Phosphoproteomics Study Based on In Vivo Inhibition Reveals Sites of Calmodulin-Dependent Protein Kinase II Regulation in the Heart

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Background—The multifunctional Ca\(^{2+}\)- and calmodulin-dependent protein kinase II (CaMKII) is a crucial mediator of cardiac physiology and pathology. Increased expression and activation of CaMKII has been linked to elevated risk for arrhythmic events and is a hallmark of human heart failure. A useful approach to determining CaMKII’s role therein is large-scale analysis of phosphorylation events by mass spectrometry. However, current large-scale phosphoproteomics approaches have proved inadequate for high-fidelity identification of kinase-specific roles. The purpose of this study was to develop a phosphoproteomics approach to specifically identify CaMKII’s downstream effects in cardiac tissue.

Methods and Results—To identify putative downstream CaMKII targets in cardiac tissue, animals with myocardial-delimited expression of the specific peptide inhibitor of CaMKII (AC3-I) or an inactive control (AC3-C) were compared using quantitative phosphoproteomics. The hearts were isolated after isoproterenol injection to induce CaMKII activation downstream of \(\beta\)-adrenergic receptor agonist stimulation. Enriched phosphopeptides from AC3-I and AC3-C mice were differentially quantified using stable isotope dimethyl labeling, strong cation exchange chromatography and high-resolution LC-MS/MS. Phosphorylation levels of several hundred sites could be profiled, including 39 phosphoproteins noticeably affected by AC3-I-mediated CaMKII inhibition.

Conclusions—Our data set included known CaMKII substrates, as well as several new candidate proteins involved in functions not previously implicated in CaMKII signaling. (J Am Heart Assoc. 2013;2:e000318 doi: 10.1161/JAHA.113.000318)

Key Words: CaMKII • mass spectrometry • phosphorylation • proteomics • transgenic mouse model
multifunctional Ca\(^{2+}\) and calmodulin-dependent protein kinase II\(^3\) (CaMKII). CaMKII is activated at elevated intracellular Ca\(^{2+}\) levels. Ca\(^{2+}\) binds calmodulin (CaM), and calcified CaM binds to the CaMKII regulatory domain.\(^4\) Both PKA and CaMKII are among the most abundant kinases in heart.\(^5\) There are 4 distinct CaMKII genes (\(x\)-\(\delta\)) that encode multiple enzyme splice variants. CaMKII\(x\) and CaMKII\(\delta\) are present in heart, and excessive CaMKII\(\delta\) activity is most implicated in myocardial disease.\(^6\),\(^7\) However, all CaMKII isoforms share highly conserved catalytic and regulatory domains and compete for overlapping substrates.

An adrenergically driven increase in CaMKII activity leads to direct phosphorylation of phospholamban (PLN) and the type II ryanodine receptor (Ryr2), at Thr17 and Ser281,\(^8\) respectively, thereby directly affecting the Ca\(^{2+}\) cycle.\(^8\),\(^9\) These events, together with CaMKII autophosphorylation at Thr287,\(^10\) are considered hallmarks of cardiac CaMKII activity. Intriguingly, both these events are intertwined with cAMP action, as PLN and Ryr2 are also phosphorylated by PKA at nearby sites Ser1611 and Ser2809,\(^12\) respectively. In addition, other cAMP pathways acting through exchange proteins activated by cAMP, which are PKA independent, have also been reported.\(^13\) Ca\(^{2+}\)/CaM-independent CaMKII activation also occurs via oxidation of a pair of regulatory domain methionines (Met281/282).\(^14\)

Heart failure is characterized by activation of the sympathetic nervous system and subsequent overstimulation of cardiac \(\beta\)-adrenergic signaling. As a consequence, CaMKII expression\(^15\) and activity\(^16\) were found to be increased in human heart failure. Mouse models with cardiac \(\beta\)-adrenergic signaling, such as proteins within the z-disc and a set of distinct sarcomeric proteins. Therefore, this study reveals interesting and novel roles for CaMKII in health and disease.

### Materials and Methods

#### Animals and Treatment Regime

All animal experiments were performed according to the ethical guidelines defined by the University of Iowa and the University Medical Centre Utrecht. All mice were bred in a C57BL/6 background. For proteomics, 2 AC3-I and 2 AC3-C mice were treated with a single bolus injection of 15 mg/kg isoproterenol (CAS# 5984-95-2; Sigma) for 30 minutes. Also 2 mice each with either transgenic peptide were treated with vehicle. The animals were euthanized, and their hearts isolated, flushed with ice-cold PBS, and immediately snap-frozen in liquid nitrogen, before storage at \(-80^\circ\)C. For Western blots, mice were treated identically.

#### Tissue Lysis, Sample Preparation, and Western Blotting

Whole hearts were taken from the \(-80^\circ\)C storage and further frozen in liquid nitrogen and subsequently pulverized in a
custom-made chilled steel mortar. For Western blotting, tissue was further lysed with RIPA lysis buffer (20 mmol/L Tris-HCl [pH 7.4], 150 mmol/L NaCl, 10 mmol/L Na2HPO4, 1% [v/v] Triton X-100, 1% [w/v] Na-deoxycholate, 0.1% [w/v] SDS, 1 mmol/L EDTA, 50 mmol/mL NaF, 2 mmol/L PMSF, and 14 μg/mL aprotinin). Isolated protein samples were loaded on SDS-PAGE for subsequent Western blotting. For detection, chemiluminescence was performed using an ECL-kit from Amersham. The following antibodies were used: anti-phospho-Cacnb2 antibodies made by Yenzyme, anti-Cacnb2 (Thr549; Neuromab), anti-phosphorylated-CaMKII (Thr 287; Upstate), anti-phospholamban (Upstate), and anti-phospho-PLN (Thr17; Santacruz).

**Strong Cation Exchange and LC-MS/MS Analysis**

Whole hearts were taken from the −80°C storage, pulverized as above, and taken up in 500 μL of lysis buffer (10 mmol/L sodium phosphate buffer [pH 7.8], 150 mmol/mL NaCl, 8 mol/L urea, 1× PhosStop tablet, and complete min protease inhibitors [both Roche Diagnostics]) and left at room temperature for 10 minutes. Samples were sonicated 3 times on ice for 1 minute with 2 minutes of resting on ice and centrifuged at 20 000 g for 15 minutes at 4°C. This procedure was repeated once, and the supernatants were combined. Protein concentrations were measured using a Bradford assay (BioRad). The individual samples were reduced with 10 mmol/L dithiothreitol for 30 minutes at 56°C and alkylated by the addition of 55 mmol/L iodoacetamide and incubation for 45 minutes at room temperature in the dark. Lysates were digested with Lys C (ratio 1:100 w/w) for 4 hours at 37°C. Samples were then diluted with 50 mmol/L ammonium bicarbonate to a final concentration of 2 mol/L urea. Trypsin (ratio 1:100 w/w) was added, and the samples were digested overnight at 37°C. Proteolytically cleaved samples were desalted and dimethylabeled on a Sep-Pak C18 column (Waters) according to Boersema et al.24 AC3-C samples were labeled light, AC3-I, heavy, and the internal standard (a mix of all samples, including non-stimulated saline mice) was tagged with the intermediate label. All samples were mixed in equal amounts prior to strong cation exchange and LC-MS/MS analysis. Strong cation exchange (SCX) separation was performed on 2 individual mouse sets consisting of AC3-I, AC3-C, and the internal standard according to Henrich et al.25 A total of 40 fractions were collected. Each mouse set was analyzed using 2-ppm mass variability and 0.2-minute retention time tolerance on precursor ion pairs. Quantitation is based on the ratio of the summed areas of 3 matched isotope patterns (a feature) across the eluting chromatographic peak of that feature. The peptide ratios are calculated using the same number of isotopes. Protein ratios are based on the median peptide ratio, with exclusion of the identified phosphopeptides. At least 2 isotopic peaks were required for inclusion, as well as a minimal signal-to-noise level of 3. Protein identifications over all 4 analyses were combined and grouped by Proteome Discoverer. Each peptide spectral match (PSM) (Mascot peptide score >25) of a phosphorylated peptide was isolated from the data in Proteome Discoverer. Site localization was performed using the pRS algorithm.27 Probability

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scores >75% were considered localized. Phospho-PSMs were then grouped according to their sequence and site(s) of phosphorylation. If multiple quantitative data points for a unique phosphopeptide (including proper site localization) were available, these were averaged, and the standard deviation was calculated. All raw and annotated data are freely available through ProteomeXchange (http://www.proteomexchange.org) under accession number PXD000174 and ProteomeXchange submission title CaMKII Cardiac Phosphoproteome. Icelogos were generated using the Icelogo software package. All Icelogos were generated with a cutoff \( P<0.01 \).28

### Immunohistochemistry

Frozen heart tissue from an AC3-I mouse was serially sectioned in 10-μm slices that were collected on aminopropyltriethoxysilane-coated glass slides. Immunohistochemistry was performed as described previously.29 Primary antibodies against Carp3 (Rabbit, Crp3 H-46 sc-98827, 1:100; SantaCruz) were used. Secondary labeling was performed with appropriate Alexa Fluor 594 (1:250) conjugated with whole IgG antibodies (Jackson Laboratories).

### Results

To establish the downstream phosphorylation targets affected by CaMKII inhibition in vivo in cardiac tissue, the phosphorylation states of mice with transgenic expression of either AC3-I or AC3-C (Figure 1A) were compared using quantitative proteomics. We used SCX-based phosphopeptide enrichment,30 dimethyl stable isotope labeling24 and high-resolution LC-MS/MS analysis (Figure 1B). AC3-I is a potent, highly specific peptide inhibitor of CaMKII, whereas AC3-C is a scrambled nonfunctional homolog peptide18,31 (Figure 1A). The AC3-I peptide mimics the autoinhibitory sequence of CaMKII (mouse Q6PHZ2, amino acids 283 to 292) by posing as a pseudosubstrate with an intact docking site (Arg284), but a deficient phosphoacceptor (Thr287 substituted by an alanine; Figure 1A). Cardiomyocyte-specific expression of AC3-I leads to potent inhibition of CaMKII in vivo as demonstrated previously.18,31 cDNA constructs of AC3-I and AC3-C fused to GFP were expressed under control of the cardiac-specific myh6 promoter. This excludes any distant effects by inhibition of CaMKII in other organs or in nonmyocardial cardiac cells that may modulate cardiac performance. Chronic expression of AC3-I has no structural myocardial phenotype, as these mice age and function normally and have equal expression of CaMKII,19 but show reduced chronotropic activation12 and preserved myocardial function18 after an isoproterenol challenge. To investigate this in more detail, we evaluated the differences in protein expression between both mouse models. Little variation was observed between AC3-I and AC3-C mouse hearts, as indicated from the 1410 quantified proteins, of which 1329 (>94%) presented a <2-fold difference between AC3-I and AC3-C (Figure 1C, Table S1), 90% of which were within 1.5-fold.

To identify downstream phosphorylation targets of CaMKII in the heart, the phosphoproteome of 2 AC3-I and 2 AC3-C mice were quantitatively compared after an intraperitoneal injection of isoproterenol (see Materials and Methods for details; Figure 1B). Prior to extensive LC-MS/MS analysis, we checked the phosphorylation of 2 known CaMKII target sites after identical isoproterenol treatment as described above: Thr17 of cardiac phospholamban (PLN)9 and Thr287 autophosphorylation of CaMKII10 (Figure 2A). Normalization to protein levels of PLN indicates that both phosphorylations were increased in the AC3-C mice. As expected, the response was blunted in the AC3-I mice. Similar trends were observed at the established Thr549 site of the β-subunit of the voltage-gated calcium channel (Cacnb2)33 (Figure 2B).

Subsequently, we enriched for cardiac phosphopeptides from the labeled mixture of AC3-C and AC3-I peptides. This led to the identification of 525 unique phosphopeptides on 282 phosphoproteins. Finally, using stringent filtering and the pRS phosphorylation site localization algorithm,27 310 confidently localized phosphorylation sites could be quantified (Table S2). Evaluation of their phosphorylation motif using Icelogo28 showed a mixture of kinase motifs, including CaMKII’s general motif RXX[pS/pT], but also the SP-directed motif and acidic-directed motifs (Figure 2C). We then isolated the putative CaMKII sites (84 in total) by selecting the motifs with an arginine or lysine residue on position −3 while disregarding those that contained a proline at position +1 ([R/K]XX[pS/pT] [noP]) and made an additional motif (Figure 2D).

Quantitative analysis of the phosphorylated peptides revealed 36 sites that were downregulated on AC3-I expression, indicative of a direct inhibitory effect of AC3-I on these particular phosphorylation sites. In contrast, another 15 sites showed a negative AC3-I/AC3-C ratio, meaning that phosphorylation at these sites was increased when CaMKII was inhibited, presumably an indirect effect of myocardial CaMKII inhibition. Satisfactorily, an Icelogo of the downregulated sites showed clear enrichment of RXXpS-based phosphosites (Figure 2E).

To further structure our quantitative phosphorylation data, we cross-referenced published reports to verify the function of each regulated phosphoprotein in our screen (Figure 3). This showed several expected functional entities, such as ion handling, gene expression, and cytoskeletal proteins involved in contractility. We also found targets related to microtubule formation and upkeep that were not previously recognized as being affected by CaMKII. As expected, we identified other signaling proteins affected by CaMKII inhibition, including the myosin light chain kinase (Ser1798 and Ser1801
downregulated). This is an interesting finding, as it suggests a direct connection between CaMKII inhibition and contractility.

Discussion

CaMKII in Health and Disease

Healthy CaMKII has thus far been implicated in 3 major physiological cardiac functions, that is, excitation-contraction coupling (ECC), excitation-transcription coupling (ETC), and "fight-or-flight" heart rate increases. As part of the ongoing efforts to understand CaMKII's function, research has mainly focused on 3 molecular nodes: (1) the regulation of ion channels (Ca$^{2+}$, Na$^+$, and K$^-$ channels), (2) the regulation of SR-Ca$^{2+}$ via the ryanodine receptor and the SERCA/PLN complex, and (3) HDAC5/NFAT/MEF2-mediated transcription. Over the past 2 decades an enormous body of work has also revealed that CaMKII is a potential driver of myocardial hypertrophy, arrhythmia, and heart failure. Both its activity and expression are increased in patients and animal models of these cardiac diseases. Also, inhibition of cardiac CaMKII in various ways protects the heart against its damaging effects reviewed in Anderson et al. In pathological hypertrophy, patients suffer from defective ECC and ETC, leading to apoptosis and arrhythmias. However, the connection between CaMKII activity and expression and heart disease is not well understood. We reasoned that an important first step toward understanding the molecular pathways affected by CaMKII was to measure direct and indirect CaMKII target phosphorylation sites after isoproterenol stimulation.

Methodology to Probe CaMKII Action Directly in the Myocardium

Monitoring molecular pathways affected by CaMKII are not trivial because the field suffers from lack of a suitable,
Cardiac Phosphorylation Targets of CaMKII  Scholten et al

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**Figure 3.** Phosphorylation sites affected by AC3-I-mediated CaMKII inhibition in mouse heart. Depicted are all upregulated (green) and downregulated (red and pink) sites categorized by specific function. Gene names are used for specific ratios observed, see Table S2.

*Means the mentioned site contains the minimum CaMKII motif (RX[XP][pT]). Boxed proteins or sites are verified CaMKII targets. For acronyms, see Table S2. CaMKII indicates calcium- and calmodulin-dependent protein kinase II; Sympo, synaptophysin-2; Lmo2, leiomodin-2; Capzb, capping protein subunit beta; Palld, Palladin; Cob1, Cordon-bleu protein-like 1; Crsp3, cysteine- and glycine-rich protein 3; IGFN, immunoglobulin-like and fibronectin type III domain-containing protein 1; Myh, myosin heavy chain; Myl, myosin light chain; MII2, histone-lysine N-methyltransferase MLL2; Eif, eukaryotic translation initiation factor; Clasp1, CLIP-associating protein 1 isoform 2; Slain, isoform 1 of SLAIN motif-containing protein; Synpo2, S300; Capzb, S263; Lmod2, S399; IGFN1, S246; Myh, S1366; Myl7 (atrat), S203; Myf2 (ventricular), S14, S15; Myzap, S435.

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Established Targets of CaMKII

Only a handful of direct phosphorylation targets of CaMKII are documented in the heart, with PLN-Thr17 being the best validated. Phosphorylation of PLN leads to aberration of its inhibitory function on SERCA2 and hence an increase in Ca\(^{2+}\) reuptake by the SR. As expected, transgenically expressed AC3-I negatively affects phosphorylation at this site, as shown previously. Additionally, and also in our screen (Figure 2B), thereby validating our approach. In addition, we found several of the known, or predicted, protein targets of cardiac CaMKII in our screen. In addition to Thr549 (Figure 2A), on Cacnb2 we identified a novel site at Ser156 (3.3-fold upregulated) that did not conform to the CaMKII motif, indicative of negative regulation at this site. Among the proteins involved in expression and translation (Figure 3) was Eif4B (eukaryotic translation initiation factor 4B), a ribosomal RNA helicase. In vitro phosphorylation screens identified Eif4B as a CaMKII substrate, although the site was lacking. Using our approach, 2 phosphorylation hot spots were detected on this protein (Figure 4), one containing Ser418, 420, 422, and 425 (Table S2) and another containing Ser495, 497, and 498. The former is functionally described as regulated at Ser422 by several kinases, including S6-kinase1 and Akt, and we did not find these sites regulated (Table S2). The latter hot spot was represented by 4 different phosphopeptides, all of which were found to be downregulated between 3- to 5-fold in AC3-I mice. In addition, Ser497 is a putative CaMKII site with an RXRXP motif. Several other eukaryotic translation initiation factors were also observed, of which Eif3c also contained a downregulated site (Ser39, 2.5-fold; Figure 4). In addition to HDAC5, these reveal a putative novel site of action where CaMKII may influence gene expression. Interestingly, in light of hypertrophy developing over a long period because of altered gene expression, these sites already responded within 30 minutes of isoproterenol stimulation.

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CaMKII Regulates Phosphorylation of Many Myofibril Proteins in the z-Disc and a-Band

AC3-I regulated sites categorized in different cellular compartments and physiological functions (Figure 3). In our phosphoproteomics screen, the sarcomere category contained the most regulated sites. Even though our analysis is somewhat biased toward the more abundant myofilament proteins, many detailed observations could be made in this compartment, of which several are described below.

Scrutinizing the literature we could annotate the exact intracellular localization of each observed phosphoprotein. A set of z-disc proteins, a-band proteins, but also costamere proteins was observed (Figure 5, Table S2). The cardiac sarcomere is strictly organized in thick filaments that contain myosin and thin filaments consisting of filamentous actin. The thin filaments align and cross-link via $\alpha$-actinin dimers at the z-disc and form a bridge to the thick filaments in the a-band (reviewed in Clark et al\textsuperscript{41}; Figure 5A). The cardiac z-disc is an intricate network of many contractile (regulatory) proteins.\textsuperscript{41} Costameric proteins link the z-disc with the sarcolemma to transmit force between these 2 regions.\textsuperscript{42}

When grouping the phosphoproteins by confirmed intracellular localization and known binding partners, a site-specific pattern became visible. Although the costameric proteins were found to be phosphorylated, none of them seemed affected by isoproterenol in AC3-I mice, whereas both the z-disc and a-band proteins were (Figure 5A). This suggests that isoproterenol-sensitive CaMKII is present or at least influences signaling nodes, specifically at these defined locations in the heart.

Titin is a major stability component of the cardiac sarcomere with its N-terminus embedded in the z-disc and its C-terminus extending into the a-band. We observed 5 different phosphopeptides on titin, harboring in total 7 quantified phosphorylation sites (Table S2). One site, Ser5070 at the z-disc part of titin, is a putative CaMKII site (RXXpS) only present in isoform 3 (A2ASS6-3), which was downregulated almost 20-fold in AC3-I mice. At the same time, at the C-terminal end, we found 2 adjacent phosphorylation sites Thr34450 (in isoform 1, A2ASS6-1) and Ser34451, of which the former was found to be 2.6-fold upregulated and the latter unchanged. These observations caused us to speculate that CaMKII directly influences the phosphorylation state of titin and thereby likely regulates sarcomere action. Further research should reveal more detailed information on the exact nature of these specific events.\textsuperscript{43,44}

We found Csrp3 (cysteine and glycine-rich protein 3, or Crp3, also called muscle lim protein) less phosphorylated in

![Figure 4](https://jaha.ahajournals.org/content/11/1/000318/F4.large.jpg)

**Figure 4.** AC3-I inhibition of cardiac CaMKII influences elongation-initiation factors. String network of the detected phosphorylated translation-elongation proteins. Depicted are the observed phosphorylation sites (P). White phosphorylations mean unaffected sites, red is downregulated sites in AC3-I mice. In more detail the 2 phosphorylation hot spots in EIF4B are depicted, including the observed phosphopeptides with their localized sites in black boldface (not affected by AC3-I) or red boldface (downregulated in AC3-I mice) and the observed AC3-I/AC3-C ratios. CaMKII indicates calcium- and calmodulin-dependent protein kinase II; EEF1D, isoform 1 of elongation factor 1-delta; Eif, eukaryotic translation initiation factor.
AC3-I murine hearts at Thr84 and Ser95 (both \( \approx 2.5 \)-fold) than in AC3-C hearts. Csrp3 is crucial to the development and maintenance of cardiac cytoarchitectural organization. Mice deficient in this protein develop severe dilated cardiomyopathy within several weeks after birth. Csrp3 localizes to the z-disc, where it binds telethonin (Tcap, phosphorylated at Ser160, unchanged). Csrp3 localizes to the nucleus and does so in failing human heart in particular. Whether this is also phosphorylation dependent is currently unknown, although it is tempting to speculate that the AC3-I inhibited sites found here combined with increased CaMKII expression and activity in failing hearts are somehow connected. Illustrative of this is the immunolabeling of Csrp3, which colocalized with the GFP-tagged transgenic AC3-I at the sarcomere in our model system (Figure 5B).

The a-band is another location where strong effects of AC3-I were observed, suggesting a direct effect of CaMKII in the contractile response (Figure 5). In addition to myosin itself (Ser1368 in isoform-6, Q02566, 2-fold downregulated), several of its master regulators were also affected. Interesting were the observations on 2 isoforms of the myosin regulatory light chain, types 2 and 7 (Myl2 and Myl7). The former is the ventricular isoform, which had an intricate phosphorylation pattern concerning Ser14, Ser15, and Ser19;
Figure 6. Compartment-specific regulation of myosin regulatory light chain. Alignment of the N-termini of Myl2 (1 to 52, ventricle) and Myl7 (1 to 60, atrium) reveals conservation of the 3 observed phosphorylation sites (yellow P and boldface) at Ser14, Ser15, and Ser19 in Myl2 and Ser22, Ser23 and Ser27 in Myl7. When comparing the quantitative data, we observed opposite regulation in both compartments as well as different consensus motifs for putative kinases at this site, suggesting that different kinases regulate these proteins in ventricles and atria. Arg20 of Myl7 (red R) is a putative CaMKII site, of which the phosphorylated form was found downregulated 2.5-fold. This arginine, and thus consensus site, is absent in Myl2. Myl indicates myosin light chain; CaMKII indicates calcium- and calmodulin-dependent protein kinase II.

**Links to Pathophysiology; Future Directions**

Increased CaMKII activity and expression are a hallmark of human heart failure, hypertrophy, and arrhythmia. Much research has been focused on the role of CaMKII in Ca\(^{2+}\) handling. However, the role of CaMKII on the myofibrils has thus far been largely unreported. Our data reveal that CaMKII inhibition has effects on many myofibrillar proteins in both the z-disc and the a-band. Interestingly, several of the AC3-I regulated (myofibril) phosphoproteins found in this study (Myh6/7, Myl2, Csrp3, PLN) are also known as genetic factors in hereditary cardiomyopathy,\(^{47}\) which often leads to heart failure. We found several more of such factors with documented hereditary mutations to be phosphorylated, although not regulated by AC3-I; these include Tpm1, MyBPC3, Tcap, Jph2, Obscn, Myoz2, Des, and LDB3. Based on our data, future research should focus on the interplay of CaMKII and perhaps other important cardiac kinases in their regulation of the sarcomere. Also, our data suggest putative novel genes to be investigated for causative cardiomyopathic mutations, for instance, in Lmod2 (Leiomodin-2), an actin capping gene involved in regulation of the thin filament length.\(^{48}\)

**Conclusions**

The study described here is the first of its kind and highlights the feasibility of performing kinase-specific quantitative phosphoproteomics directly in cardiac mouse tissue. Using the transgenic model allowed us to isolate a small subset of 39 CaMKII-regulated phosphoproteins, including exact site localization, which provides a valuable resource for future research into CaMKII’s role in the healthy but also the diseased heart.

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**Disclosures**

None.
References


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