RESULTS

GSK-3β Modulates LDA In Vivo

We used a cardiomyocyte-specific inducible GSK-3β knockout mouse to assess whether GSK-3β affects myofilament function in vivo. To evaluate knockdown efficiency, we prepared 2 sets of protein samples from the LV, those processed from whole tissue and those enriched for the myofilament (see Figure S1 for specificity of this enrichment).[39] We detected significant GSK-3β in the myofilament-enriched samples from GSK-3β$^{fl/fl}$/Cre- tamoxifen-treated control mice (Figure 1A through 1C) suggesting there is a pool of GSK-3β which localizes to the myofilament. In the GSK-3β$^{fl/fl}$/Cre+ tamoxifen-treated knockout mice, there was an ≈70% reduction in GSK-3β in both the whole tissue and myofilament-enriched samples (Figure 1A through 1C). At 12 weeks of age (used for all studies), there were no in vivo functional or structural differences between control and knockout mice as assessed by echocardiography (Table S2), except a mild hypertrophy in the anterior wall, consistent with GSK-3β's role as an inhibitor of hypertrophy.

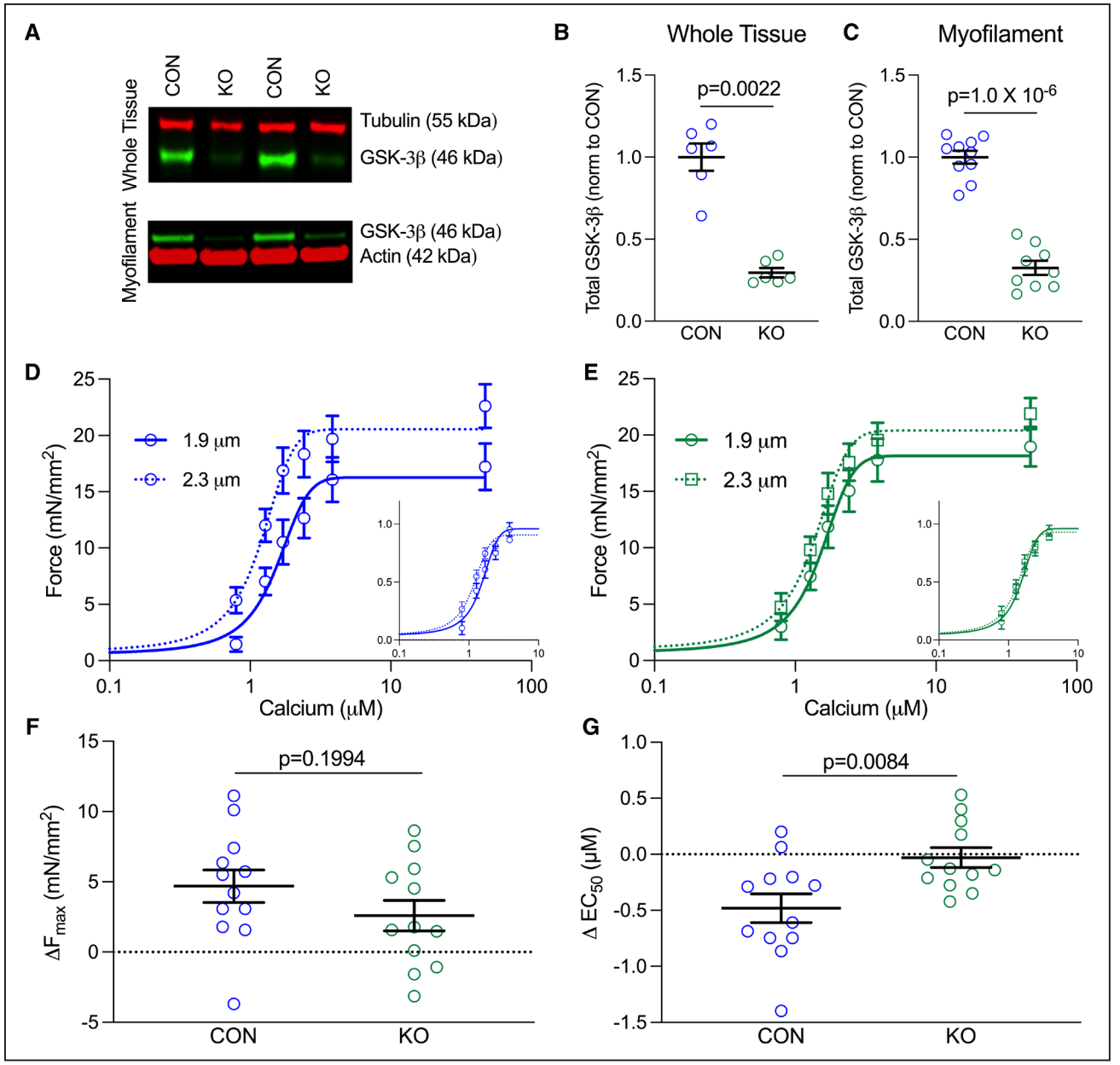

Figure 1. Genetic ablation of GSK-3β (glycogen synthase kinase 3β) results in loss of length-dependent activation.
A, Example western blots of GSK-3β in left ventricular (LV) whole tissue lysis (**top**) and myofilament-enriched (**bottom**) samples from GSK-3β knockout (KO) and littermate control (CON) mice. **B**, Quantification of GSK-3β in whole tissue (P=0.0022, n=6/group) and (**C**) myofilament-enriched (P=1.0×10^{-6}, n values: CON=10, KO=9) samples. GSK-3β was normalized to either tubulin (whole tissue) or actin (myofilament) and further normalized to CON, shown as mean±SEM. P values were calculated by Mann-Whitney t test for the whole tissue comparison. **D**, Mean force as a function of calcium concentration and fitted curves for skinned myocytes from CON and (**E**) KO mice in which curves were measured at a sarcomere length (SL) of 1.9 μm and then 2.3 μm. Normalized curves are shown in the **bottom right** of each graph to emphasize shifts in calcium sensitivity. **F**, Delta F_{max} and (**G**) EC_{50} for CON and GSK-3β KO mice between SL 1.9 and 2.3 μm (P=0.0084 by unpaired t test, n=12 myocytes from 4 CON and 3 KO mice).

We previously found decreased GSK-3β activity in a dog model of ventricular mechanical dyssynchrony[13] that has out-of-phase stress-strain relationships. Thus, we hypothesized GSK-3β may be involved in a mechano-transduction pathway at the myofilament, one of the most critical being LDA.[40] Thus, we performed force-calcium measurements at a SL of 1.9 μm (short) then stretched the myocyte to SL=2.3 μm (long) in skinned myocytes isolated from LV myocardium from control and knockout mice (n=4–5 mice/group, 2–4 cells/mouse; Figure 1D and 1E). Stretching control myocytes increased both F_{max} and calcium sensitivity (P=6.4×10^{-4}, P=4.97×10^{-4}, respectively, via 2-way repeated measures ANOVA, Figure 1F and 1G, Table S3), the expected effect of LDA. Conversely, myocytes from GSK-3β knockout mice exhibited no significant change in either F_{max} or EC_{50} with

stretch. Additionally, there was no statistical difference in calcium sensitivity between the 2 groups at the short SL (P=0.33). Thus, GSK-3β is critical for the ability of the myofilament to adequately respond to stretch, and its genetic removal ablates LDA.

To ensure this was a direct effect of GSK-3β, we next tested whether exogenous GSK-3β would similarly enhance calcium sensitivity at long SLs while having no impact at short SLs. To avoid potential rundown from 4 subsequent activations, we performed paired pre-GSK-3β and post-GSK-3β treatments separately at short and long SLs in control and knockout mice. Exogenous GSK-3β treatment (0.1 μg for 15 minutes) had no effect in myocytes at a short SL (n=8 cells from 3 mice/group), but increased calcium sensitivity when the myocytes were stretched to a long SL (n=11 myocytes from 4 mice; P=0.037 by paired t test; Figure S2). Interestingly, when analyzed by 2-way ANOVA, there was no interaction between genotype and treatment, hence the data were analyzed by a paired t test. This result indicates exogenous GSK-3β can enhance LDA in both control and knockout myocytes and suggests the functional impact of GSK-3β is not saturated in normal hearts.

GSK-3β Is Required for Maintaining Passive Tension

We next sought to explore the mechanism of GSK-3β's impact on LDA. We first showed by Western blot that phosphorylation sites on cardiac troponin I (S23/24) and cMyBP-C (S273, S282, and S302) that can impact LDA[41] are unchanged in the GSK-3β knockout mice (Figure S3). There is evidence LDA is impacted by changes in interfilament spacing, which describes the distance between thick and thin filaments. When the sarcomere is stretched, interfilament lattice spacing decreases, promoting force generation.[42] Using synchrotron small-angle x-ray diffraction, we measured interfilament lattice spacing (d10) in isolated, skinned papillary muscles in relaxing solution at both short and long SLs. Lattice spacing significantly decreased with stretch, but there were no statistical differences between GSK-3β control and knockout fibers (Figure 2A), eliminating it as a plausible mechanism.

As the molecular spring responsible for passive tension[43] and resting SL, titin acts as a stretch sensor for LDA. For example, transgenic expression of a more compliant titin isoform depressed LDA.[28] To assess whether titin may be contributing to GSK-3β's modulation of LDA, we assessed passive tension in control and knockout myocytes. Myocytes from knockout mice had significantly decreased passive tension at long SLs (2.4 and 2.6 μm, P=0.0083 and P=0.016 by unpaired t test, n=4 mice/group, 3–4 cells/mouse, Figure 2B, Table S4). If GSK-3β indeed modulates LDA via passive tension, recombinant GSK-3β should be able to increase passive tension similar to its effect on calcium sensitivity at long SLs. We performed passive tension experiments in control and GSK-3β knockout myocytes before and after treatment with exogenous GSK-3β (0.1 μg GSK-3β for 5 minutes). GSK-3β treatment significantly increased passive tension in both control and knockout myocytes (calculated by paired t test, n=3 mice/group, 4–5 cells/mouse, Figure 2C and 2D).

In addition to passive tension and consistent with a more compliant titin, resting SL was also increased in GSK-3β knockout myocytes compared with control myocytes (n=3 mice/group, 30 cells/mouse, P=0.024 by Mann-Whitney test, Figure 2E and 2F). Together, these results indicate that loss of GSK-3β from the myofilament results in a more compliant titin, which is known to depress LDA.

There are 2 isoforms of titin, the stiff N2B and more compliant N2BA,[44] and it is possible the observed change in passive tension was due to a switch in the relative expression of these 2 isoforms. We prepared[45] LV samples from control and knockout mice and quantified the ratio of N2BA/N2B (n=5-6/group, Figure 2G) and found the isoform composition to be unchanged. Thus, the decrease in passive tension in knockout cells was likely due to reduced phosphorylation of GSK-3β's myofilament targets.

Exogenous GSK-3β Cannot Rescue Function in the Absence of cMyBP-C

We next determined whether genetic removal of a protein in the LDA pathway, specifically, cMyBP-C, would block the ability of exogenous GSK-3β to rescue calcium sensitivity at a long SL. LDA requires MyBP-C,[46] whose C terminus binds titin[47] and N terminus binds the thin filament.[48,49] Importantly, we observed no evidence of a direct interaction of GSK-3β and cMyBP-C (ie, no change in cMyBP-C phosphorylation in the knockout mice), so here we are testing whether GSK-3β's functional impact lies upstream of cMyBP-C.

We used 2 genetically engineered mouse lines, one with complete ablation of cMyBP-C (cMyBP-C knockout)[30] and one transgenic expressing a truncated cMyBP-C lacking the N-terminal C0 and C1 domains that interact with the thin filament (ΔC0–C1f).[31] Both strains have been previously studied, although we confirmed their cMyBP-C expression profiles here (Figure S4A). We measured force-calcium relationships before and after exogenous GSK-3β treatment (n=3 mice/group, 3 cells/mouse). Although GSK-3β increased calcium sensitivity at long SLs in Con myocytes, it failed to affect calcium sensitivity in the cMyBP-C knockout or cMyBP-C$^{\Delta C0C1f}$ mice (Figure S4B through S4G). Together, these findings indicate cMyBP-C, and particularly the N terminus, is downstream of GSK-3β's titin-based modulation of calcium sensitivity. However, the precise role of cMyBP-C in LDA has not

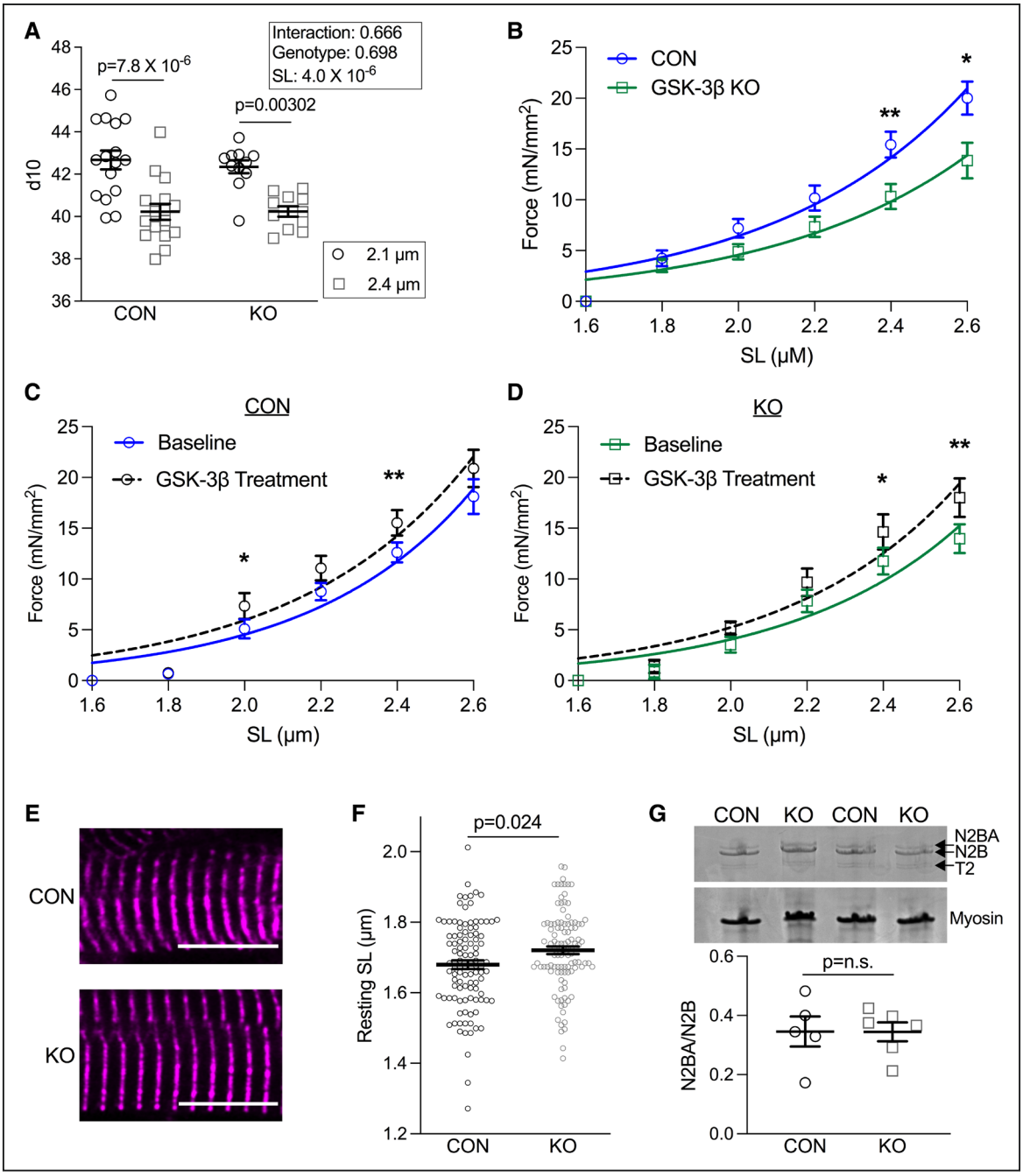

Figure 2. GSK-3β (Glycogen synthase kinase 3β) knockout (KO) mice have increased titin compliance.
A, Summary d10 (lattice spacing) collected by x-ray diffraction data from skinned papillary muscle fibers from control (CON; 16 fibers from 6 animals) and GSK-3β KO mice (11 fibers from 4 animals). Fibers were measured at sarcomere length (SL) 2.1 and 2.4 μm (effect of SL P=4.0×10^{-6} by repeated measures 2-way ANOVA with Sidak multiple pair-wise comparison test). **B**, Passive tension measured in CON and GSK-3β KO mice at SLs from 1.6 to 2.6 μm. (P values determined by unpaired parametric t test, with the exception of data collected at 2.0, which was determined by Mann-Whitney t test.) Passive tension measured in (**C**) CON (n=3 mice/4–5 cells per mouse; P: 1.8=0.88^{W}, 2.0=0.022^{W}, 2.2=0.060^{P}, 2.4=0.011^{W}, 2.6=0.15^{P}) and (**D**) KO mice (n=3 mice/4–5 cells per mouse; P: 1.8=0.69^{W}, 2.0=0.080^{W}, 2.2=0.20^{P}, 2.4=0.043^{P}, 2.6=0.0073^{P}) at baseline and with GSK-3β treatment. P values were determined by paired t test. **E**, Representative images of fixed tissue from CON and KO mice in which the z-disc is designated with α-actinin (n=30 cells/animal from 3 animals/group). Scale bar =10 μm. **F**, Summary data of resting SL (μm; P=0.024 by Mann-Whitney t test) shown as means±SEM. **G**, Representative Coomassie stained gel with quantification below of CON and GSK-3β KO mice in which titin isoforms N2BA, N2B, degraded titin (T2), and myosin are indicated by arrows (n=5–6 mice/group). P value was determined by Mann-Whitney test. ^{P} indicates parametric test; and ^{W}, nonparametric Wilcoxon test.

been fully elucidated, so it is possible cMyBP-C acts in a separate parallel pathway to the titin-based mechanism and that GSK-3β is involved in both.

GSK-3β Phosphorylates Z-Disc Proteins

To further understand the mechanism by which GSK-3β modulates LDA, we next sought to identify the myofilament

phospho-targets of GSK-3β. Mass spectrometry analysis of phospho-enriched myofilament samples from GSK-3β control (n=4) and knockout mice (n=5) identified 981 phospho-sites on 305 proteins (Table S5). Using cut-offs of $P<0.05$ and $\log_2 FC<-0.5$, we found decreased phosphorylation at 9 S/T residues on 8 proteins in the knockout mice, including the structural z-disc protein abLIM-1,[50] and z-disc affiliated proteins Supervillin,[51] Synaptopodin,[52] and SPEG (striated muscle preferentially expressed protein kinase) (Figure 3A and 3B), suggesting they are GSK-3β targets. Two phosphorylation sites increased ($\log_2 FC>0.5$, HSPB6 (heat shock protein beta-6) and B3AT [band 3 anion transport protein]). We did not detect changes in phosphorylation of any thin filament proteins known to impact calcium sensitivity as well as any changes in titin phosphorylation that could be linked to the decreased passive tension.

The mass spectrometry results indicated GSK-3β's myofilament targets are primarily at the z-disc in vivo, which we verified with immunofluorescence on LV skinned myocytes from control and knockout mice (n=3), using antibodies against phosphorylated serine/threonine residues, and α-actinin. As a positive control, a subset of control myocytes was incubated with alkaline phosphatase. We used the α-actinin channel to delineate the z-disc and create a region of interest to quantify the pSer/Thr signal. The area of z-disc pSer/Thr staining significantly decreased in GSK-3β knockout mice compared with control animals ($P=0.0091$, Figure 3C and 3D). Decreased phosphorylated z-disc area was also observed in cells incubated with alkaline phosphatase ($P=1.0\times10^{-6}$).

Phosphorylation at Y216 Targets GSK-3β to the Myofilament

Consistent with its targets, we also found GSK-3β localizes to the myofilament z-disc. Myocytes isolated from human nonfailing LV myocardium were skinned before plating/fixing to remove the confounding presence of the cytosolic pool of GSK-3β. While total GSK-3β displayed a mild colocalization with α-actinin, pY216 GSK-3β displayed a much stronger localization to the z-disc. Comparatively, pS9 GSK-was not at the z-disc and instead appeared to localize to the intercalated disc (Figure 4A). This intercalated disc localization was not unique to the pS9 antibody, however, as all GSK-3β antibodies localized to the intercalated disc (Figure S5). Immunofluorescence images for IgG and secondary antibody only controls to test for nonspecific binding in human myocytes are shown in Figure S6.

That phosphorylation at Y216 on GSK-3β increased its affinity for the myofilament was further shown by immunoprecipitation experiments in human nonfailing LV myocardium enriched for the myofilament. Using total GSK-3β, pS9 GSK-3β, or pY216 GSK-3β as bait, total and pS9 GSK-3β did not pull-down myofilament proteins, while pY216 GSK-3β showed high levels of the primary myofilament proteins (Figure 4B). Because of the strong association of myofilament proteins for each other, this result does not indicate a specific binding partner of pY216 GSK-3β. As total GSK-3β should include pY216 GSK-3β, it was surprising there was little interaction between total GSK-3β and the myofilament. One explanation is that Y216 phosphorylation makes up a small percentage of total GSK-3β, however, it is also possible this is due to differences between the primary antibodies.

To confirm whether phosphorylation at Y216 is necessary and sufficient for GSK-3β's association with the myofilament, we generated myc-tagged adenoviruses (so the same primary antibody could be used in each) for wild-type GSK-3β, phospho-null (Y216F) GSK-3β, and phospho-mimetic (Y216E) GSK-3β and transduced them into neonatal rat ventricular myocytes. Probing for the myc-tag revealed equal expression in the transduced neonatal rat ventricular myocytes (Figure 4C and 4D). Coimmunoprecipitation in myofilament-enriched samples showed Y216E GSK-3β strongly bound to the myofilament, while almost no binding was observed with the phospho-null Y216F GSK-3β (Figure 4E and 4F). A modest amount of myc-wild-type GSK-3β also associated with the myofilament, which we attributed to endogenous Y216 phosphorylation, as shown by Western blot (Figure 4G).

AbLIM-1 Interacts With Titin at the Z-Disc and Is Required for Normal LDA

Of the GSK-3β phospho-targets we identified by mass spectrometry, abLIM-1 was of specific interest because (1) multiple phosphorylation sites on the protein were decreased in the knockout mice indicating strong regulation by GSK-3β (Figure 5A), (2) we previously identified abLIM-1 as a target of GSK-3β in a dog model of HF,[13] and (3) LIM domains are involved in stress sensing.[53] No statistical differences in protein (n=5; Figure 5B and 5C) or transcript levels (n=4; Figure S7A) of abLIM-1 were detected in knockout mice. Immunofluorescence in control and knockout myocytes showed abLIM-1 localizes to the z-disc (Figure 5D) as previously shown[50] and is solely a sarcomeric protein, as abLIM-1 was only detected in the myofilament fraction and absent from the soluble, primarily cytosolic, fraction (n=3 control, 4 knockout; Figure S7B and S7C). Immunofluorescence images for IgG and secondary antibody only controls to test for nonspecific binding in mouse myocytes are shown in Figure S8.

The N terminus of titin, specifically the Z1Z2 domains and z-repeats, localizes to the z-disc.[54] The Z1Z2 domains bind to TCAP (telethonin), a complex that is important for stress sensing at the z-disc[55] and involved in maintaining passive tension.[56] As passive tension was decreased in the GSK-3β knockout mice, we hypothesized abLIM-1 interacts with the Z1Z2 domains of titin at the z-disc. We

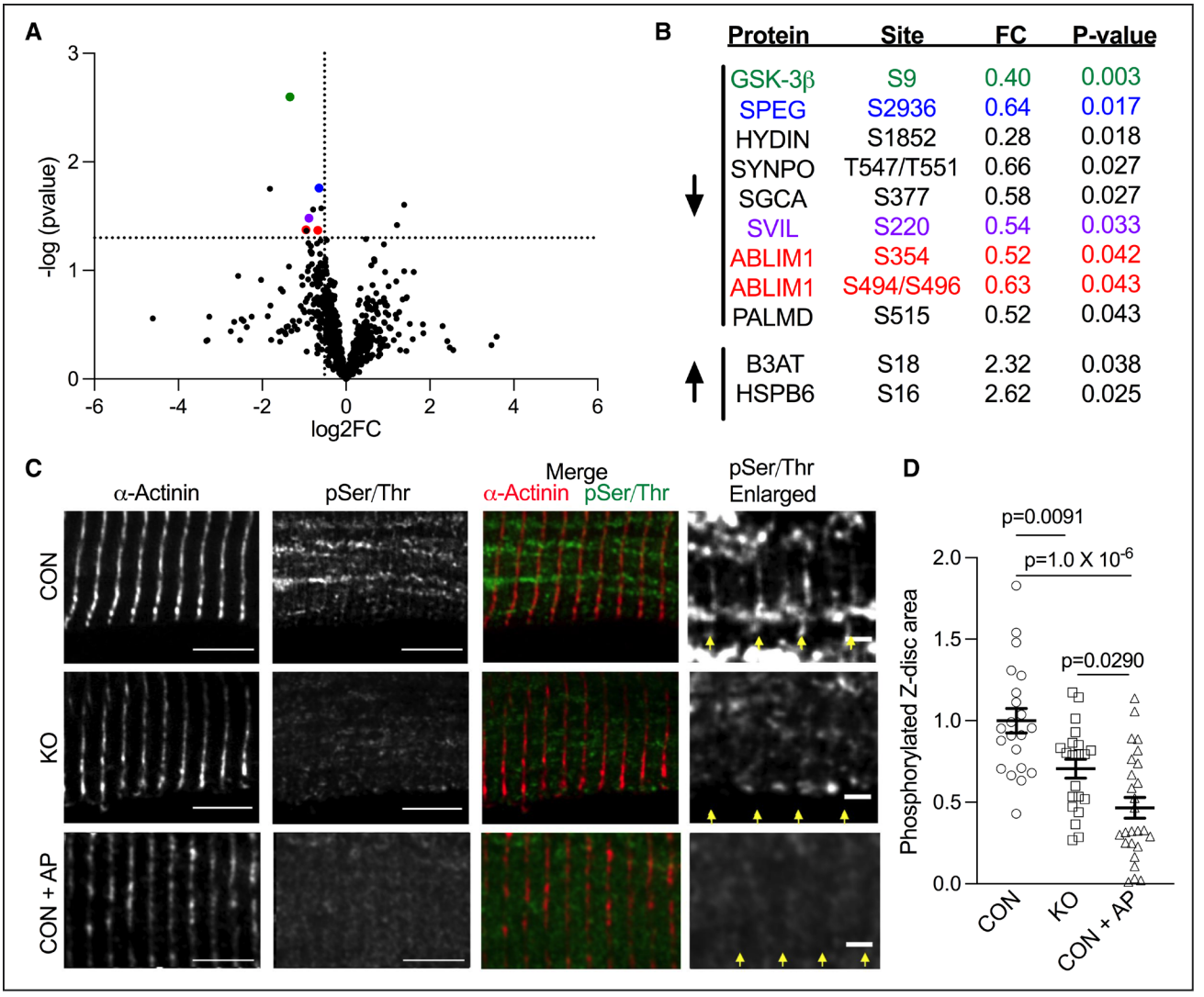

Figure 3. GSK-3β (Glycogen synthase kinase 3β) phosphorylates z-disc proteins.
A, Volcano plot depicting phosphorylation sites in control (CON; n=4) and GSK-3β knockout (KO; n=5) mice identified by mass spectrometry. Cutoffs for *P* value are displayed at *P*=0.05 and for a fold change of a 30% decrease. **B**, Significantly downregulated and upregulated sites. Proteins shown to localize to the z-disc are color coded to their respective points on the volcano plot. **C**, Representative immunofluorescence from CON and GSK-3β KO LV tissue in which samples were probed for the z-disc marker α-actinin (red in merged image) and phosphorylated serine/threonine residues (green in merged image). A subset of CON tissue was preincubated with alkaline phosphatase (AP) to act as a positive control. Scale bar=5 μm (2 μm in enlarged images). Yellow arrows delineate z-discs. **D**, Quantification of z-disc area containing pSer/Thr staining (normalized to control). n=3 mice, 21–26 cells/group, 10 z-discs/cell. *P* values were calculated via 1-way ANOVA with Tukey multiple comparison test. ABLIM1 indicates actin binding LIM domain protein 1; B3AT, band 3 anion transport protein; HSPB6, heat shock protein beta-6; HYDIN, hydrocephalus-inducing protein homolog; PALMD, palmdelphin; SGCA, sarcoglycan alpha; SPEG, striated muscle preferentially expressed protein kinase; SVIL, supervillin; and SYNPO, synaptopodin.

performed coimmunoprecipitation experiments in which we incubated recombinant GST-tagged abLIM-1 with Z1Z2 peptides (a kind gift from Dr Siegfried Labeit). Using the GST-tag on abLIM-1 as bait, we were able to identify Z1Z2 in the elutants via mass spectrometry (Figure 5E), indicating that abLIM-1 can bind the Z1Z2 domains on titin. To determine whether this interaction can be regulated by GSK-3β, we treated abLIM-1 with GSK-3β and found this ablated its interaction with Z1Z2 (n=4–5 samples/group; *P*=0.0188 by ANOVA as previous studies have indicated IP-MS/MS (immunoprecipitation-mass spectrometry) experiments in the myofilament can be analyzed by parametric statistical tests[57]).

We next sought to determine mechanistically whether abLIM-1 is involved in LDA signaling in the myocyte. We generated engineered human heart tissues (EHT)[35] by seeding decellularized myocardium with human induced pluripotent stem cell cardiomyocytes and allowed them to mature for 2 weeks. The EHTs were then treated with either scrambled siRNA or abLIM-1 siRNA, which resulted in a 30% knockdown of abLIM-1 (*P*=0.032, Figure 5F and 5G). The abLIM-1 treatment did not result in alteration of cross-sectional area of the EHTs (Figure 5H). EHTs were paced at 1 Hz in Tyrodes solution while simultaneously measuring isometric twitch force. To measure LDA, the EHT was slacked to −10% of the

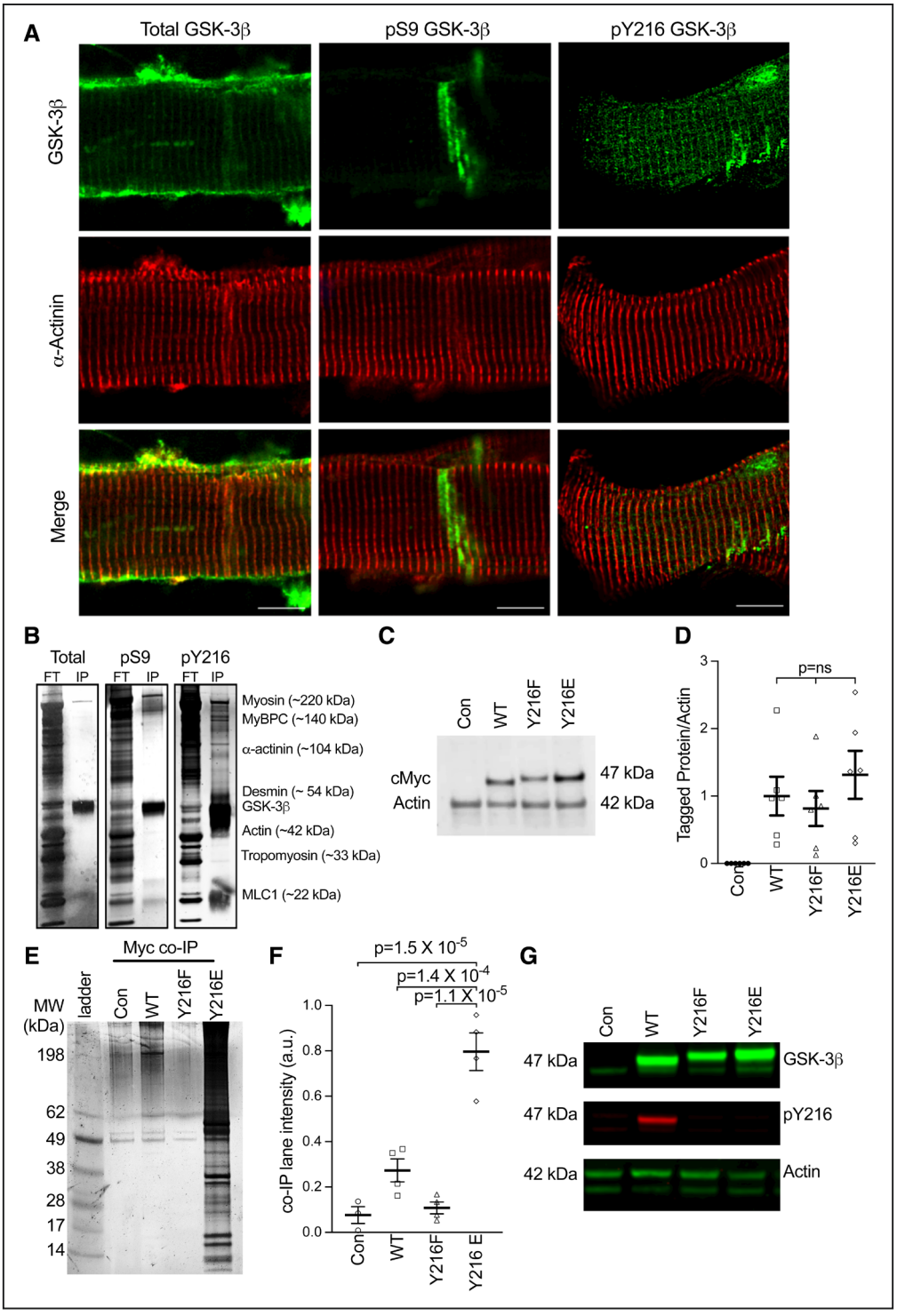

Figure 4. GSK-3β (Glycogen synthase kinase 3β) associates with the myofilament which is mediated by Y216 phosphorylation. **A**, Representative immunofluorescence of human nonfailing (NF) ventricular myocytes. Total, pS9, and pY219 GSK-3β are depicted in green and counterstained with α-actinin (red). Scale bars=10 μm. **B**, Silver-stained gel depicting flow through (FT) and eluted immunoprecipitation (IP) for myofilament-enriched NF sample coimmunoprecipitated with either total, pS9, or pY216 GSK-3β antibodies. Labels on the **right** correspond to hypothesized myofilament proteins and molecular weights. **C**, Example Western blot for cMyc for control (CON; untransduced) neonatal rat ventricular cardiomyocytes (NRVMs) and those transduced with Myc-tagged wild-type (WT) GSK-3β, Y216F GSK-3β, and Y216E GSK-3β. **D**, Summary data from data in **B**, means±SEM (n= 6/group). *P* values were calculated via ordinary 1-way ANOVA with Tukey multiple comparisons. **E**, Representative silver-stained gel depicting coimmunoprecipitation of proteins pulled down (Myc co-IP) with a myc-tag antibody in control, WT, Y216F, and Y216E groups. **F**, Summary data of co-IP experiment, showing total lane intensity (mean±SEM; n=4/group, statistics by nonparametric 1-way ANOVA [Kruskal-Wallis] with Dunn multiple comparisons). **G**, Representative Western blot from **E**, depicting total GSK-3β, pY216, and actin in myofilament-enriched NRVMs. MLC1 indicates myosin light chain.

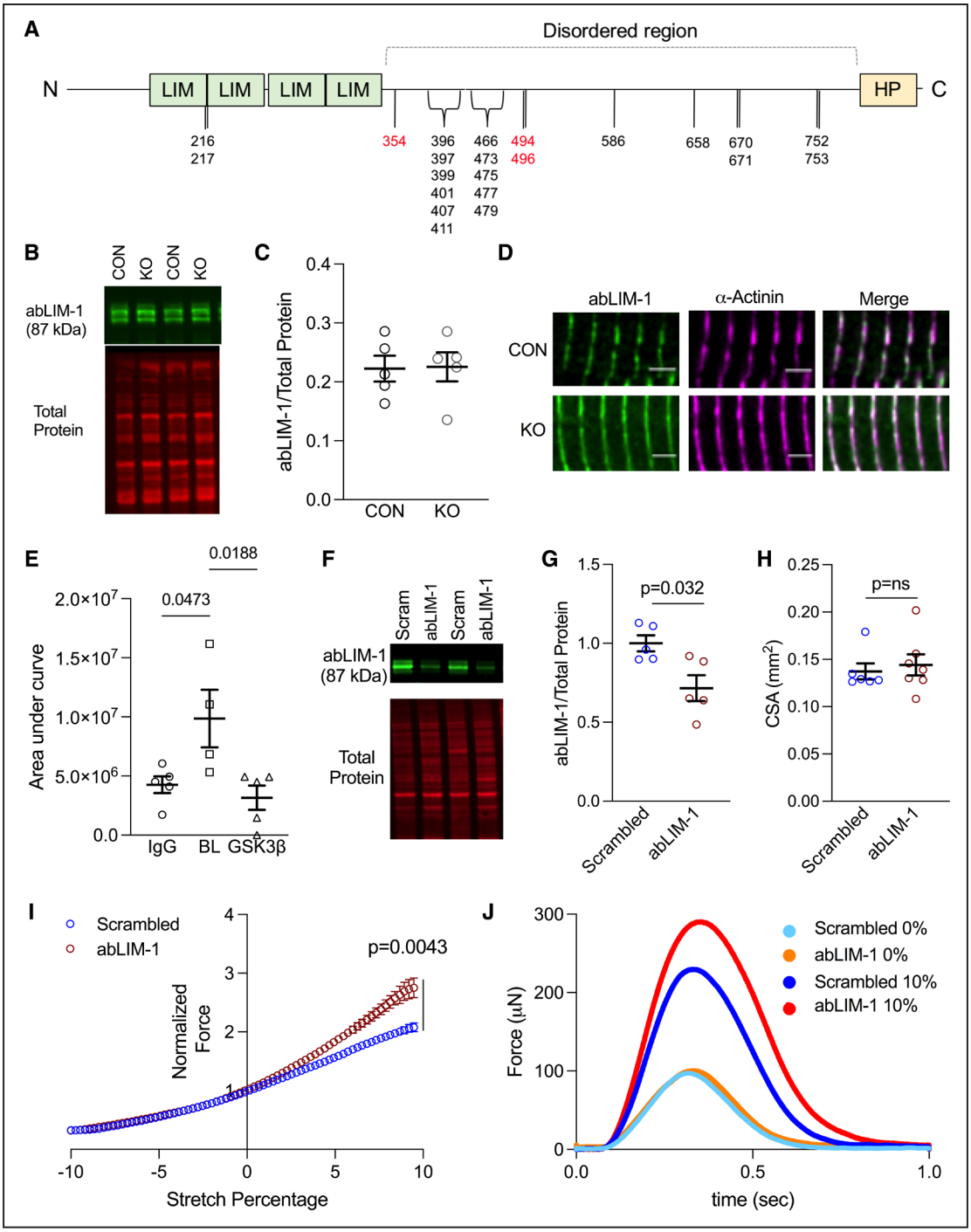

Figure 5. AbLIM-1 (Actin-binding LIM protein 1) localizes to the z-disc and modulates passive stiffness via titin.
A, Graphic of abLIM-1 domains and phosphorylation sites. Sites depicted in black are unchanged in GSK-3β (glycogen synthase kinase 3β) knockout (KO) mice, and sites depicted in red were reduced. **B**, Representative Western blot of abLIM-1 in whole tissue lysis of control (CON) and GSK-3β KO mice. **C**, abLIM-1 normalized to total protein in CON and KO mice (n=5). Statistics were calculated via Mann-Whitney test (**D**) abLIM-1 (green) and α-actinin (pink) staining in CON and GSK-3β KO mouse left ventricle (LV). **E**, Quantification (area under the curve) of Z1Z2 titin peptides nonspecifically bound (IgG control) and pulled down by GST-tagged abLIM-1, at baseline (BL) and with GSK-3β pretreatment (GSK) obtained by mass spectrometry. n values are as follows: IgG=5, BL=4, and GSK-3β=5. *P* values were calculated via 1-way ANOVA and Tukey multiple comparison test. **F**, Representative western blot of abLIM-1 and total protein stain in scrambled and abLIM-1 engineered heart tissues (EHTs). **G**, Quantification of abLIM-1/total protein, normalized to scrambled (n=5, *P*=0.032). *P* value was calculated by Mann-Whitney test. **H**, Cross-sectional area (CSA) of scrambled and abLIM-1 EHTs. *P* value was calculated via unpaired Mann-Whitney test. **I**, Twitch forces in EHTs treated with either scrambled (blue) or abLIM-1 (red) siRNA. Twitch forces were measured at 72 steps between a −10% (of total EHT length) slack to +10% stretch. Twitch forces are normalized to 0% stretch (n values: Scrambled=6, abLIM-1=7). *P*=0.0043 refers to the difference between groups at 10% stretch, calculated by unpaired *t* test. **J**, Representative traces of force (μN) at 0% (light blue = scrambled, orange = abLIM-1) and 10% (dark blue = scrambled, red = abLIM-1) stretch in a scrambled and abLIM-1 siRNA treated EHT.

tissue culture length and then stretched to +10%, all while continuing 1 Hz pacing (n=6 scrambled, 7 abLIM-1). Both scrambled and abLIM-1 siRNA treated EHTs exhibited increased twitch force with stretch, however, the EHTs with reduced abLIM-1 showed enhanced length sensitivity compared with the scrambled group, with a divergence occurring around 2% stretch (Figure 5I). The greatest difference in force occurred at 10% stretch (P=0.0043; (Figure 5J). These experiments indicate that abLIM-1 binds titin at the z-disc to depress passive tension and thus LDA, and that its phosphorylation by GSK-3β relieves this inhibition and results in normal function.

Myofilament GSK-3β Is Decreased in HF Samples With Dampened LDA

We next determined whether myofilament-localized GSK-3β plays a role in human HF. We used LV myocardium from human nonfailing rejected donor hearts (n=19) and explanted hearts from patients with HF (n=22, demographics in the Table). These 41 samples were collected from 2 biobanks (Loyola University Chicago and Cleveland Clinic). Myofilament GSK-3β was significantly reduced in myocardium from patients with HF compared with nonfailing hearts, while whole tissue GSK-3β remained unchanged when normalized to either the total protein stain (Figure 6A and 6B) or the actin band of the total protein stain (Figure S9). No statistical differences were detected in phosphorylated GSK-3β (pS9 and pY216 normalized to total GSK-3β) in either whole tissue or the myofilament (Figure S10A through S10E).

Despite observing no statistical difference in whole tissue GSK-3β between the groups, we selected the samples with the highest and lowest levels of whole tissue GSK-3β (n=5–7 cells/ 3 hearts per group) and measured force-calcium relationships at long SL. There were no statistical differences between these groups (Figure S11), supporting that whole tissue (primarily cytosolic) GSK-3β is not responsible for modulating sarcomeric function.

Last, we tested whether HF patients with diminished sarcomere-localized GSK-3β had depressed LDA as the mouse model would predict. We measured force-calcium relationships at both short and long SLs in nonfailing and HF LV (n=3 hearts/group, 3 cells/heart). Both nonfailing and HF groups experienced an increase in F_{max} with stretch; however, while we observed increased calcium sensitivity in nonfailing myocytes when increasing SL, this effect was entirely absent in the HF group (Figure 6C through 6F, Table S6). These data suggest z-disc localized GSK-3β is lost in human HF, which correlates with a loss of LDA.

Table. Patient Characteristics for NF and HF Samples

	NF	HF	*P* value
n	19	22	…
Age, y	58.3±8.6	53.9±2.7*	0.21
% White	94.7%	72.2%†	0.063
% Female	42.1%	40.9%	0.94
% Ischemic	0%	63.6%	<0.0001
LVEF, %	62.1±2.2%‡	21.4±2.0%§	<0.0001

Age, gender, ethnicity, ischemia, and LVEF for NF and HF LV samples. *P* values were calculated as follows: age (unpaired *t* test), ethnicity (χ^2), sex (χ^2), ischemia (χ^2), LVEF (Mann-Whitney test). HF indicates heart failure; LV, left ventricle; LVEF, left ventricular ejection fraction; and NF, nonfailing.

Superscripts represent categories with missing values

*4 missing values,

†4 missing values,

‡7 missing values, and

§11 missing values. Missing values have been excluded from statistical analysis.

DISCUSSION

GSK-3β is a central kinase in multiple critical signaling pathways in the cardiomyocyte, and recent evidence[13,58] suggests it also regulates sarcomere contractile function, although direct evidence is lacking. To address this gap, we used an inducible cardiomyocyte-specific GSK-3β knockout mouse model. We discovered GSK-3β is essential for normal LDA, the ability of the myocyte to respond to stretch and a critical component of the Frank-Starling mechanism, resulting in calcium desensitization at longer SLs. Mechanistically, this is via decreased titin-based passive tension observed in the knockout mice, which is a critical length sensor for LDA. GSK-3β localizes to the sarcomere z-disc via its own phosphorylation at Y216 and phosphorylates several z-disc proteins, including abLIM-1. Almost nothing is known about abLIM-1 in the heart, but we have found that it localizes to the z-disc where it can bind to the Z1Z2 domains of titin, and this binding is blocked by GSK-3β phosphorylation. Furthermore, abLIM-1 acts as an inhibitor of passive tension and LDA, which is relieved by GSK-3β. Importantly, in LV myocardium from human patients with HF there was reduced sarcomeric GSK-3β (but not whole tissue GSK-3β) and diminished LDA compared with nonfailing samples. As reduced LDA in HF is detrimental, altering Y216 phosphorylation to restore sarcomeric GSK-3β localization is a potential therapeutic strategy for restoring the Frank-Starling mechanism in patients with HF.

GSK-3β Modulates LDA Via Titin

The Frank-Starling law of the heart states that the stroke volume of the LV increases as LV volume increases,[59] allowing the heart to respond to changes in volume on a beat-to-beat basis. This behavior stems from the response of cardiomyocytes to stretch, which results in an increase in force production, termed LDA. LDA, and thus the Frank-Starling mechanism, can become depressed in HF.[23–27] While most studies are conducted

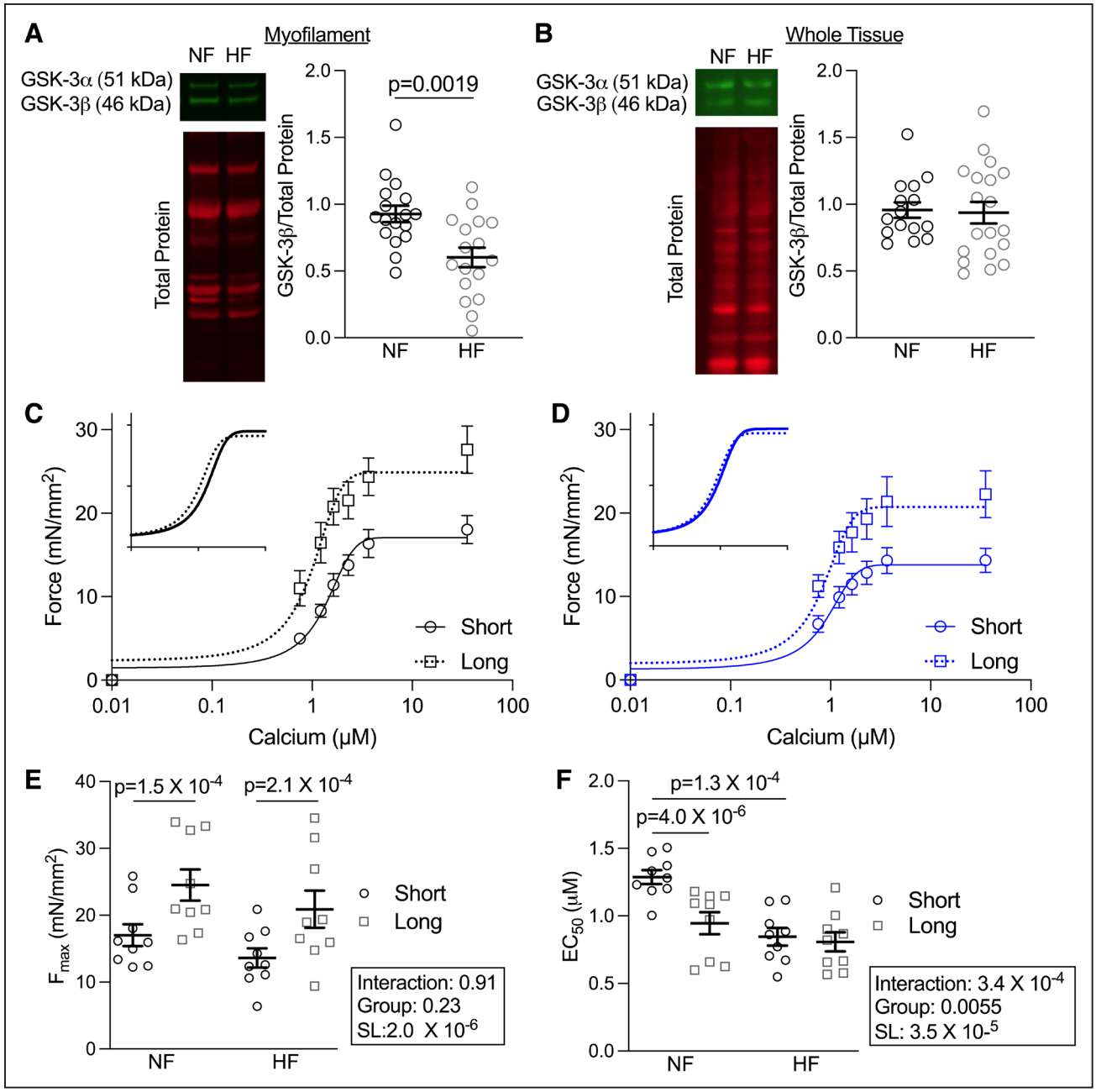

Figure 6. Myofilament-localized GSK-3β (glycogen synthase kinase 3β) and length-dependent activation (LDA) is reduced in human heart failure (HF).

A, Example blots and quantification of myofilament-enriched (n=17, *P*=0.0019 by unpaired *t* test) and (**B**) whole tissue preparation (n values: nonfailing [NF]=15, HF=19) of left ventricle (LV) from human NF and HF samples. **C**, Mean force as a function of calcium concentration and fitted curves for skinned myocytes from the LV of NF and (**D**) patients with HF from which measurements were taken at short (1.9 μm) and long (2.3 μm) sarcomere length (SL). **E**, F_{max} and (**F**) EC_{50} depicted as mean±SEM (n=9 myocytes from 3 patients). *P* values were calculated from 2-way repeated measures ANOVA with pair-wise comparisons.

in patients with end-stage HF, animal models have shown that this mechanism may be lost in the earlier stages, before hypertrophy and fibrosis.[5] Despite the fact it was initially discovered over a century ago—the molecular mechanisms of LDA are still unclear, which may explain why some studies have found the Frank-Starling mechanism to be unaltered in HF.[26,27] There are several hypotheses supported by the existing work, and these may not be exclusive, since such a critical behavior could warrant redundant pathways to operate. There are currently 3 primary mechanisms for explaining LDA[20]: (1) decreased interfilament spacing from sarcomeric stretch brings myosin heads closer to actin to increase the likelihood of crossbridge formation, (2) phosphorylation/regulation of thin filament proteins with stretch increases calcium sensitivity, and (3) stretch is sensed by titin

strain, resulting in structural rearrangement that favors crossbridge formation.

We were able to rule out the first 2 mechanisms by which GSK-3β regulates LDA, as lattice spacing was not altered in GSK-3β knockout mice and phospho-proteomics did not identify any phosphorylation sites connected to LDA or calcium sensitivity.[20] Instead, our data implicate the titin-based mechanism. Titin's ability to create passive tension directly correlates with an enhanced LDA response[28,60,61]; and myocytes with a loss of titin compliance have a blunted LDA just as we observed in the GSK-3β knockout mice. Additionally, incubation with recombinant GSK-3β increased passive tension. How, then, does GSK-3β modify titin compliance? GSK-3β did not affect titin itself, as MS revealed GSK-3β did not phosphorylate any titin residues, and there was no change in titin isoforms. Thus, GSK-3β must regulate the interaction between titin and z-disc proteins.

GSK-3β Facilitates LDA Via abLIM-1

While a great deal remains unknown about titin's structure, function, and interacting partners at the z-disc, there are studies that support the hypothesis that altering titin's interaction with z-disc proteins can alter passive tension. For example, the Z1Z2 domains of titin, which are located in its z-disc region, are highly compliant in isolation, but are stabilized via z-disc binding partners.[54]

Of the GSK-3β phosphorylated proteins identified in our phospho-proteomics screen, we were most interested in abLIM-1. abLIM-1 has been the focus of very few studies, only one of which identified it in the heart.[50] However, several proteins of the same family, which contain LIM domains, such as MLP (muscle LIM protein) and FHL1 and FHL2 (Four and a half LIM domain protein) have been shown to be stress/strain sensors.[62] Indeed, this work establishes abLIM-1 as a critical z-disc protein in maintaining normal function. First, abLIM-1 interacts with the Z1Z2 domains of titin in vitro which also interact with TCAP, an interaction important for stress sensing, maintaining passive tension,[55,56] and likely LDA although this has not yet been shown.

Similar to TCAP, abLIM-1 is also required for normal stress sensing at the z-disc, as siRNA-induced reduction in abLIM-1 significantly increased LDA. The direction of this impact was somewhat surprising, as we anticipated facilitation or enhancement of LDA by abLIM-1, and that its reduction would lead to depressed LDA. However, these results show that abLIM-1 is not simply required for the LDA mechanism to proceed but modulates the response of the cell to stretch by acting as a brake on stretch sensing and LDA. This regulatory paradigm is not unprecedented, as there are other proteins in cardiac excitation-contraction coupling that inhibit activity in a phosphorylation-dependent manner, such as cMyBP-C and phospholamban. In fact, our experiments with recombinant Z1Z2 and abLIM-1 show that GSK-3β ablates their interaction and relieves abLIM-1's inhibition of passive tension and LDA. These results introduce a new player in sarcomere mechanosensing, and future studies are warranted to understand the structural basis for abLIM-1's novel functional role and how its phosphorylation alters this function.

GSK-3β Localizes Through pY216

It is likely that declining myofilament GSK-3β contributes to decreased LDA and Frank-Starling mechanism observed in the failing human heart.[5,24,26] As GSK-3β can directly enhance calcium sensitivity at long SLs, it is an intriguing candidate for a HF therapeutic. Indeed, a therapeutic that could increase calcium sensitivity at long SLs while not affecting it at short or resting SLs would be highly beneficial, especially since LDA takes place within 5 ms[21] so it can regulate calcium sensitivity during a single beat. A straight-forward calcium sensitizer would increase contractility but would also slow relaxation. However, a therapeutic that increased calcium sensitivity at long SLs when the heart is filled with blood just before systole would enhance contractility, and as the LV chamber volume decreases during systole and myocyte SL shortens calcium sensitivity would decrease, aiding relaxation. Indeed, compounds that aim to enhance sarcomere-based contractility have frequently resulted in depressed relaxation,[63] creating a benefit-cost tradeoff.

However, GSK-3β is such a promiscuous signaling kinase[64] that broadly elevating GSK-3β would likely result in catastrophic off-target effects. Thus, the ability to manipulate strictly myofilament-localized GSK-3β is imperative, which we found is regulated by its phosphorylation at Y216. Several kinases have been reported to phosphorylate Y216, including Fyn (tyroine-protein kinaes fyn),[65] PYK2 (protein-tyrosine kinase 2-beta),[66] MEK1 (dual specificity mitogen-activated protein kinase 1),[67] and as an auto-phosphorylation event.[68] Generally, tyrosine kinases phosphorylate fewer targets than serine/threonine kinases,[69] thus the large number of kinases targeting Y216 suggests dynamic regulation. Whether targeting the Y216 phosphorylation site directly or by modulating the activity of an upstream kinase would be a useful therapeutic approach to restore myofilament GSK-3β and thus LDA in HF will need to be established in future studies.

Conclusions

We have identified a novel mechanism by which GSK-3β regulates LDA, arising from its z-disc localization and phosphorylation targets, most likely abLIM-1, a new player in sarcomere function. While LDA and the Frank-Starling mechanism were described well over a century ago, many aspects of the mechanism remain unresolved. This study reveals new kinase regulation of LDA and suggests myofilament GSK-3β is a strong therapeutic

candidate to rescue the depressed LDA that is observed in HF. Furthermore, these results add to the small number of studies demonstrating that the z-disc is not merely a structural scaffold for the thin filaments but is also capable of regulating active contraction.

ARTICLE INFORMATION

Received May 4, 2021; revision received January 20, 2022; accepted February 7, 2022.

Affiliations

Department of Cell and Molecular Physiology, Loyola University Stritch School of Medicine, Maywood, IL (M.J.S.-D., M.P., T.G.M., N.A.M., E.P., J.A.K.). Center for Synchrotron Radiation Research and Instrumentation and Department of Biological Sciences, Illinois Institute of Technology, Chicago (W.M., H.M.G., T.I.). Department of Bioengineering, Yale University, New Haven, CT (S. Shao, S. Shen, S.G.C.). Division of Cardiovascular Health and Disease, Department of Internal Medicine, Heart, Lung, and Vascular Institute, University of Cincinnati, OH (M.K., S. Sadayappan). Department of Pharmacology, Cardiovascular Research Institute, UC Davis School of Medicine, CA (J.L.M.). Department of Cardiovascular and Metabolic Sciences, Cleveland Clinic, OH (C.S.M.). Department of Cellular and Molecular Physiology, Yale School of Medicine, New Haven, CT (S.G.C.).

Acknowledgments

We thank the patients and organ donors who donated the samples used in this project. This research used resources of the Advanced Photon Source, a US Department of Energy (DOE) Office of Science User Facility operated for the DOE Office of Science by Argonne National Laboratory under Contract No. DE-AC02-06CH11357.

Sources of Funding

This work was supported by the National Institutes of Health (R01HL136737 to Dr Kirk; 9P41GM103622 to T. Irving; R01HL130357, R01HL105826, R01AR078001, and R01HL143490 to S. Sadayappan), the American Heart Association (831515 to M.J. Stachowski-Doll; 111POST7210031 to M. Papadaki, 19TPA34830084 to S.S., and 14SDG20380148 to Dr Kirk.), and the National Science Foundation (1653160 to S.G. Campbell). This study used equipment obtained with the support of a shared instrumentation grant S10OD028449 from the National Institutes of Health to Loyola University Chicago.

Disclosures

Dr Kirk and S. Sadayappan provided consulting and conducted collaborative studies with various pharmaceutical companies, but all such work is unrelated to the content of this article. S.G.S. holds equity ownership in Propria LLC, which has licensed technology used in the research reported in this publication. The other authors report no conflicts.

Supplemental Material

Supplemental Methods
Tables S1–S6
Figures S1–S11

REFERENCES

1. Sakaguchi T, Takefuji M, Wettschureck N, Hamaguchi T, Amano M, Kato K, Tsuda T, Eguchi S, Ishihama S, Mori Y, et al. Protein kinase N promotes stress-induced cardiac dysfunction through phosphorylation of myocardin-related transcription factor a and disruption of its interaction with actin. *Circulation*. 2019;140:1737–1752. doi: 10.1161/CIRCULATIONAHA.119.041019
2. Ai X, Curran JW, Shannon TR, Bers DM, Pogwizd SM. Ca2+/calmodulin-dependent protein kinase modulates cardiac ryanodine receptor phosphorylation and sarcoplasmic reticulum Ca2+ leak in heart failure. *Circ Res*. 2005;97:1314–1322. doi: 10.1161/01.RES.0000194329.41863.89
3. Fuller SJ, Osborne SA, Leonard SJ, Hardyman MA, Vaniotis G, Allen BG, Sugden PH, Clerk A. Cardiac protein kinases: the cardiomyocyte kinome and differential kinase expression in human failing hearts. *Cardiovasc Res*. 2015;108:87–98. doi: 10.1093/cvr/cvv210
4. Lee DI, Zhu G, Sasaki T, Cho GS, Hamdani N, Holewinski R, Jo SH, Danner T, Zhang M, Rainer PP, et al. Phosphodiesterase 9A controls nitric-oxide-independent cGMP and hypertrophic heart disease. *Nature*. 2015;519:472–476. doi: 10.1038/nature14332
5. Komamura K, Shannon RP, Ihara T, Shen YT, Mirsky I, Bishop SP, Vatner SF. Exhaustion of Frank-Starling mechanism in conscious dogs with heart failure. *Am J Physiol*. 1993;265:H1119–H1131. doi: 10.1152/ajpheart.1993.265.4.H1119
6. Woulfe KC, Gao E, Lal H, Harris D, Fan Q, Vagnozzi R, DeCaul M, Shang X, Patel S, Woodgett JR, et al. Glycogen synthase kinase-3beta regulates post-myocardial infarction remodeling and stress-induced cardiomyocyte proliferation in vivo. *Circ Res*. 2010;106:1635–1645. doi: 10.1161/CIRCRESAHA.109.211482
7. Zhai P, Sadoshima J. Glycogen synthase kinase-3β controls autophagy during myocardial ischemia and reperfusion. *Autophagy*. 2012;8:138–139. doi: 10.4161/auto.8.1.18314
8. Morisco C, Zebrowski D, Condorelli G, Tsichlis P, Vatner SF, Sadoshima J. The Akt-glycogen synthase kinase 3beta pathway regulates transcription of atrial natriuretic factor induced by beta-adrenergic receptor stimulation in cardiac myocytes. *J Biol Chem*. 2000;275:14466–14475. doi: 10.1074/jbc.275.19.14466
9. Haq S, Choukroun G, Kang ZB, Ranu H, Matsui T, Rosenzweig A, Molkentin JD, Alessandrini A, Woodgett J, Hajjar R, et al. Glycogen synthase kinase-3beta is a negative regulator of cardiomyocyte hypertrophy. *J Cell Biol*. 2000;151:117–130. doi: 10.1083/jcb.151.1.117
10. Antos CL, McKinsey TA, Frey N, Kutschke W, McAnally J, Shelton JM, Richardson JA, Hill JA, Olson EN. Activated glycogen synthase-3b suppresses cardiac hypertrophy in vivo. *PNAS*. 2001;99:907–912. doi: 10.1073/pnas.231619298
11. Matsuda T, Zhai P, Maejima Y, Hong C, Gao S, Tian B, Goto K, Takagi H, Tamamori-Adachi M, Kitajima S, et al. Distinct roles of GSK-3a and GSK-3b phosphoryltion in the heart under pressure overload. *PNAS*. 2008;105:20900–20905. doi: 10.1073/pnas.0808315106
12. Hirotani S, Zhai P, Tomita H, Galeotti J, Marquez JP, Gao S, Hong C, Yatani A, Avila J, Sadoshima J. Inhibition of glycogen synthase kinase 3beta during heart failure is protective. *Circ Res*. 2007;101:1164–1174. doi: 10.1161/CIRCRESAHA.107.160614
13. Kirk JA, Holewinski RJ, Kooij V, Agnetti G, Tunin RS, Witayavanitkul N, de Tombe PP, Gao WD, Van Eyk J, Kass DA. Cardiac resynchronization sensitizes the sarcomere to calcium by reactivating GSK-3β. *J Clin Invest*. 2014;124:129–138. doi: 10.1172/JCI69253
14. Gordon AM, Homsher E, Regnier M. Regulation of contraction in striated muscle. *Physiol Rev*. 2000;80:853–924. doi: 10.1152/physrev.2000.80.2.853
15. Robinson P, Griffiths PJ, Watkins H, Redwood CS. Dilated and hypertrophic cardiomyopathy mutations in troponin and alpha-tropomyosin have opposing effects on the calcium affinity of cardiac thin filaments. *Circ Res*. 2007;101:1266–1273. doi: 10.1161/CIRCRESAHA.107.156380
16. Ren X, Hensley N, Brady MB, Gao WD. The genetic and molecular bases for hypertrophic cardiomyopathy: the role for calcium sensitization. *J Cardiothorac Vasc Anesth*. 2018;32:478–487. doi: 10.1053/j.jvca.2017.05.035
17. Wolff MR, Buck SH, Stoker SW, Greaser ML, Mentzer RM. Myofibrillar calcium sensitivity of isometric tension is increased in human dilated cardiomyopathies: role of altered beta-adrenergically mediated protein phosphorylation. *J Clin Invest*. 1996;98:167–176. doi: 10.1172/JCI118762
18. Papadaki M, Holewinski RJ, Previs SB, Martin TG, Stachowski MJ, Li A, Blair CA, Moravec CS, Van Eyk JE, Campbell KS, et al. Diabetes with heart failure increases methylglyoxal modifications in the sarcomere, which inhibit function. *JCI Insight*. 2018;3:121264. doi: 10.1172/jci.insight.121264
19. Kass DA, Solaro RJ. Mechanisms and use of calcium-sensitizing agents in the failing heart. *Circulation*. 2006;113:305–315. doi: 10.1161/CIRCULATIONAHA.105.542407
20. de Tombe PP, Mateja RD, Tachampa K, Ait Mou Y, Farman GP, Irving TC. Myofilament length dependent activation. *J Mol Cell Cardiol*. 2010;48:851–858. doi: 10.1016/j.yjmcc.2009.12.017
21. Mateja RD, de Tombe PP. Myofilament length-dependent activation develops within 5 ms in guinea-pig myocardium. *Biophys J*. 2012;103:L13–L15. doi: 10.1016/j.bpj.2012.05.034
22. Gill RM, Jones BD, Corbly AK, Ohad DG, Smith GD, Sandusky GE, Christe ME, Wang J, Shen W. Exhaustion of the Frank-Starling mechanism in conscious dogs with heart failure induced by chronic coronary microembolization. *Life Sci*. 2006;79:536–544. doi: 10.1016/j.lfs.2006.01.045
23. Sequeira V, Wijnker PJ, Nijenkamp LL, Kuster DW, Najafi A, Witjas-Paalberends ER, Regan JA, Boontje N, Ten Cate FJ, Germans T, et al.

Perturbed length-dependent activation in human hypertrophic cardiomyopathy with missense sarcomeric gene mutations. *Circ Res*. 2013;112:1491–1505. doi: 10.1161/CIRCRESAHA.111.300436
24. Schwinger RH, Böhm M, Koch A, Schmidt U, Morano I, Eissner HJ, Uberfuhr P, Reichart B, Erdmann E. The failing human heart is unable to use the Frank-Starling mechanism. *Circ Res*. 1994;74:959–969. doi: 10.1161/01.res.74.5.959
25. Kitzman DW, Higginbotham MB, Cobb FR, Sheikh KH, Sullivan MJ. Exercise intolerance in patients with heart failure and preserved left ventricular systolic function: failure of the Frank-Starling mechanism. *J Am Coll Cardiol*. 1991;17:1065–1072. doi: 10.1016/0735-1097(91)90832-t
26. Holubarsch C, Ruf T, Goldstein DJ, Ashton RC, Nickl W, Pieske B, Pioch K, Lüdemann J, Wiesner S, Hasenfuss G, et al. Existence of the Frank-Starling mechanism in the failing human heart. Investigations on the organ, tissue, and sarcomere levels. *Circulation*. 1996;94:683–689. doi: 10.1161/01.cir.94.4.683
27. Weil J, Eschenhagen T, Hirt S, Magnussen O, Mittmann C, Remmers U, Scholz H. Preserved Frank-Starling mechanism in human end stage heart failure. *Cardiovasc Res*. 1998;37:541–548. doi: 10.1016/s0008-6363(97)00227-7
28. Ait-Mou Y, Hsu K, Farman GP, Kumar M, Greaser ML, Irving TC, de Tombe PP. Titin strain contributes to the Frank-Starling law of the heart by structural rearrangements of both thin- and thick-filament proteins. *Proc Natl Acad Sci USA*. 2016;113:2306–2311. doi: 10.1073/pnas.1516732113
29. Lynch TLt, Ismahil MA, Jegga AG, Zilliox MJ, Troidl C, Prabhu SD, Sadayappan S. Cardiac inflammation in genetic dilated cardiomyopathy caused by MYBPC3 mutation. *J Mol Cell Cardiol*. 2017;102:83–93. doi: 10.1016/j.yjmcc.2016.12.002
30. McConnell BK, Jones KA, Fatkin D, Arroyo LH, Lee RT, Aristizabal O, Turnbull DH, Georgakopoulos D, Kass D, Bond M, et al. Dilated cardiomyopathy in homozygous myosin-binding protein-C mutant mice. *J Clin Invest*. 1999;104:1235–1244. doi: 10.1172/JCI7377
31. Lynch TLt, Kumar M, McNamara JW, Kuster DWD, Sivaguru M, Singh RR, Previs MJ, Lee KH, Kuffel G, Zilliox MJ, et al. Amino terminus of cardiac myosin binding protein-C regulates cardiac contractility. *J Mol Cell Cardiol*. 2021;156:33–44. doi: 10.1016/j.yjmcc.2021.03.009
32. Fischetti R, Stepanov S, Rosenbaum G, Barrea R, Black E, Gore D, Heurich R, Kondrashkina E, Kropf AJ, Wang S, et al. The BioCAT undulator beamline 18ID: a facility for biological non-crystalline diffraction and X-ray absorption spectroscopy at the Advanced Photon Source. *J Synchrotron Radiat*. 2004;11:399–405. doi: 10.1107/S0909049504016760
33. Jiratrakanvong J, Shao J, Menendez M, Li X, Li J, Ma W, Agam G, Irving T. MuscleX: software suite for diffraction X-ray imaging V1.13.1. 2018.
34. Ma W, Gong H, Irving T. Myosin head configurations in resting and contracting murine skeletal muscle. *Int J Mol Sci*. 2018;19:2643. doi: 10.3390/ijms19092643
35. Schwan J, Kwaczala AT, Ryan TJ, Bartulos O, Ren Y, Sewanan LR, Morris AH, Jacoby DL, Qyang Y, Campbell SG. Anisotropic engineered heart tissue made from laser-cut decellularized myocardium. *Sci Rep*. 2016;6:32068. doi: 10.1038/srep32068
36. Sewanan LR, Schwan J, Kluger J, Park J, Jacoby DL, Qyang Y, Campbell SG. Extracellular matrix from hypertrophic myocardium provokes impaired twitch dynamics in healthy cardiomyocytes. *JACC Basic Transl Sci*. 2019;4:495–505. doi: 10.1016/j.jacbts.2019.03.004
37. Kirk JA, Chakir K, Lee KH, Karst E, Holewinski RJ, Pironti G, Tunin RS, Pozios I, Abraham TP, de Tombe P, et al. Pacemaker-induced transient asynchrony suppresses heart failure progression. *Sci Transl Med*. 2015;7:319ra207. doi: 10.1126/scitranslmed.aad2899
38. Martin TG, Myers VD, Dubey P, Dubey S, Perez E, Moravec CS, Willis MS, Feldman AM, Kirk JA. Cardiomyocyte contractile impairment in heart failure results from reduced BAG3-mediated sarcomeric protein turnover. *Nat Commun*. 2021;12:2942. doi: 10.1038/s41467-021-23272-z
39. Arrell DK, Neverova I, Fraser H, Marbán E, Van Eyk JE. Proteomic analysis of pharmacologically preconditioned cardiomyocytes reveals novel phosphorylation of myosin light chain 1. *Circ Res*. 2001;89:480–487. doi: 10.1161/hh1801.097240
40. Konhilas JP, Irving TC, de Tombe PP. Frank-Starling law of the heart and the cellular mechanisms of length-dependent activation. *Pflugers Arch*. 2002;445:305–310. doi: 10.1007/s00424-002-0902-1
41. Kumar M, Govindan S, Zhang M, Khairallah RJ, Martin JL, Sadayappan S, de Tombe PP. Cardiac Myosin-binding Protein C and Troponin-I phosphorylation independently modulate myofilament length-dependent activation*. *J Biol Chem*. 2015;290:29241–29249. doi: 10.1074/jbc.M115.686790
42. Martyn DA, Adhikari BB, Regnier M, Gu J, Xu S, Yu LC. Response of equatorial x-ray reflections and stiffness to altered sarcomere length and myofilament lattice spacing in relaxed skinned cardiac muscle. *Biophys J*. 2004;86:1002–1011. doi: 10.1016/S0006-3495(04)74175-2
43. Linke WA. Sense and stretchability: the role of titin and titin-associated proteins in myocardial stress-sensing and mechanical dysfunction. *Cardiovasc Res*. 2008;77:637–648. doi: 10.1016/j.cardiores.2007.03.029
44. Hutchinson KR, Saripalli C, Chung CS, Granzier H. Increased myocardial stiffness due to cardiac titin isoform switching in a mouse model of volume overload limits eccentric remodeling. *J Mol Cell Cardiol*. 2015;79:104–114. doi: 10.1016/j.yjmcc.2014.10.020
45. Lahmers S, Wu Y, Call DR, Labeit S, Granzier H. Developmental control of titin isoform expression and passive stiffness in fetal and neonatal myocardium. *Circ Res*. 2004;94:505–513. doi: 10.1161/01.RES.0000115522.52554.86
46. Hanft LM, Fitzsimons DP, Hacker TA, Moss RL, McDonald KS. Cardiac MyBP-C phosphorylation regulates the Frank-Starling relationship in murine hearts. *J Gen Physiol*. 2021;153:e202012770. doi: 10.1085/jgp.202012770
47. Freiburg A, Gautel M. A molecular map of the interactions between titin and myosin-binding protein C. Implications for sarcomeric assembly in familial hypertrophic cardiomyopathy. *Eur J Biochem*. 1996;235:317–323. doi: 10.1111/j.1432-1033.1996.00317.x
48. Harris SP, Belknap B, Van Sciver RE, White HD, Galkin VE. C0 and C1 N-terminal Ig domains of myosin binding protein C exert different effects on thin filament activation. *Proc Natl Acad Sci USA*. 2016;113:1558–1563. doi: 10.1073/pnas.1518891113
49. Mun JY, Previs MJ, Yu HY, Gulick J, Tobacman LS, Beck Previs S, Robbins J, Warshaw DM, Craig R. Myosin-binding protein C displaces tropomyosin to activate cardiac thin filaments and governs their speed by an independent mechanism. *Proc Natl Acad Sci USA*. 2014;111:2170–2175. doi: 10.1073/pnas.1316001111
50. Roof DJ, Hayes A, Adamian M, Chishti AH, Li T. Molecular characterization of abLIM, a novel actin-binding and double zinc finger protein. *J Cell Biol*. 1997;138:575–588. doi: 10.1083/jcb.138.3.575
51. Hedberg-Oldfors C, Meyer R, Nolte K, Abdul Rahim Y, Lindberg C, Karason K, Thuestad IJ, Visuttijai K, Geijer M, Begemann M, et al. Loss of supervillin causes myopathy with myofibrillar disorganization and autophagic vacuoles. *Brain*. 2020;143:2406–2420. doi: 10.1093/brain/awaa206
52. Asanuma K, Kim K, Oh J, Giardino L, Chabanis S, Faul C, Reiser J, Mundel P. Synaptopodin regulates the actin-bundling activity of α-actinin in an isoform-specific manner. *J Clin Invest*. 2005;115:1188–1198. doi: 10.1172/JCI23371
53. Hoshijima M. Mechanical stress-strain sensors embedded in cardiac cytoskeleton: Z disk, titin, and associated structures. *Am J Physiol Heart Circ Physiol*. 2006;290:H1313–H1325. doi: 10.1152/ajpheart.00816.2005
54. Kollár V, Szatmári D, Grama L, Kellermayer MS. Dynamic strength of titin's Z-disk end. *J Biomed Biotechnol*. 2010;2010:838530. doi: 10.1155/2010/838530
55. Gregorio CC, Trombitás K, Centner T, Kolmerer B, Stier G, Kunke K, Suzuki K, Obermayr F, Herrmann B, Granzier H, et al. The NH2 terminus of titin spans the Z-disc: its interaction with a novel 19-kD ligand (T-cap) is required for sarcomeric integrity. *J Cell Biol*. 1998;143:1013–1027. doi: 10.1083/jcb.143.4.1013
56. Lee EH, Gao M, Pinotsis N, Wilmanns M, Schulten K. Mechanical strength of the titin Z1Z2-telethonin complex. *Structure*. 2006;14:497–509. doi: 10.1016/j.str.2005.12.005
57. Rudolph F, Fink C, Hüttemeister J, Kirchner M, Radke MH, Lopez Carballo J, Wagner E, Kohl T, Lehnart SE, Mertins P, et al. Deconstructing sarcomeric structure-function relations in titin-BioID knock-in mice. *Nat Commun*. 2020;11:3133. doi: 10.1038/s41467-020-16929-8
58. Kuster DW, Sequeira V, Najafi A, Boontje NM, Wijnker PJ, Witjas-Paalberends ER, Marston SB, Dos Remedios CG, Carrier L, Demmers JA, et al. GSK3β phosphorylates newly identified site in the proline-alanine-rich region of cardiac myosin-binding protein C and alters cross-bridge cycling kinetics in human: short communication. *Circ Res*. 2013;112:633–639. doi: 10.1161/CIRCRESAHA.112.275602
59. Solaro RJ. Mechanisms of the Frank-Starling law of the heart: the beat goes on. *Biophys J*. 2007;93:4095–4096. doi: 10.1529/biophysj.107.117200
60. Cazorla O, Wu Y, Irving TC, Granzier H. Titin-based modulation of calcium sensitivity of active tension in mouse skinned cardiac myocytes. *Circ Res*. 2001;88:1028–1035. doi: 10.1161/hh1001.090876
61. Methawasin M, Hutchinson KR, Lee EJ, Smith JE 3rd, Saripalli C, Hidalgo CG, Ottenheijm CA, Granzier H. Experimentally increasing titin compliance

in a novel mouse model attenuates the Frank-Starling mechanism but has a beneficial effect on diastole. *Circulation*. 2014;129:1924–1936. doi: 10.1161/CIRCULATIONAHA.113.005610
62. Li A, Ponten F, dos Remedios CG. The interactome of LIM domain proteins: the contributions of LIM domain proteins to heart failure and heart development. *Proteomics*. 2012;12:203–225. doi: 10.1002/pmic.201100492
63. Hajjar RJ, Schmidt U, Helm P, Gwathmey JK. Ca++ sensitizers impair cardiac relaxation in failing human myocardium. *J Pharmacol Exp Ther*. 1997;280:247–254.
64. Beurel E, Grieco SF, Jope RS. Glycogen synthase kinase-3 (GSK3): regulation, actions, and diseases. *Pharmacol Ther*. 2015;148:114–131. doi: 10.1016/j.pharmthera.2014.11.016
65. Lesort M, Jope RS, Johnson GV. Insulin transiently increases tau phosphorylation: involvement of glycogen synthase kinase-3beta and Fyn tyrosine kinase. *J Neurochem*. 1999;72:576–584. doi: 10.1046/j.1471-4159.1999.0720576.x
66. Hartigan JA, Xiong WC, Johnson GV. Glycogen synthase kinase 3beta is tyrosine phosphorylated by PYK2. *Biochem Biophys Res Commun*. 2001;284:485–489. doi: 10.1006/bbrc.2001.4986
67. Takahashi-Yanaga F, Shiraishi F, Hirata M, Miwa Y, Morimoto S, Sasaguri T. Glycogen synthase kinase-3beta is tyrosine-phosphorylated by MEK1 in human skin fibroblasts. *Biochem Biophys Res Commun*. 2004;316:411–415. doi: 10.1016/j.bbrc.2004.02.061
68. Wang QM, Fiol CJ, DePaoli-Roach AA, Roach PJ. Glycogen synthase kinase-3 beta is a dual specificity kinase differentially regulated by tyrosine and serine/threonine phosphorylation. *J Biol Chem*. 1994;269:14566–14574.
69. Stachowski MJ, Holewinski RJ, Grote E, Venkatraman V, Van Eyk JE, Kirk JA. Phospho-proteomic analysis of cardiac dyssynchrony and resynchronization therapy. *Proteomics*. 2018;18:e1800079. doi: 10.1002/pmic.201800079