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Phospho-Proteomic Analysis of Cardiac Dyssynchrony and Resynchronization Therapy

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Abstract

Cardiac dyssynchrony arises from conduction abnormalities during heart failure and worsens morbidity and mortality. Cardiac Resynchronization Therapy (CRT) re-coordinates contraction using bi-ventricular pacing, but the cellular and molecular mechanisms involved remain largely unknown. We aimed to determine how dyssynchronous heart failure (HF_{dys}) alters the phospho-proteome and how CRT interacts with this unique phospho-proteome by analyzing Ser/Thr and Tyr phosphorylation. Phospho-enriched myocardium from dog models of Control, HF_{dys}, and CRT was analyzed via mass spectrometry. There were 209 regulated phospho-sites among 1,761 identified sites. Compared to Con and CRT, HF_{dys} was hyper-phosphorylated and tyrosine phosphorylation was more likely to be involved in signaling that increased with HF_{dys} and was exacerbated by CRT. For each regulated site, the most-likely targeting-kinase was predicted, and CK2 was highly specific for sites that were “fixed” by CRT, suggesting activation of CK2 signaling occurs in HF_{dys} that is reversed by CRT, which was supported by western blot analysis. These data elucidated signaling networks and kinases that may be involved and deserve further study. Importantly, we have identified a possible role for CK2 modulation in CRT. This may be harnessed in the future therapeutically to compliment CRT, improving its clinical effects.

Keywords

CK2; dyssynchrony; heart failure; phosphorylation

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Introduction

In a healthy heart, the cardiac myocardium is stimulated by the Purkinje conduction system to coordinate the timing of contraction throughout the left ventricle. This coordination optimizes stroke volume, the volume of blood ejected from the left ventricle with each beat. Almost half of heart failure patients develop conduction abnormalities [1], the most prevalent being a left bundle branch block (LBBB). The LBBB causes a temporal delay in the activation of the left ventricular free wall (late activated region) compared to the septum (early activated region), which reduces systolic efficiency as a portion of the blood is shifted within the chamber between these regions, as opposed to being ejected into the circulatory system [2, 3]. Heart failure patients with cardiac dyssynchrony display worsened systolic function, morbidity, and mortality than heart failure patients with synchronous contraction [4].

Cardiac Resynchronization Therapy (CRT) is a pacemaker-based treatment for heart failure with cardiac dyssynchrony [5, 6] that involves bi-ventricular pacing. While pharmaceutical efforts to improve systolic function result in short term improvement, CRT is the only clinical treatment that improves systolic function while increasing cardiac work and promoting long term survival [6–8]. Unfortunately, 30 - 40% of CRT recipients do not respond to the therapy with clinical improvements, and efforts to improve this “non-responder rate” by examining global mechanics have been unsuccessful [9, 10]. We hypothesized the answer may be in the cellular and molecular mechanisms that underlie dyssynchrony and CRT, which remain to be fully elucidated.

Ourselves and others have used canine models of dyssynchrony and CRT to assess the cellular and molecular mechanisms underlying the disease and its treatment. For example, changes have been found in calcium handling proteins (including phospholamban, Serca2A, and Na^+ - Ca^{2+} exchanger levels in human and canine) [11, 12], β -adrenergic signaling through regulator g-protein signaling (RGS) proteins [13, 14], intermediate filaments [15], mitochondrial function [16] and others [17–19]. We have explored the role of a specific kinase driven pathway in dyssynchrony and CRT. In a canine model, glycogen synthase kinase 3 β (GSK-3 β) was shown to be deactivated in dyssynchrony and reactivated in CRT and that this reactivation coincided with a restoration of myofilament contraction metrics [20, 21]. This previous work indicated that phosphorylation events play a key role in how dyssynchrony and CRT impact cellular function.

Here, our goal was to compile a profile of the serine, threonine, and tyrosine phosphoproteome of healthy, heart failure with cardiac dyssynchrony (HF_{dyss}), and CRT canine models. We then used this data to examine potential signaling pathways and kinases that are distinctly activated by dyssynchrony and CRT, to further help to elucidate the mechanisms involved in the disease and treatment. Long-term, this data could help decrease the non-responder rate, identify drug targets that could mimic the clinical benefits of CRT for those patients who are not eligible for the therapy (i.e., do not have dyssynchrony), or remove the necessity of an implanted device.

Methods

Animal Models

All animal studies were approved by the Johns Hopkins Animal Care and Use Committee and performed by trained personnel. Control (Con) dogs received no intervention. Heart failure with cardiac dyssynchrony (HF_{dys}) and Cardiac Resynchronization Therapy (CRT) dog models received left bundle branch block (LBBB) via radio-frequency ablation to induce dyssynchrony and a pacemaker implant (Medtronic, Minneapolis, MN). Animals were given one week to recover and then the pacing protocol was initiated. Both HF_{dys} and CRT were tachypaced at 200 bpm to induce heart failure for the entire 6-week protocol. The pacing originated from the atrium for the entire six weeks in the HF_{dys} group, but in the CRT group the first three weeks were atrial and the second three weeks was bi-ventricular to resynchronize the hearts (Figure 1A). At the end of the protocol, the animals were anesthetized with pentobarbital, intubated, and the heart was excised and immediately placed in ice-cold cardioplegia. Further details of the model have been published previously [14, 20, 22].

Sample Preparation

Tissue from left ventricular lateral wall was used to prepare all samples (n : Con: 4, HF_{dys}: 4, CRT: 4). Each tissue sample was split into three parts (Figure 1B), one enriched for myofilament (via triton-based extraction) [23], one myofilament depleted (tissue homogenized in 10 mM Tris (pH 7.5), 250 mM sucrose, 1 mM EDTA spun at 500 rcf for 1 minute and discard pellet) and one whole tissue homogenate and phospho-tyrosine enriched (antibody-based approach). All buffers included protease and phosphatase inhibitors. The resulting 36 samples were reduced, alkylated, and digested overnight using trypsin (Promega, Madison, WI) at 100 ug/1mg protein. The reaction was stopped with 10% TFA and samples were individually de-salted on Oasis HLB cartridges (Water, 30 mg) and eluted in 300 μ L of 80% ACN, 5% TFA, 1M Glycolic acid. Each sample was then incubated in 50 μ L titanium dioxide (TiO₂) slurry (30 mg/mL, Glycen Corp, Columbia, MD) at room temperature on a shaker overnight. Then the TiO₂ beads were washed twice with 200 μ L of 80% ACN, 5% TFA, once with 200 μ L of 80% ACN, 0.1% TFA, and eluted in 180 μ L of 30% ACN/ 1% NH₄OH and neutralized with 200 μ L of 10% FA. Samples were then desalted on Oasis HLB μ -elution plates (Waters) and eluted in 80% ACN, 0.1% FA, dried in speedvac, then resuspended in 0.1% FA for LC MS/MS analysis.

Mass Spectrometry – Phospho-Proteome

Dried samples were resuspended in 0.1% FA and analyzed by LC-MS/MS on an EASY-nLC 1000 (mobile phase A was 0.1% FA in water and mobile phase B was 0.1 % FA in ACN) connected to an Orbitrap Elite (Thermo) equipped with a nanoelectrospray ion source. Peptides for each sample were loaded in duplicate onto a Dionex Acclaim® PepMap100 trap column (Thermo, 75 μ m $\times$ 2 cm, C18 3 μ m 100Å) and separated on a Dionex Acclaim® PepMap RSLC analytical column (Thermo, 50 μ m $\times$ 15 cm, C18 2 μ m 100Å) at a flow rate of 300 nL/min using a linear gradient of 2-30% B for 120 minutes, 30-98% B for 5 minutes, then holding at 98% for 10 minutes. The nano-source capillary temperature was set to 275 °C and the spray voltage was set to 2 kV. MS1 scans were acquired in the Orbitrap Elite

at a resolution of 60,000 FWHM (400-1600 m/z) with an AGC target of 1×10^6 ions over a maximum of 500 ms. MS2 spectra were acquired for the top 20 ions from each MS1 scan in normal scan mode in the ion trap with a target setting of 1×10^4 ions, an accumulation time of 50 ms, and an isolation width of 2 Da. The normalized collision energy was set to 35% and one microscan was acquired for each spectra. Monoisotopic precursor selection was enabled and only MS1 signals exceeding 1000 counts triggered the MS2 scans, with +1 and unassigned charge states not being selected for MS2 analysis. Dynamic exclusion was enabled with a repeat count of 2, repeat duration of 30 seconds and exclusion duration of 90 seconds.

Mass Spectrometry – Total Proteome

Samples were prepared as for phospho-proteomics, but without TiO_2 enrichment, from all three groups ($n = 3$ per group). LC/MS/MS analysis was performed using an Ultimate 3000 nano LC (Thermo Scientific) connected to an Orbitrap LUMOS mass spectrometer (Thermo Scientific) equipped with an EasySpray ion source. Peptides were loaded onto a PepMap RSLC C18 column (2 μm , 100 $\text{\AA}$, 75 μm i.d. $\times$ 25 mm, Thermo) using a flow rate of 300 nL/min for 15 min at 1% B (mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1 % formic acid in acetonitrile) after which point they were separated with a linear gradient of 5-20% B for 45 minutes, 20-35% B for 15 minutes, 35-85% B for 3 min, holding at 85% B for 5 minutes and re-equilibrating at 1% B for 17 minutes at 400 nL/min. The nano-source capillary temperature was set to 300°C and the spray voltage was set to 1.8 kV. MS1 scans were acquired in the Orbitrap at a resolution of 240,000 Hz with advanced peak detection (APD) from mass range 400-1600 m/z. For MS1 scans the AGC target was set to 4×10^5 ions with a max fill time of 50 ms. MS2 spectra were acquired using the TopSpeed method with a total cycle time of 3 seconds and an AGC target of 1×10^4 and a max fill time of 50 ms, and an isolation width of 1.6 Da. Precursor ions were fragmented using HCD with a normalized collision energy of 30% and analyzed using rapid scan rates in the ion trap. Monoisotopic precursor selection was enabled and only MS1 signals exceeding 5000 counts triggered the MS2 scans, with +1 and unassigned charge states not being selected for MS2 analysis. Dynamic exclusion was enabled with a repeat count of 1 and exclusion duration of 15 seconds.

Analysis

Phospho-enriched Raw MS/MS data originating from the Orbitrap Elite were batch searched based on biological sample using the Sorcerer 2TM-SEQUEST® algorithm (Sage-N Research, Milpitas, CA, USA) using default peak extraction parameters. Data were searched against the most recent version of the canine Uniprot database using the following criteria: Fixed modification: +57 on C (carbamidomethyl); Variable modification: +16 on M (oxidation), +80 on S,T,Y (phosphorylation); Enzyme: Trypsin with 2 max missed cleavages; Parent Tolerance: 50 ppm; Fragment tolerance: 1.00 Da. Post-search analysis was performed using Scaffold 3 version 3.6.2 (Proteome Software, Inc., Portland, OR) with protein and peptide probability thresholds set to 95% and 90%, respectively, and one peptide required for identification. Protein and peptide false discovery rates were calculated automatically using Scaffold's probabilistic method and were equal to or less than 1% for all samples using the above thresholds. Spectral counts were summed from each enrichment

and each technical duplicate run for each biological replicate ($n = 4$ dogs per group), such that there was a total of 12 samples. Phosphosite localization was verified using Scaffold PTM version 2.1.1 (Proteome) and only Phosphosites observed with 5 or more total spectral counts at >80% site localizations were included in the final list.

Total protein sample raw data was searched using the Sorcerer Sequest search engine (Sagen) with the following search parameters: Full Trypsin cleavage, static modification of +57Da on Cysteine, variable modification of +16 on Methionine, MS1 error of 10 ppm, MS2 error of 1 Da. Data was post processed using Scaffold 4.

The phosphosites were then analyzed using GPS 2.1 (Group-based Prediction System, ver 2.1) [24] software for computational prediction of kinase-specific phosphorylation sites at high threshold and only the top scoring kinase for each observed phosphorylation site were reported. Data were analyzed using Ingenuity Pathway Analysis (QIAGEN, Redwood City).

Principal component analysis (PCA) on the Ser/Thr and Tyr datasets was done separately, using ClustVis [25].

Western Blot

LV tissue was prepared using a homogenizer in cell lysis buffer (Cell Signaling, Danvers, MA), and further solubilized by adding 1% SDS. Samples were blotted using anti-CK2- α (1:1000, Invitrogen). Total protein was stained for using REVERT Total Protein Stain (LICOR). Blots were scanned using an Odyssey Infrared Imager (Li-Cor Biosciences, Lincoln, NE).

Statistical Analysis

One-way ANOVA was used to compare spectral counts between the three groups (Control, HF_{dys}, CRT), with Holm-Sidak used for post-hoc tests. Data are presented as mean $\pm$ standard error of the mean (S.E.M.). Significance is indicated by $p < 0.05$. Phosphorylation sites with an ANOVA $p < 0.05$ were considered an “altered” within the three groups (Con, HF_{dys}, CRT) and the pattern of significant post-hoc tests for each site determined the “Category of Change” for each site. If an altered phosphorylation site also had a significant change in the total expression for that protein and the pattern of changes were the same between the phosphorylation site and total protein, the phosphorylation site was removed from further analysis.

Results

The *in vivo* effects of dyssynchrony and CRT in these models, published previously, show that echocardiographic and hemodynamic analysis confirmed dyssynchrony and significant heart failure as evidenced by geometric remodeling and systolic dysfunction [13, 20, 22]. All dogs responded to CRT, which resynchronizes contraction and improves function.

Analysis of the three experimental groups (Con, HF_{dys}, CRT), $n = 4$ dogs per group, revealed changes in the level of phosphorylation of serine, threonine, and tyrosine sites, networks in which these proteins are involved, and the specific kinase families

phosphorylating these sites. The complete list of all Ser/Thr and Tyr identified sites can be found in Supplemental Table 1 and Supplemental Table 2. There were 21% and 29% more phospho-Ser/Thr spectra assigned to the HF_{dys} group compared to the Con and CRT groups, respectively (Fig 1C, $p=0.04$). These differences suggest HF_{dys} is a hyper-phosphorylated state, and CRT acts to restore phosphorylation levels back towards a healthy baseline. However, there was no significant difference in total spectra assigned for phosphorylated Tyr sites between experimental groups (Fig 1C).

By one-way ANOVA, we identified 209 phosphorylation sites which were quantitatively altered between the three groups. Three of the 209 phosphorylation sites were removed because the change in phosphorylation level was due to changes in the total protein level (Supplemental Table 3). Based on the pattern of post-hoc statistical tests comparing spectral counts from each group against the other groups individually, each of these 209 phospho-sites was assigned to one “Category of Change” (20 sites could not be assigned because despite a significant ANOVA, no post-hoc tests were significant). For example, if the post-hoc tests for a site indicated a difference between Con and both HF_{dys} and CRT but no difference between HF_{dys} and CRT, it would be assigned to the “HF_{dys} – Not Reversed” Category of Change (Fig 2A). Because the assignment is based on a significant magnitude of change, not direction, each category includes phosphorylation sites that both increased and decreased between the groups (Fig 2A). There were four “Categories of Change”: “HF_{dys} – Not Reversed” (44 sites, 25 decreased in HF_{dys} and 19 increased in HF_{dys}) indicates sites that are either increased or decreased in HF_{dys}, but not affected by CRT, “Reversed by CRT” (95 sites; 17 decreased in HF and 78 increased in HF) represents sites that are significantly changed in HF_{dys} and are restored by CRT, “CRT Only” (21 sites; 7 decreased in CRT and 14 increased in CRT) are sites that were unaltered in HF_{dys}, but were affected by CRT, and “Exacerbated by CRT” (26 sites: 1 decreased in HF and 25 increased in HF) sites that are increased or decreased by HF_{dys} and are further increased or decreased in the same direction by CRT (Fig 2A, 2B). There were more Ser/Thr sites in every category of change, except “Exacerbated by CRT” in which there were over four times more Tyr sites (Fig 2C). PCA analysis of both Ser/Thr and Tyr sites reveals that the three groups, Con, HF_{dys}, and CRT, each segregate independently (Fig 2D).

Ingenuity Pathway Analysis was performed separately on the proteins phosphorylated in each category of change, and “HF_{dys} – Not Reversed” and “CRT Only” are shown in Fig 3A and Fig 3B, respectively. In the “HF_{dys} – Not Reversed” network, myofilament and myofilament-associated proteins such as myosin, titin (TTN), actin (ACTA1, ACTG2), tropomyosin (TPM1), and desmin (DES) were identified. Other proteins found in this network include Protein Kinase C (PRKCD), neural precursor cell expressed developmentally down-regulated protein 9 (NEDD9), and tensin 1 (TNS1). In the “Reversed by CRT” network, desmin (DES) and actin (ACTA2) were identified, as well as proteins involved in other sarcomeric roles such as scaffolding, (spectrin beta (SPTBN1)), stress resistance and actin organization (Heat shock protein 10 (HSPE1)), and myofilament protein tethering (myopalladin (MYPN)). Also in this network were cardiomyopathy associated 5 (CMYA5) and cardiac LIM domain protein (CSRP3), both of which are associated with cardiomyopathies.

The kinases predicted to phosphorylate the largest number of altered sites (ANOVA $p < 0.05$) are shown for the Ser/Thr sites (Fig 4A) and Tyr sites (Fig 4B), as well as which “Category of Change” these sites were assigned. Thus, these figures represent the *number* of phosphorylation sites which exhibited a quantitative change in the *level* of phosphorylation by semi-quantitative spectral counting. Most of the predicted kinases for the altered Ser/Thr phosphorylation sites were in one of seven groups: ACG-SGK (Serum and Glucocorticoid induced kinase), ACG-PKC/PKG (Protein Kinase C/G), CMGC-ERK (Extracellular signal Regulated kinase), CMGC-p38, CMGC-JNK (c-Jun N-terminal kinase), Other-CK2 (previously casein kinase II), Other-AUR (aurora kinases) (Fig 4A). For 28% of the identified sites, no Ser/Thr kinase could be predicted using a high confidence cutoff. Of the predicted kinases, JNK and p38 were found in all four categories of change. In contrast to the Ser/Thr kinases, there were a greater number of predicted Tyr kinases, none of which was predicted for more than three individual Tyr phosphorylation sites. Furthermore, none of the Tyr kinases were enriched in any category of change (Fig 4B).

Predicted targets of CK2 were very specific for the “Reversed by CRT” category. To validate whether CK2 expression mirrored the broad changes in its predicted targets, we performed western blot analysis on LV tissue from the same dog samples for α -CK2 (CK2 is constitutively active, so we did not analyze its phosphorylation sites). There was a trend ($p = 0.09$ by one-way ANOVA, $n = 4$ per group) towards an increase in CK2 expression in HF_{dys} compared to Con (Con: 1.00 ± 0.21 , HF_{dys}: 1.91 ± 0.38) that was reversed by CRT (1.27 ± 0.11) (Fig 5).

Discussion

Our goal was to catalogue the phosphorylation changes in the left ventricle of canine models of Con, HF_{dys}, and CRT, and perform pathway and kinase analysis to identify processes that are likely being altered in the disease and its treatment. Generally, HF_{dys} is hyper-phosphorylated by Ser/Thr kinases compared to Con and CRT hearts, which agrees with previous studies that suggest broad kinase activation and hyper-phosphorylation occurs during heart failure [26–29]. Importantly, CRT reduced and thus restored overall phosphorylation. While the clinical efficacy of CRT has been known for some time, it was nonetheless unexpected that it should reverse such a broad fingerprint of heart failure. However, CRT did not reverse every HF_{dys}-induced increase in phosphorylation, nor was that its only effect, as it also changed the overall pattern of phosphorylation. CRT often exacerbated HF_{dys} related changes or induced unique phosphorylation events, as has been observed in other studies of CRT [13]. The PCA analysis supports this, showing that CRT moves away from HF_{dys} and towards the Control group, but is still distinct from both groups.

The “Reversed by CRT” contained almost twice as many Ser/Thr sites as Tyr sites, while the “Exacerbated by CRT” group was composed almost entirely of Tyr sites. Studies suggest Tyr phosphorylation is very low at baseline and is increased during specific signaling events [30]. Thus, one possible explanation for the observed pattern is that protective tyrosine phosphorylation signaling is increased in HF_{dys} and is further augmented in CRT. However,

this explanation would require a redistribution of tyrosine phosphorylation, as overall tyrosine phosphorylation was unchanged.

We previously showed in single-cell force experiments that HF_{dys} reduces myofilament functional metrics and CRT restores them [20]. Our current experiment yielded evidence of the role of myofilament protein phosphorylation in HF and CRT. We detected approximately half (6 of 14) of the myofilament GSK-3 β phosphorylation sites previously hypothesized to play a role in CRT's recovery effect to be altered significantly in our groups in this study [20]. That we did not detect all the previously identified sites is most likely due to a difference in approach between this study and the previous one. Here we were interested in the broad ser/thr and tyr phospho-proteome, while in the previous study we were interested only in myofilament ser/thr phosphorylation and enriched accordingly. Also of interest was that GSK-3 β itself was not predicted to target a large percentage of the total altered phospho-proteome. This could be seen as a cautionary tale that more kinase targets does not necessarily directly correlate to functional significance.

Furthermore, proteins classified previously as myofilament and myofilament-associated [20, 31] were present in all four categories of change, but the most, twenty-one, were detected as significant in the "Reversed by CRT" category. Proteins such as tensin-1 and actin-binding LIM protein 1 [20] and desmin [15], previously hypothesized to be targets of phosphorylation in CRT or HF_{dys} were detected as significant in the "Reversed by CRT" category, supporting that phosphorylation of these proteins alter cellular mechanics during CRT. In addition, twenty proteins such as titin and myosin had multiple phosphorylation sites that changed between experimental groups, often in different categories of change and different directions (increased or decreased phosphorylation). These changes within the same protein indicate that CRT exerts its benefits through both reversal of HF_{dys} signaling and unique phosphorylation events that may integrate to improve function.

Another goal of this study was to investigate the role of specific kinases in HF_{dys} and CRT. Several Ser/Thr kinases were predicted to target many sites that were altered amongst the three groups (<10 sites), while each predicted Tyr kinase targeted very few altered sites (<4 sites). As Ser/Thr kinases are suggested to make up about 80% of all kinases [32] we anticipated that each individual kinase would target fewer sites. However, it appears, in HF_{dys} and CRT at least, that Ser/Thr kinases tend to be involved in broad activation pathways, while Tyr kinases are more limited, specific, and targeted. A kinase could not be predicted for a large portion of the detected Tyr phosphorylation sites, thus hampering our analysis and interpretation.

Several predicted kinases stood out in our analysis: JNK, p38, and CK2 (formerly casein kinase II). Both JNK and p38 are known to be activated by stress [33], and p38 has been linked to hypertrophy and apoptosis [34, 35], so their activation in HF_{dys} is anticipated. As such, in a previous study using the same canine models used here, p38 activity differed between the lateral wall and septum in HF_{dys}, and both the activity and difference between the two regions were decreased by CRT [22], agreeing with our findings.

The protein kinase CK2 was the most prevalent and specific kinase predicted in the “Reversed by CRT” category of change. All of the significantly changed sites we predicted to be targets of CK2 exhibited increased phosphorylation in HF_{dys} and a return towards control values with CRT, in comparison to sites attributed to other kinases which experienced both increases and decreases in phosphorylation within the same group. Western blot analysis of CK2 showed a similar pattern – an increase in expression in HF_{dys} that was reversed by CRT. CK2 has been observed to not change in expression between human non-failing and end stage heart failure patients, however this study had few non-failing controls ($n=2$) and the heart failure group ($n=3$) included both ischemic and dilated cardiomyopathy patients [36]. CK2 has been implicated in the hypertrophic response in the heart [37, 38], which agrees with our data here showing an increase in disease and a reversal with treatment. However, the mechanisms by which CK2 is implicated in hypertrophy have been proposed for both apoptotic and most recently, an anti-apoptotic mechanism in which CK2 inhibits Caspase-3 and promotes Cardiotrophin 1 (CT1) [39]. However, other studies suggest CK2 may also be anti-hypertrophic [40].

A recent study suggested that CK2 targets comprise over 20% of the entire phospho-proteome [41]. The promiscuous nature of CK2 made it surprising that its targets were so specific to the “Reversed by CRT” category. Perhaps there is a regulatory mechanism involved in CK2 modulation, such as altered localization, that is not yet understood. This same regulatory mechanism may be responsible for the contradictory pro- and anti-hypertrophic properties of CK2 [37, 38, 40]. We know little regarding how CK2 is regulated, although it is often considered a “master regulator”. Thus, CK2 may be responsible for controlling other kinases in CRT and thus an even much larger portion of our data set. Further studies into the role of CK2 in dyssynchrony and CRT are necessary to discover whether it could be therapeutically harnessed.

While this study is the first phospho-proteomic analysis of cardiac dyssynchrony and CRT, there are limitations. First, we have attributed changes in phosphorylation sites to a change in kinase expression/activity. However, it is also possible that phosphorylation at a specific residue could change due to a change in phosphatase expression/activity. Currently, phosphatases are comparatively poorly understood and predicting a phosphatase for a given phosphorylation site cannot be reliably done. Second, we have identified critical kinases by highlighting those with a large change in the number of targets. As discussed earlier, GSK-3 β sites did not represent a large portion of the phospho-proteome that was altered, but previous functional and mechanistic studies have confirmed its importance. Thus, we are likely missing other critical signaling components that may have very few, but essential, phosphorylation targets. The proposed list of important kinases presented here should be considered extensive, but not exhaustive.

Through the examination of phosphorylation events in cardiac dyssynchrony and CRT, we have identified several interesting aspects of the signaling networks involved in the disease and its treatment. Unsurprisingly, kinase activity and targeting is significantly altered between control, disease, and treated canine hearts. Despite nearly equal number of altered Ser/Thr and Tyr phosphorylation sites, far more Ser/Thr sites were present in every category of change, except “Changed by CRT”. Thus, targeting Tyr kinases may reproduce some of

the unique characteristics of CRT. Multiple networks and pathways identified here as key elements in HF_{dys} and CRT are validated by previous targeted work with these models. Lastly, we have evidence that CK2 may be an important modulator in CRT. This may be harnessed in the future as a therapeutic target to compliment CRT, improving its clinical effects or reducing the non-responder rate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance

Phospho-proteomic profiling in an established, pre-clinical large animal model of cardiac dyssynchrony and cardiac resynchronization therapy (CRT) revealed several important findings. First, CRT moved the overall phospho-proteome away from dyssynchronous heart failure and towards healthy control hearts but was still distinct from both groups. Second, hyperphosphorylation from broad kinase activation, a common fingerprint of heart failure, is largely reversed by CRT. Third, we identified several signaling pathways that are altered by CRT that could be leveraged to improve the low response rate. Lastly, we discovered a possible “master regulator kinase”, CK2, which is activated by dyssynchrony and de-activated by CRT.

Figure 1: Schematics of the dog model pacing schedule and the mass spectrometry work flow. (A) The HF_{dys} (dyssynchronous heart failure) and CRT (Cardiac Resynchronization Therapy) models received an implanted pacemaker and radio-frequency ablation of the left bundle branch (to generate a left bundle branch block, LBBB, and dyssynchrony). After a one-week recovery period, the HF_{dys} and CRT groups were subjected to six weeks atrial tachypacing (200 bpm), which induced heart failure. After three weeks of pacing, the CRT model (which had been the same as HF_{dys} to this point) was switched from atrial to bi-ventricular (Bi-V) pacing to recoordinate contraction (200 bpm, so underlying heart failure

was not altered). Control dogs received no intervention. **(B)** Tissue samples (LV lateral wall, $n = 4$ per group) were split into three parts, one enriched for myofilament, one myofilament-depleted, and one whole tissue homogenate and phospho-tyrosine enriched. Samples were TiO2 phospho-enriched, prepared for MS via the usual methods, and run on an Orbitrap Elite. Samples were then searched with Scaffold and Sorcerer, and significant changes between groups were determined using one-way ANOVA and Holm-Sidak post-hoc tests. The kinase for each site was predicted using Group-based Prediction System 2.1. **(C)** Number of assigned MS/MS spectra for each animal ($n=4$ /group) for Ser/Thr (black) and Tyr (grey) sites.

Figure 2: Altered phosphorylation sites were placed into categories of change based on pattern of differences.

(A) Based on the pattern of significant post-hoc tests for each phosphorylation site, each was placed into a category of change. Each circle represents Con (N), HF_{dys} (D) or CRT (C), showing the patterns of change that were included in each category. For example, in “HF_{dys}-Not reversed” phosphorylation can be decreased from non-failing in both dyssynchrony and CRT or increased from non-failing in both dyssynchrony and CRT. (B) Of the 11% of the phospho-proteome that was altered (left pie chart), the right pie chart indicates the

percentage of total phosphorylation sites changed in HF_{dys} and CRT, divided into individual categories of change. **(C)** Number of significantly altered sites phosphorylated for Ser/Thr or Tyr sites in the four categories of change. **(D)** Principal component analysis for Ser/Thr (left) and Tyr (right) phosphorylation data. Unit variance scaling was applied to phosphorylation data, SVD with imputation was used to calculate principal components.

Figure 3: Ingenuity Analysis for “HF_{dys}- Not Reversed” and “CRT only” categories.
(A) Proteins with (*) had phosphorylation sites changed in HF_{dys} and not reversed by CRT.
(B) Proteins with (*) had phosphorylation sites that were altered only in the CRT group.
 Legend for interactions and protein types is shown to the right. The networks were generated using QIAGEN’s Ingenuity Pathway Analysis (IPA[®], QIAGEN Redwood City, www.qiagen.com/ingenuity).

Figure 4: Kinases and number of predicted targets for each category of change.

The number of phosphorylation sites that shared the same top predicted (A) Ser/Thr or (B) Tyr kinase. SGK, serum/glucocorticoid regulated kinase 1; PKC/PKG, protein kinase C/G; ERK, extracellular regulated kinase; p38, p38 mitogen activated protein kinase; JNK, c-Jun N-terminal kinase; CK2, casein kinase 2; AUR, aurora kinase.

Figure 5: Western blot analysis agreed with kinase prediction for CK2 levels in the three groups. (A) Representative CK2 blot for Con, HF_{dys}, and CRT (top) and total protein stain (bottom) for normalization. (B) Mean, SEM, and individual data points for CK2 normalized to total protein stain ($n = 4$ per group) and then normalized to the mean of the Con group. The one-way ANOVA trended towards significance ($p = 0.09$) suggesting an increase in CK2 levels in HF_{dys} that was reversed by CRT.