Cardiac resynchronization sensitizes the sarcomere to calcium by reactivating GSK-3β

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Cardiac resynchronization therapy (CRT), the application of biventricular stimulation to correct discoordinate contraction, is the only heart failure treatment that enhances acute and chronic systolic function, increases cardiac work, and reduces mortality. Resting myocyte function also increases after CRT despite only modest improvement in calcium transients, suggesting that CRT may enhance myofilament calcium responsiveness. To test this hypothesis, we examined adult dogs subjected to tachypacing-induced heart failure for 6 weeks, concurrent with ventricular dyssynchrony (HF dys) or CRT. Myofilament force-calcium relationships were measured in skinned trabeculae and/or myocytes. Compared with control, maximal calcium-activated force and calcium sensitivity declined globally in HF dys; however, CRT restored both. Phosphatase PP1 induced calcium desensitization in control and CRT-treated cells, while HF dys cells were unaffected, implying that CRT enhances myofilament phosphorylation. Proteomics revealed phosphorylation sites on Z-disk and M-band proteins, which were predicted to be targets of glycogen synthase kinase-3β (GSK-3β). We found that GSK-3β was deactivated in HF dys and reactivated by CRT. Mass spectrometry of myofilament proteins from HF dys, animals incubated with GSK-3β–confirmed GSK-3β–dependent phosphorylation at many of the same sites observed with CRT. GSK-3β restored calcium sensitivity in HF dys, but did not affect control or CRT cells. These data indicate that CRT improves calcium responsiveness of myofilaments following HF dys through GSK-3β reactivation, identifying a therapeutic approach to enhancing contractile function.

Introduction

For the past 30 years or more, the quest to develop treatments that directly improve systolic performance of the failing heart while also conferring long-term survival benefits has been met with disappointment (1). Agents that stimulate the β1-adrenergic receptor or enhance cyclic AMP (e.g., PDE3 inhibitors) work in the short term, but have worsened chronic outcomes. This has led some to question whether safe and effective systolic therapy for the failing heart is an oxymoron (2). A counterexample for this conclusion to be reconsidered is the device treatment known as cardiac resynchronization therapy (CRT). CRT restores coordinate contraction in heart failure (HF) patients who also have ventricular dyssynchrony due to conduction abnormalities. Its improvement of systolic function occurs within 1 beat (3) and is associated with negligible or even reduced myocardial oxygen consumption, indicating improved chamber efficiency (4, 5). While dyssynchrony is an independent risk factor in patients with HF, successful CRT reduces mortality (6, 7), perhaps to levels even below those of HF patients without dyssynchrony (8, 9).

The effects of CRT were initially attributed to its impact on chamber mechnoenergetics (3). However, recent studies by our and other laboratories have revealed many changes at the cellular and molecular levels as well, and these present a unique profile for CRT unmatched by other HF treatments to date. For example, marked heterogeneity of regional gene expression is reversed (10), the cytoprotective Akt pathway is stimulated and is coupled with reduced apoptosis (11, 12), calcium handling and associated ultrastructure of the sarcoplasmic reticulum improve (13–15), mitochondrial efficiency and ATP synthase activity are enhanced via a redox switch (16), ion channel function regulating the action potential is improved (17, 18), and resting as well as β-adrenergic–stimulated myocyte function are markedly restored by a mechanism involving regulators of G protein signaling (19, 20). Interestingly, while CRT substantially improves resting myocyte function throughout the ventricle, this is not consistently matched by increases in the calcium transient (17, 20), suggesting CRT may also enhance myofilament responses to calcium. This is intriguing, as small molecule HF therapies that target the myofilament are of growing interest (1, 21–23).

Accordingly, we tested the hypothesis that myofilament function is depressed in dyssynchronous HF (HF dys) and improved by CRT. Given that myofilament Ca²⁺ sensitivity is modulated by thin-filament protein phosphorylation (24–26) and prior data reveal CRT can modify several pertinent kinases and phosphatases (11), we speculated that CRT affects myofilament protein phosphorylation as an underlying mechanism. To investigate this, we studied a canine model combining tachypacing-induced HF with dyssynchrony or CRT (19, 20). Heart failure was induced by 6 weeks of rapid pacing. HF dys combined left bundle branch ablation with atrial tachypacing. For resynchronized HF (CRT), the same procedure was used for the initial 3 weeks, followed by rapid biventricular stimulation. These and other model modifications utilized in this study are summarized in Figure 1. Echocardiographic and hemodynamic

Conflict of interest: Jonathan A. Kirk and David A. Kass have a patent pending regarding the use of temporary dyssynchrony pacing to improve cardiac function in failing hearts.

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research article

Figure 1
Timeline of pacing protocols in each of the canine models: control, HFdys, CRT, HFsync, V3A3 (3 weeks RV pacing to induce dyssynchrony, 3 weeks atrial pacing to restore synchrony), and AVA (HFsync with 2 weeks of induced transient dyssynchrony via RV pacing). The HFdys and CRT models receive LBBB via radio-frequency ablation. After a 1-week recovery period from the pacemaker implant, each pacing protocol lasts 6 weeks before the animal is sacrificed.

Features of these models have been reported (19, 20) and are summarized for the current set of animals in Supplemental Table 1 (supplemental material available online with this article; doi:10.1172/JCI69253DS1). Trabecular and/or myocyte sarcomere function were assessed and proteomics used to identify phosphorylation changes linked to enhanced myofilament function from CRT.

Results

**CRT globally restores myofilament function.** Steady state force-calcium data for skinned myocytes from the LV lateral endocardium are shown in Figure 2A. Myofilament function was markedly depressed in HFdys, with maximum calcium-activated force ($F_{\text{max}}$) declining nearly 50% compared with normal controls (controls: 37.9 ± 3.5 mN mm$^{-2}$, n = 15 myocytes from 6 dogs, HFdys: 21.5 ± 3.0 mN mm$^{-2}$, n = 16 myocytes from 6 dogs, P < 0.001; Figure 2B). HFdys also exhibited Ca$^{2+}$ desensitization as quantified by a rise in EC$_{50}$ (controls: 2.34 ± 0.13 μM, HFdys: 3.04 ± 0.17 μM, P = 0.003; Figure 2B). CRT remarkably and completely restored both parameters of myofilament function to normal ($F_{\text{max}}$: 40.3 ± 2.60 mN mm$^{-2}$, EC$_{50}$: 2.18 ± 0.13 μM, n = 16 myocytes from 7 dogs, P < 0.001 vs. HFdys, P = NS vs. controls).

Contractile dyssynchrony with a left-bundle delay results in marked differences in regional stress (high in lateral wall due to early stretch/late contraction, lower in septum/RV with early contraction/late stretch). To determine whether this influenced CRT modulation of myofilament function, we studied skinned trabecula from the RV free wall (Figure 2, C and D) and single myocytes from the LV septum (Supplemental Figure 1). Both preparations revealed similarly depressed sarcomere function, as found in the LV free wall for HFdys, and marked improvement with CRT. Thus, the precise nature of cyclical regional stress or strain temporal pattern was not a primary determinant of myofilament changes in either condition.

We next tested whether CRT alters crossbridge mechanoenergetics by simultaneously assessing isolated fiber bundle ATP utilization and force development. The ATP required to achieve a given crossbridge tension (tension cost) and the rate offorce redevelopment upon sudden stress release (crossbridge kinetics, $k_d$) were similarly depressed in HFdys and CRT (Figure 2, E and F). The number of attached crossbridges during tension development, assessed by dynamic stiffness, was unchanged from control values by either HFdys or CRT (Figure 2G). Thus, CRT did not affect the mechanoenergetics of the crossbridge.

**Importance of resynchronization.** The recovery of myofilament function with CRT was not merely due to contraction being synchronous, as sarcomere function was also markedly depressed in HF that was always synchronous (HFsync). As with HFdys, $F_{\text{max}}$ was much reduced in HFsync (21.08 ± 3.22 mN mm$^{-2}$, $P = 0.001$ vs. controls and CRT, $P =$ NS vs. HFdys, n = 15 myocytes from 5 dogs; Supplemental Figure 2). However, whereas HFdys cells displayed a rise in EC$_{50}$, those from HFsync had a lower value (i.e., sensitized to Ca$^{2+}$: 1.70 ± 0.11 μM, $P = 0.007$, 0.036, 0.003 vs. controls, CRT, and HFdys respectively).

These data indicated that improved myofibrillar function required preexisting dyssynchrony that was subsequently reversed. To test this concept further, we modified the HFsync model to include a 2-week period of dyssynchrony induced by RV pacing during weeks 3 and 4 (AVA model [atrial, ventricular, atrial pacing]) (19). Compared with HFsync, AVA myocardium had a greater $F_{\text{max}}$ (AVA: 23.4 ± 1.9 mN mm$^{-2}$, n = 10 trabeculae from 5 dogs, HFsync: 16.5 ± 1.9, n = 22 trabeculae from 8 dogs, $P < 0.05$ vs. HFsync, $P =$ NS vs. controls) and EC$_{50}$ returned to normal control values (Figure 3, A and B).

CRT involved direct ventricular myocardial stimulation that might have triggered improved myofibrillar function. To test this, we studied an alternative “CRT” model that used rapid RV free-wall pacing (no left bundle branch block [LBBB]) was performed) to generate dyssynchrony and HF and then subsequently resynchronized the hearts by switching to rapid atrial pacing, avoiding the need for any direct ventricular stimulation. The results for this model (V3A3) were very similar to those with CRT (Figure 3, C and D), indicating direct pacemaker stimulation of the LV was not required.

**Altered maximum force is due to myocyte hypertrophy.** The decline in $F_{\text{max}}$ (normalized to myocyte cross-sectional area [CSA]) in both HFdys and HFsync could result from proteolytic cleavage of myofibrillar proteins such as troponin I (TnI) (27) or isoform switching (e.g., troponin T [TnT]; ref. 28). However, silver stained 1D gels and Western blots revealed neither protein cleavage products nor isoform switching in these models (Supplemental Figure 3). Normalization of force to CSA is generally performed, since one assumes bigger cells will have more force-generating units. Yet while control, HFdys, and CRT cells had similar raw (nonnormalized) peak force, CSA differed markedly (Figure 4D). Specifically, HFdys had twice the CSA of controls, but this was reversed by CRT (controls: 251 ± 36, HFdys: 465 ± 64, CRT: 268 ± 44 μm$^2$, $P < 0.012$, 0.015 HFdys vs. controls and CRT, respectively; Figure 4, A and B). To rule out potential selection bias in cells for which physiological data had been measured, we measured CSA from all cells in a given microscopic field (n = 150 myocytes from 3 dogs). This confirmed that HFdys myocytes were enlarged and that CRT reversed this ($P < 0.001$, Figure 4C). In trabecula, $F_{\text{max}}$ was also depressed (Figure 2C), but CSA was similar among the models (Figure 4E).
Ca$^{2+}$ sensitization is mediated by myofilament phosphorylation. Figure 5A displays example force-calcium data from RV trabeculae prior to and following incubation with the phosphatase PP1. Dephosphorylation did not change $F_{\text{max}}$ in any group (Supplemental Figure 4A). However, EC$_{50}$ increased (desensitized) in control ($\Delta$EC$_{50}$: 1.09 ± 0.22 μM, n = 6) and CRT ($\Delta$EC$_{50}$: 0.70 ± 0.23 μM, n = 5) trabeculae, but was unaltered in HF$_{dys}$ ($\Delta$EC$_{50}$: -0.06 ± 0.14 μM, n = 5, $P < 0.003$, 0.04 vs. controls and CRT, respectively; Figure 5B). PP1-induced calcium desensitization without a change in $F_{\text{max}}$ was also observed in V3A3 and AVA models, indicating phosphorylation was involved with their improved Ca$^{2+}$ sensitivity as well (Supplemental Figure 4).

Ca$^{2+}$ sensitivity is regulated by phosphorylation of the regulatory thin-filament proteins TnI, TnT, myosin-binding protein C (MyBPC), and myosin light chain 2 (MLC2). Table 1 lists the phosphorylation sites involved, their respective influence on Ca$^{2+}$ sensitivity, and directional changes in each due to HF (if reported). Previous studies show that phosphorylation of TnI at S22/S23 (PKA) (29) and S43/S45 (PKC) (30, 31) desensitize, whereas phosphorylation at T144 (PKC) (31, 32) or S150 (AMP-kinase, PAK1) (33, 34) increase sensitivity (the latter only reported in vitro). Overall, Tnl phosphorylation declined similarly in HF$_{dys}$ and CRT (Figure 5C), and reduced S22/S23 targeting was directly confirmed by immunoblot (Supplemental Figure 5). This supports a Ca$^{2+}$ sensitization effect (as opposed to desensitization observed in HF$_{dys}$, e.g., Figure 2A). MyBPC phosphorylated at S273/S282/S302 (35) (by PKA and other kinases; ref. 36) or MLC2 phosphorylation at S15/S19 increases Ca$^{2+}$-sensitivity (37). These posttranslational modifications (PTMs) declined to the same extent in both failure models (Figure 5, D and E) and so could not be responsible for the differences between HF$_{dys}$ and CRT. Finally, TnT phosphorylation at T206 (38) (PKC) or S278/ T287 (39) (Rho-kinase II) is linked to sensitization, but these sites were not altered in the models (Figure 5F).
The lack of differential changes in all of these sites was further confirmed by mass spectrometry (Supplemental Table 2) and/or 2D differential gel electrophoresis (Supplemental Figure 6).

Z-disk and M-band proteins are phosphorylated by CRT; role of GSK-3β. Given that the known phospho-protein targets did not appear to be involved, we turned to mass spectrometry to broadly interrogate the myofilibrillar subproteome. A total of 1187 phosphorylation sites on 166 myofilament and myofilament-associated proteins were detected, and CRT significantly altered 33 of these sites (P < 0.05, quantified by normalized spectral counts; Table 2). Based on the PP1 results, we focused on residues exhibiting increased phosphorylation with CRT, yielding 15 sites on 13 proteins (Table 2 and Supplemental Figures 7-21). Figure 6A summarizes this analysis. The majority of modified sites resided in proteins expressed in the Z-disk and M-band regions. Though such proteins were incubated with 18O-labeled ATP in the absence or presence of recombinant active GSK-3β for 30 minutes at 30°C and prepared for proteomic analysis. Table 3 lists the phosphorylated amino acid residues targeted by GSK-3β that were also found to increase with CRT. Four of the 7 GSK-3β sites revealed by silico analysis were confirmed, and 4 additional sites altered by CRT but not predicted by bioinformatics were revealed as GSK-3β targets as well. This supports the notion that GSK-3β converts a subset of the HFdys phospho-proteome into one similar to CRT.

To determine whether the phosphorylated residues increased by CRT were indeed targets of GSK-3β, we performed a second mass spectrometry experiment. The myofilament subproteome obtained from HFdys dogs was incubated with 18O-labeled ATP in the absence or presence of recombinant active GSK-3β for 30 minutes at 30°C and prepared for proteomic analysis. Table 3 lists the phosphorylated amino acid residues targeted by GSK-3β that were also found to increase with CRT. Four of the 7 GSK-3β sites revealed by silico analysis were confirmed, and 4 additional sites altered by CRT but not predicted by bioinformatics were revealed as GSK-3β targets as well. This supports the notion that GSK-3β converts a subset of the HFdys phospho-proteome into one similar to CRT.

To test whether GSK-3β myofilament phosphorylation could be responsible for the sensitizing affect, myofilament function was examined in HFdys myocytes before and after exposure to recombinant active GSK-3β for 30 minutes. Treated cells exhibited a decline in EC50 similar to that observed with CRT (P < 0.001 HFdys vs. pretreatment, P = NS versus CRT; Figure 6, B and C). In

Figure 3
Myofilament function can be improved via a brief period of induced dyssynchrony. (A and B) Both maximum force and calcium sensitivity are improved in the HFsync model when the middle 2 weeks of the pacing protocol is made dyssynchronous (AVA model). (C and D) When dyssynchrony is induced via RV pacing (instead of radio frequency ablation of the left bundle branch) and resynchronization is restored by moving the pacing site to the atrium (V3A3), the effects of CRT are recapitulated. Dyssynchrony caused a decrease in Fmax, which was increased by V3A3. Dyssynchrony desensitized the myofilament response to calcium (increase in EC50), which was also reversed in the V3A3 model. *P < 0.05 vs. HFsync or HFdys.

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Figure 4
The apparent decrease in Fmax is due to myocyte hypertrophy. (A) Skinned myocytes from controls, HFdys, and CRT. (B) CSA (calculated by measuring both width and depth and assuming an elliptical shape) was significantly increased in HFdys, but restored to normal levels in CRT. The unbiased approach also showed that HFdys myocytes were hypertrophied, and this was reversed by CRT (n = 150 myocytes from 3 dogs per group). Fmax is typically normalized to CSA, but if it is not, the myocytes generate the same amount of force between the groups, indicating that the force producing capacity of the myocytes is not altered; they are just bigger. There was no difference in trabecular muscle size, despite the fact that a decrease in Fmax was also observed. This suggests that there are fewer myocytes in HFdys in a constant area of myocardium. *P < 0.05 vs. control; #P < 0.05 vs. HFdys by 1-way ANOVA.
contrast, myocytes from CRT hearts were unchanged by GSK-3β, consistent with existing stimulation of this pathway and underlying sensitization. Control myocytes were also unaffected by GSK-3β. Fmax was unaltered by GSK-3β in any of the groups, agreeing with our finding that Fmax is primarily affected by myocyte geometry in these models.

To determine whether differential effects on calcium sensitivity were specific to GSK-3β, we exposed HF dys and CRT myocytes to PKA and Akt, 2 kinases involved in GSK-3β signaling. As expected, PKA modestly increased Fmax while decreasing calcium sensitivity (increased EC50), and it did so similarly in both groups (no interaction between group and treatment) (Supplemental Figure 22). This result is consistent with reduced TnI and MyBP C phosphorylation at PKA-targeted sites in MyBP C. Bottom: both HF dys and CRT showed decreased phosphorylation at S273 and S282 (n = 3). (E) Top: Western blot against S15 phosphorylation on MLC2 and phospho-serine motif sites on MLC2. Bottom: HF dys (n = 4) and CRT (n = 4) were decreased from controls (n = 3), but no different from each other at S15, and there was no change in overall serine phosphorylation. (F) Top: phospho-motif antibodies for serine and threonine sites and total Tn T. Bottom: there was very little phosphorylation of Tn T in the 3 groups, with no differences among them (n = 3). *P < 0.05 vs. control by 1-way ANOVA.

Figure 5
Calcium sensitization is mediated by phosphorylation, but not at the known targets. (A) Representative force-calcium curves for controls, HF dys, and CRT before protein phosphatase-1 (PP1) and after treatment. (B) While there was no change in Fmax, PP1 caused a desensitization in the controls (n = 6) and CRT (n = 5) groups, but had no effect on HF dys (n = 5). (C) Top: phospho-Tag gel blotted for Tn I. Bottom: there was an increase in the unphosphorylated form and a decrease in the biphosphorylated form of Tn I in the HF dys and CRT groups (n = 3), indicating overall dephosphorylation of Tn I in HF. 0P , unphosphorylated; 1P , phosphorylation state 1; 2P , phosphorylation state 2. (D) Top: Western blot with antibodies against 3 phosphorylation sites on MyBP C. Bottom: both HF dys and CRT showed decreased phosphorylation at S273 and S282 (n = 3). (E) Top: Western blot against S15 phosphorylation on MLC2 and phospho-serine motif sites on MLC2. Bottom: HF dys (n = 4) and CRT (n = 4) were decreased from controls (n = 3), but no different from each other at S15, and there was no change in overall serine phosphorylation. (F) Top: phospho-motif antibodies for serine and threonine sites and total Tn T. Bottom: there was very little phosphorylation of Tn T in the 3 groups, with no differences among them (n = 3). *P < 0.05 vs. control by 1-way ANOVA.
on GSK-3β; Supplemental Figure 23, D and E). These PKA and Akt data further support the specificity of the CRT myofilament response to GSK-3β phosphorylation of myofilament proteins.

Time course and offsetting effects on Ca²⁺ sensitivity by dyssynchrony. The observation that Ca²⁺ sensitivity declined with HFdys, but rose with HFsync suggested differential phosphorylation might mediate dysfunction due to dyssynchrony. To test this further, we examined the time course of changes in both models, performing studies at 3 weeks in myocytes isolated from endocardial biopsies. After 3 weeks of pacing, both models showed a similar decline in Fmax; however, there was dissociation of Ca²⁺ sensitivity (Figure 7, A and B), with a fall in EC₅₀ in HFsync (sensitization), but no change in HFdys. There were no further declines in HFsync at week 6, whereas EC₅₀ rose in HFdys. These data support an early sensitization factor in both models that is gradually offset by desensitization solely in HFdys (Figure 7C). This could relate to the decline in TnI phosphorylation by week 3 that remained similar reduced at week 6 in both models (Figure 7, D and E). However, the ratio of phospho/total GSK-3β was unaltered at either time in HFsync, whereas there was a gradual rise in the ratio in HFdys (enzyme inactivation) concordant with the gradual increase in EC₅₀. These results support the notion that phospho/total GSK-3β may serve as a biomarker for dyssynchrony with relevance to contractile function.

Discussion

We report that HFdys exhibits depressed myofilament responsiveness to calcium in both early and late activated regions and that CRT restores this to virtually normal levels. The mechanism appears to involve reactivation of GSK-3β and phosphorylation of Z-disk and M-band proteins as well as reversal of myocyte hypertrophy with depressed maximal force-per myocyte CSA. Similar myofilament functional improvement is attained if synchronous failing hearts are subjected to a period of dyssynchrony, confirming the key role of recoordinating contraction to this response. These results identify a mechanism by which CRT enhances systolic function beyond that previously attributed to chamber mechanics (3), adrenergic regulation (19, 20), and calcium homeostasis (17). The findings further suggest new approaches for improving myofilament function in the broader HF population.

| Table 1 |
| Myofilament phosphorylation sites that are known to modulate calcium sensitivity |

<table>
<thead>
<tr>
<th>Protein</th>
<th>Phosphorylation sites</th>
<th>Kinase</th>
<th>Effect on Ca²⁺ sensitivity</th>
<th>Phosphorylation change in HF</th>
<th>References</th>
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<td>PKC</td>
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<td>PKA, others</td>
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<td>S15/S19</td>
<td>MLCK</td>
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The table provides the direction of a change in calcium sensitivity due to phosphorylation, the direction that phosphorylation is modified by HF, and how this in turn impacts calcium sensitivity.

| Table 2 |
| Myofilament phosphorylation sites increased by CRT |

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<th>Protein</th>
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<td>S513</td>
<td>0.98</td>
<td>8</td>
<td>0.039</td>
<td>GTPGSSPYAsPR</td>
<td>Binds tropomyosin</td>
<td>CDK5, p38</td>
</tr>
</tbody>
</table>

The peptide sequence is shown, with the phosphorylated site indicated in lowercase. Possible kinases were determined using the Human Protein Reference Database and NetPhosK. Loc prob, Localization probability as determined by the A-Score algorithm using scaffold PTM; THRAP-3, thyroid hormone receptor–associated protein 3; SORB-2, sorbin and SH3 domain–containing protein 2; LDB3, LIM domain–binding protein 3; LMO7, LIM domain only protein 7.
Dyssynchrony generates regional heterogeneity of wall stress in the ventricle, with areas of increased or decreased load corresponding, respectively, to late or early activation. Despite these differences, similar depression of myofilament function and its recovery by CRT were observed in both regions. This indicates that the precise pattern of stress (or strain) is less important than the presence of spatial heterogeneity of these properties. Global chamber modifications with both dyssynchronous and resynchronized HF are not unique to myofilament function, but have also been observed with respect to antiapoptosis signaling coupled to Akt and GSK-3β (11), ion channel function (17), and adrenergic regulation (19).

Successful CRT converts hearts with a prior history of dyssynchrony into those with coordinate contraction. This temporal sequence was central to improving myofibrillar function just as it was to improving myocardial responsiveness to β-adrenergic stimulation (19). Failing hearts that were never dyssynchronous (HFsync) had worse myofilament function than those that were resynchronized, the latter including CRT, V3A3, and AVA models. The presence of dyssynchrony differentially affected calcium sensitivity. Enhanced Ca²⁺ sensitivity as observed with HFsync has been reported in human HFsync (40), whereas reduced Ca²⁺ sensitivity as with HFdys was compatible with experimental data from failing hearts with myocardial infarction (24). These differences were previously attributed to difficulties in preserving the phosphorylation state of myofilament proteins in human explant tissue samples (41). However, the current data controls for this (dogs do not receive different medications and tissue is harvested almost immediately after sacrifice), suggesting instead that regional stress-strain heterogeneity, as occurs with electromechanical dyssynchrony or regional infarction, plays a role. Disparities in Ca²⁺ sensitivity between HFsync and HFdys may underlie the greater adverse risks (increased morbidity and mortality) observed in HF patients with dyssynchrony (42, 43).

The AVA study that exposed hearts to temporary dyssynchrony also increased Fmax over HFsync and suggests what we believe is a novel translational approach for improving myofibrillar function in the failing heart. For example, patients with HFsync might benefit from a defined exposure to RV pacing–induced dyssynchrony that is then terminated to activate ameliorative changes. Alter-

Figure 6
Increased phosphorylation at GSK-3β sites in CRT mediated the calcium-sensitizing effect. (A) Schematic of the cardiac sarcomere showing Z-disk (green), titin (gray) thick filament (blue), M-line (black), and thin filament (red). The proteins (and sites) that were identified as increased with CRT are shown, with their best-known localization. Asterisks indicate matches with GSK-3β consensus sequence. (B) Representative force-calcium relationships in controls (top), HFdys (middle), and CRT (bottom) before (solid lines) and after GSK-3β treatment (dashed lines). (C) There was a calcium-sensitizing effect of GSK-3β in HFdys (n = 8), bringing it to the same level as controls and CRT. GSK-3β had no effect on controls (n = 5) or CRT myocytes (n = 8). *P < 0.05 vs. control baseline value.
For example, in HF patients treated with LV assist devices (LVAD), several prior studies, though they were not specifically highlighted. Cell hypertrophy (45), and CRT restores both to normal (11). Similar results have appeared in HFdys amplifies p38 and CaMKII activity that can stimulate hypertrophy (44), establishing molecular signaling, such as GSK-3\(\beta\) inactivation, that triggers subsequent changes after hearts are subsequently resynchronized. This phenomenon may reflect a form of dysynchrony-induced “preconditioning” (44), establishing molecular signaling, such as GSK-3\(\beta\) inactivation, that triggers subsequent changes after hearts are subsequently resynchronized. For example, in HF patients treated with LV assist devices (LVAD), several prior studies, though they were not specifically highlighted. Cell hypertrophy (45), and CRT restores both to normal (11). Similar results have appeared in HFdys amplifies p38 and CaMKII activity that can stimulate hypertrophy (44), establishing molecular signaling, such as GSK-3\(\beta\) inactivation, that triggers subsequent changes after hearts are subsequently resynchronized. For example, in HF patients treated with LV assist devices (LVAD), several prior studies, though they were not specifically highlighted. Cell hypertrophy (45), and CRT restores both to normal (11). Similar results have appeared in HFdys amplifies p38 and CaMKII activity that can stimulate hypertrophy (44), establishing molecular signaling, such as GSK-3\(\beta\) inactivation, that triggers subsequent changes after hearts are subsequently resynchronized. For example, in HF patients treated with LV assist devices (LVAD), several prior studies, though they were not specifically highlighted. Cell hypertrophy (45), and CRT restores both to normal (11). Similar results have appeared in HFdys amplifies p38 and CaMKII activity that can stimulate hypertrophy (44), establishing molecular signaling, such as GSK-3\(\beta\) inactivation, that triggers subsequent changes after hearts are subsequently resynchronized. For example, in HF patients treated with LV assist devices (LVAD), several prior studies, though they were not specifically highlighted. Cell hypertrophy (45), and CRT restores both to normal (11). Similar results have appeared in HFdys amplifies p38 and CaMKII activity that can stimulate hypertrophy (44), establishing molecular signaling, such as GSK-3\(\beta\) inactivation, that triggers subsequent changes after hearts are subsequently resynchronized.
Our study leaves unanswered the exact identity of which GSK-3β target or targets are responsible for improved calcium sensitivity by CRT. The M-band and Z-disk are highly structured regions in the sarcomere, and protein substitution cannot be achieved by diffusion in skinned cell preparations. Thus, direct confirmation will require site mutagenesis and genetic modifications. Ongoing efforts to translate the canine model to the mouse will be an important next step to dissecting the pathways, since gene delivery and/or transgenic models are more practical. This will also allow genetic knockdown of GSK-3β to confirm the myofilament substrates. The canine model is highly translational but poses its own limitations, since tachypacing is required throughout the protocol to generate HF. This impacts global functional parameters such as stroke volume or ejection fraction, which decline at fast heart rates independently of contractility changes (58). This likely explains why such variables and chamber volumes were similar among the models despite differences in myofilament contractile function, whereas the less load-sensitive parameter dP/dt max/IP (IP, instantaneous developed pressure at time of dP/dt max) was improved by CRT.

The myofilament is rapidly becoming an attractive target for enhancing systolic function in HF (21). Over the past 10 to 20 years, investigators have explored drugs to improve Ca2+ responsiveness, though such efforts have yet to yield a broadly successful therapy. A new approach to enhancing myofilament ATPase activity was reported (21) and is currently in clinical trials (23), though chronic efficacy remains unknown. In contrast, CRT is already a successful treatment with documented long-term benefits on morbidity and mortality. The present analysis attempts to reverse engineer it, unmasking an unanticipated yet striking improvement in myofilament function by a mechanism likely to be both safe and effective. At present, we do not know whether there is a single Z-disk or M-band protein target or whether several are involved, but the results strongly suggest GSK-3β as a key signaling kinase, revealing what we believe to be a novel role for this enzyme in the sarcomere. The ability to mimic Ca2+-sensitizing effects of CRT by augmenting GSK-3β activation in HF dys myocytes and/or exposing the heart to brief periods of sustained dyssynchrony may help forge new paths for HF treatment.

Methods
Canine pacing models. Details of the canine models have been previously reported (19, 20). All dogs except controls received a pacemaker implant (Medtronic). HF dys and CRT models both received left bundle branch radio-frequency ablation at time of pacemaker implant, while in 3 other models (HF sync, AVA, S22/S23 TnI phosphorylation was decreased in both HF dys and HF sync by 3 weeks, with no additional decrease between 3 and 6 weeks (n = 5 per time point). (E) Phospho GSK-3β was unchanged in HF sync over the 6 weeks, but linearly increased in HF dys; P < 0.01 via multivariate regression analysis. *P < 0.05 vs. controls; #P < 0.05 vs. HF sync at 3 weeks.
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V3A3), the left bundle remained intact. The location and timing of pacing varied among the experimental models and are detailed in Figure 1. All dogs were provided 7 to 10 days for full recovery prior to initiating 6 weeks of tachypacing (200 bpm). After 3 weeks of pacing, a subset of dogs was placed under anesthesia and a 7F bioprobe (Cordis Corp.) was used to collect 5–7 biopsies from the endocardium near the RV apex under fluoroscopic guidance.

At terminal study, animals were anesthetized with pentobarbital, hearts excised under ice-cold cardioplegia, and tissue prepared for analysis. Supplemental Table 1 provides global hemodynamics and echocardiographic data for each model — revealing the presence of cardiac failure at the chamber level compared with normal controls. All models display chamber dilation, systolic, and diastolic dysfunction, though the CRT model had improved systolic function (dP/dtmax/IP). Other details are provided in Supplemental Methods and Supplemental Table 1.

Myofilament function. Right heart trabeculae were isolated and skinned overnight in the presence of 1% Triton X-100 and protease (Sigma-Aldrich) and phosphatase inhibitors (Roche Diagnostics). Trabeculae were attached to a force transducer (Si Heidelberg) and a stationary hook. Force was measured as bath Ca2+ concentration was increased from 0 to saturating conditions. In a subset of studies, trabeculae were subsequently treated with the phosphatase PPI (25,000 mU/ml × 60 minutes; New England Biolabs) and a second force-calcium relation determined.

As trabeculae could not be isolated from the LV free wall, studies were also performed in isolated myocytes. Companion studies from the LV septum were also conducted. For myocyte analysis, flash-frozen endomyocardium was homogenized in Triton X-100 and protease and phosphatase inhibitors and the mixture disaggregated by low-speed pulverization to generate a skinned myocyte preparation. Myocytes were then attached to a force transducer and motor arm. Once attached, the protocol was similar to that used in trabeculae. Subsets of myocytes were exposed to either GSK-3β (10 μg/ml, 20 minutes; Sigma-Aldrich), PKA (0.125 units/ml, 20 minutes; Sigma-Aldrich), or Akt (10 μg/ml, 20 minutes; Sigma-Aldrich). Force-calcium data were collected before and after treatment.

Force-calcium data were fit to the Hill equation: F = Fmax × Ca2+/EC50 + 1, yielding Fmax, calcium sensitivity (Ca2+ required to achieve 50% maximal force; EC50), and cooperativity (Hill coefficient, h).

Phos-Tag and Western blots. Tissue samples from control, HFem, and CRT hearts were processed in the presence of protease and phosphatase inhibitors to obtain a myofilament-enriched protein sample (Supplemental Methods). Samples were run on phos-Tag gels (Wako Pure Chemical Industries) and transferred to a membrane; cardiac Tnl was detected with the BI-7 Tnl antibody (Spectral Diagnostics). For other proteins, immunoblots were performed using MyBP-C antibodies against phospho-S273, phospho-S282, phospho-S302, and total (gift from Sakthivel Sadayappan, Loyola University, Chicago, Illinois, USA), MLC-2 phospho-antibodies against phospho-S282, phospho-S302, and total (from Sakthivel Sadayappan, Loyola University, Chicago, Illinois, USA), and/or total GSK-3β (Santa Cruz Biotechnology Inc.). Blots were scanned using an Odyssey Infrared Imager (Li-Cor Biosciences) and analyzed using Odyssey Application software (v3.0.30, Li-Cor Biosciences). Several of these antibodies had no prior publications supporting their use in canine heart tissue. In these instances, antibodies were optimized and verified by comparing Western blots performed in alternate species (mouse). Fortunately, the myofilament proteins share very high homology across mammalian species.

Mass spectrometry. Six HFem and 6 CRT LV lateral wall myocardial samples were myofilament enriched (1 mg protein in 8M urea, 1% SDS) and digest in trypsin (Promega Corp.). Alternatively, myofilament-enriched samples from HFem tissue were split into 2 equal parts, one incubated in ATP γ-32P-O-labeled (Cambridge Isotope Laboratories Inc.) relax buffer, and the second incubated in labeled relax buffer plus 4 μg of GSK-3β (n = 3). The labeled relax buffer contained a 1:1 ratio of unlabeled and 32P-labeled ATP. The samples were incubated at 30°C for 30 minutes.

All myofilament samples were fractionated by strong-cation exchange to decrease sample complexity and increase sequence coverage. Each fraction was then individually phospho enriched using titanium oxide affinity chromatography. Phospho-peptides were analyzed on an Agilent 1200 Nano-LC system (Agilent Technologies) connected to an LTQ-Orbitrap mass spectrometer (Thermo Scientific) equipped with a nanoelectrospray ion source.

All raw MS/MS data was searched using the Sorcerer 2-SEQUEST algorithm (Sage-N Research). Additional analysis was done using Scaffold PTM (v1.1.3; Proteome Software). For studies with GSK-3β treatment, MS data were searched using SEQUEST, with labeled phosphorylation that included an additional 6.01 Da shift due to the 32P-labeled phosphate group. Kinase consensus amino acid sequences were predicted using NetPhosK (http://cbs.dtu.dk/services/NetPhosK/) and the Human Protein Reference Database (hprd.org).

Further details are provided in the Supplemental Methods.

Statistics. Values were compared between the groups using 1-way ANOVA, with post hoc comparisons made with the Holm-Sidak method using SigmaPlot (v11.1, Systat Software Inc.). MS was quantified based on label-free spectra counts. Normalized spectral counts were compared using Student’s t test. GSK-3β phosphorylation relationships were tested using multivariate regression analysis using Systat (Systat Software, Inc.). All tests were 2-tailed with a P value of less than 0.05 considered significant. All values are presented as mean ± SEM.

Study approval. All animal studies were approved by the Johns Hopkins Animal Care and Use Committee and performed by trained personnel.

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