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Comprehensive assessment of chamber-specific and transmural heterogeneity in myofilament protein phosphorylation by top-down mass spectrometry

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Abstract

The heart is characterized by a remarkable degree of heterogeneity, the basis of which is a subject of active investigation. Myofilament protein post-translational modifications (PTMs) represent a critical mechanism regulating cardiac contractility, and emerging evidence shows that pathological cardiac conditions induce contractile heterogeneity that correlates with transmural variations in the modification status of myofilament proteins. Nevertheless, whether there exists basal heterogeneity in myofilament protein PTMs in the heart remains unclear. Here we have systematically assessed chamber-specific and transmural variations in myofilament protein PTMs, specifically, the phosphorylation of cardiac troponin I (cTnI), cardiac troponin T (cTnT), tropomyosin (Tpm), and myosin light chain 2 (MLC2). We show that the phosphorylation of cTnI and α Tm vary in the different chambers of the heart, whereas the phosphorylation of MLC2 and cTnT does not. In contrast, no significant transmural differences were observed in the phosphorylation of any of the myofilament proteins analyzed. These results highlight the

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Disclosures

None.

importance of appropriate tissue sampling—particularly for studies aimed at elucidating disease mechanisms and biomarker discovery—in order to minimize potential variations arising from basal heterogeneity in myofilament PTMs in the heart.

Keywords

Transmural Heterogeneity; Chamber Heterogeneity; Phosphorylation; Mass Spectrometry; Myofilament

1. Introduction

The heart is characterized by a remarkable degree of heterogeneity, which underlies normal pump function and is also altered in heart failure [1–6]. Contractile heterogeneity, in particular, has taken on increased significance with the demonstration that transmural changes in myocardial contractility not only occur in the failing heart [1–3], but may also be predictive of adverse cardiac events [7, 8]. Myocardial contractility is governed by a number of factors, including the size and duration of Ca^{2+} transients, as well as the intrinsic properties of the contractile apparatus, which depend primarily on the expression of myofilament protein isoforms and post-translational modifications (PTMs) [9–12].

Myofilament protein PTMs have emerged as a key mechanism regulating cardiac contractility in health and disease [9–12]. Previous studies in humans, as well as large and small animals, have shown that aging and pathological cardiac conditions induce contractile heterogeneity across the free wall of the left ventricle (LV) that correlates with differences in the modification status of myofilament proteins [1, 2, 5]. Yet, there is conflicting evidence in the literature regarding the existence of basal heterogeneity in myofilament PTMs. Although several groups have observed transmural variations in myofilament PTMs across the LV free wall in small animals [4, 13–15], such heterogeneity does not appear to be present in humans and large animals under basal conditions [1, 2, 16]. Comparatively, very little is known regarding the existence of chamber-specific variations in myofilament protein PTMs in the heart [17]. Knowledge of basal chamber-specific or transmural variations in myofilament protein PTMs will undoubtedly be essential for understanding cardiac physiology, elucidating PTM-associated disease mechanisms, and biomarker discovery.

Top-down mass spectrometry (MS) is a powerful tool for the comprehensive assessment of protein PTMs [18–20]. Intact proteins are analyzed in top-down MS, which allows for the detection of the entire complement of protein PTMs simultaneously without *a priori* knowledge [18–20]. Furthermore, the addition of PTMs to intact proteins has relatively little influence on their physiochemical properties, thus, allowing for the reliable quantification of modified and un-modified protein forms present within the same spectrum [18–20]. Herein, we have utilized quantitative top-down MS to systematically assess chamber-specific and transmural variations in myofilament protein PTMs in the hearts of healthy pigs, which currently represent the gold standard model system for human cardiovascular diseases [21]; with a special focus on the phosphorylation of cardiac troponin I (cTnI), cardiac troponin T (cTnT), tropomyosin (Tpm), and myosin light chain 2 (MLC2).

2. Methods

A detailed Materials and Methods section is provided in the Supplemental Material.

2.1 Tissue procurement

Pig heart tissue was obtained from Yorkshire domestic pigs (approximately 3 months of age) as approved by the University of Wisconsin Animal Care and Use Committee. Excised hearts were quickly sectioned into the LV, right ventricle (RV), left atrium (LA), and right atrium (RA), flash frozen in liquid nitrogen, and stored at -80°C for later use. For experiments examining chamber-specific heterogeneity in myofilament protein phosphorylation, the LV samples consisted predominantly of mid-myocardium (MYO) with little or no sub-endocardium (ENDO) or sub-epicardium (EPI). For experiments examining transmural heterogeneity in myofilament protein phosphorylation, the free wall of the LV was further sectioned into thirds with the inner most third, the middle third, and the outer most third representing the ENDO, MYO, and EPI, respectively, prior to flash freezing.

2.2 Immunoaffinity purification of cardiac troponin complex

Cardiac troponin complex was isolated from pig myocardial tissue by immunoaffinity purification as previously described [22].

2.3 Preparation of myofilament extracts

Myofilament proteins were extracted from pig myocardial tissue using a two-step extraction procedure as previously described [17, 23].

2.4 Offline and online top-down high-resolution MS and tandem MS (MS/MS)

For MS analysis of cTnI and cTnT, desalting and offline MS analysis were carried out as previously described [22]. Top-down MS and MS/MS analyses of MLC2 and myosin light chain 1 (MLC1) were also carried out as previously described [23], with minor modifications. For online MS analysis of Tpm, myofilament extracts were diluted 1:1 (v/v) with mobile phase A (mobile phase A: 0.1% formic acid in water; mobile phase B: 0.1% formic acid in methanol) prior to liquid chromatography (LC)-MS. Myofilament extracts were separated using a Dionex U3000 LC system (Thermo Scientific, Boston, MA, USA) equipped with a home-packed PLRP column (PLRP-S, 200 mm $\times$ 500 μm , 10 μm , 1000 $\text{\AA}$; Varian, Lake Forest, CA, USA) and a gradient going from 20% B to 90% B over 55 min, at a flow rate of 12.5 $\mu\text{L}/\text{min}$. The Dionex U3000 LC system was coupled online with a 12T Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (Bruker Daltonics, Billerica, MA, USA) using the Bruker electrospray ionization source. Samples were introduced into the mass spectrometer using a capillary voltage and an endcap offset of -4.5 kV and -5 kV, respectively. A resolving power of 250,000 (at m/z 400) and a fixed ion accumulation time of 0.02 seconds were used for spectral acquisition. Mass spectra obtained using the methods described above were highly reproducible (Supp. Fig. 1).

2.5 Protein identification

Identification of the atrial isoforms of MLC2 and MLC1 from pig was carried out as previously described [23].

2.6 Western blot

Myofilament extracts were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked with Protein-Free Blocking Buffer (Thermo Scientific) and blotted with antibodies against cTnI (Thermo Scientific) and cTnI phosphorylated at Ser22/23 (Cell Signaling Technology, Beverly, MA, USA). Western blots were analyzed using ImageJ.

2.7 Quantitative analysis

Offline and online mass and tandem mass spectra were analyzed using in-house developed MASH Suite software [24] and Bruker Data Analysis software, respectively, and manually validated to ensure data accuracy. The most abundant relative molecular masses and monoisotopic masses are reported for intact proteins and fragment ions, respectively. The relative abundances of each protein form, as well as the total protein phosphorylation, were calculated based on the signal intensities from the mass spectra as previously described [17, 23, 25, 26], taking into account oxidized protein forms and those associated with non-covalent adducts.

2.8 Statistical analysis

Data are from at least three biological replicates ($n=3$), each with two technical replicates. For the chamber analysis, the relative abundance of each protein form and the total protein phosphorylation values from the EPI, MYO, and ENDO were included as additional replicates for the LV ($n=12$ for LV in the chamber comparison). The relative abundance data were analyzed in R 3.2.1 using linear mixed effects models with random intercepts incorporating 2 main effects (either chamber or transmural layer and phosphorylation type) and their interaction. One-way ANOVA was used to evaluate the statistical significance of variance for the total protein phosphorylation in either the different chambers of the heart, or in the different layers of the LV free wall. The Mann-Whitney U test was used for group comparison of the Western blot data. All values are reported as mean $\pm$ SEM. Differences between means were considered significant at $p < 0.05$.

3. Results

3.1 cTnI phosphorylation varies in a chamber-specific but not transmural manner in the heart

To determine whether cTnI phosphorylation varies in the different chambers of the heart, cTnI was immunoaffinity purified from the LV, RV, LA, and RA of healthy pig hearts, and analyzed by top-down MS. Three major cTnI protein forms, with relative molecular masses that matched closely with those previously reported for un-phosphorylated, mono-phosphorylated ($_p$ cTnI), and bis-phosphorylated ($_{pp}$ cTnI) cTnI from pig [22, 23], were detected in all four chambers of the heart (Fig. 1A). Several additional protein forms,

including cTnI containing the V116A polymorphism reported previously [22], as well as oxidized cTnI and cTnI associated with non-covalent adducts, were also observed (Fig. 1A). MS-based quantification of the cTnI protein forms in each chamber revealed significant differences in the relative abundances of un-phosphorylated cTnI, p cTnI, and pp cTnI (Fig. 1B, Supplemental Results). Calculation of the total phosphorylation revealed that cTnI phosphorylation was significantly greater in the ventricles than in the atria (Fig. 1C, Supplemental Results).

Next, cTnI from the EPI, MYO, and ENDO of the LV free wall was analyzed using top-down MS to determine whether cTnI phosphorylation also varies transmurally in the heart. Un-phosphorylated cTnI, p cTnI, and pp cTnI were the predominant cTnI protein forms detected by top-down MS in the EPI, MYO and ENDO (Fig. 1D). Additionally, minor protein forms corresponding to cTnI associated with non-covalent adducts were also observed (Fig. 1D). Quantitative top-down MS analysis revealed no significant differences in either the relative abundances of cTnI protein forms, or total cTnI phosphorylation, across the LV free wall (Figs. 1E and F, Supplemental Results).

Western blot analysis of cTnI-Ser22/23 phosphorylation, which we have previously shown are the only sites basally phosphorylated in pig myocardium [22], was used to confirm the results of the top-down MS analysis. cTnI-Ser22/23 phosphorylation was significantly greater in the LV than in the LA and RA (Fig. 2A), which corresponds well with the significantly increased relative abundance of pp cTnI in the LV (compared to the LA and RA) observed by MS analysis (Fig. 1B). Likewise, cTnI-Ser22/23 phosphorylation in the RV was increased relative to the LA and RA by Western blot (Fig. 2A); however, the difference between the RV and RA was not statistically significant as it was based on the MS analysis (Fig. 1B). Similarly, cTnI-Ser22/23 phosphorylation, as assessed by Western blot, was not significantly different across the layers of the LV free wall (Fig. 2B), which is also in good agreement with the results of our top-down MS analysis (Fig. 1E). Collectively, these results clearly demonstrate that basal cTnI phosphorylation varies in a chamber-specific but not transmural manner in the heart.

3.2 cTnT phosphorylation does not vary in the different chambers of the heart or across the LV free wall

We next sought to determine whether the phosphorylation of cTnT varies in the different chambers of the heart. Two cTnT protein forms, with relative molecular masses similar to those previously published for un-phosphorylated and mono-phosphorylated (p cTnT) cTnT from pig [23], were detected by top-down MS in all four chambers of the heart (Fig. 3A). Additional protein forms corresponding to cTnT associated with non-covalent adducts were also observed in the spectra (Fig. 3A). Quantification of cTnT protein forms revealed that the relative abundances of cTnT and p cTnT did not differ significantly between the LV, RV, LA, and RA (Fig. 3B, Supplemental Results). Likewise, total cTnT phosphorylation was similar across the chambers of the heart (Fig. 3C, Supplemental Results).

Similarly, top-down MS analysis of cTnT phosphorylation across the LV free wall showed no significant differences in either the relative abundances of cTnT protein forms or total cTnT phosphorylation in the EPI, MYO, or ENDO (Figs. 3D–F, Supplemental Results).

These results clearly show that cTnT phosphorylation does not vary in a chamber-specific or transmural manner in the heart.

3.3 Tpm phosphorylation exhibits chamber-specific but not transmural heterogeneity in the heart

We next examined Tpm phosphorylation in the atria and ventricles to determine whether there is chamber-specific heterogeneity in Tpm phosphorylation in the heart. Three Tpm protein forms, with relative molecular masses similar to those previously reported for un-phosphorylated Tpm1.1 (α Tm), mono-phosphorylated Tpm1.1 ($p\alpha$ Tm), and un-phosphorylated Tpm2.2 (β Tm) from pig [23, 27], were detected by top-down MS analysis in the LV, RV, LA, and RA (Fig. 4A). Minor protein forms corresponding to oxidized α Tm and $p\alpha$ Tm, respectively, were also observed in the spectra (Fig. 4A). Quantification of the Tpm protein forms in spectra from the different chambers of the heart revealed significant differences in the relative abundances of un-phosphorylated α Tm, $p\alpha$ Tm, and un-phosphorylated β Tm (Fig. 4B, Supplemental Results). Overall, the total phosphorylation of α Tm was increased in the atria relative to the ventricles (Fig. 4C, Supplemental Results), although the difference in total phosphorylation between the RA and LV did not reach statistical significance ($p = 0.050013$) (Fig. 4C).

Top-down MS analysis of Tpm in the EPI, MYO, and ENDO revealed no significant differences in either the relative abundances of Tpm protein forms, or total α Tm phosphorylation, across the free wall of the LV (Figs. 4D–F, Supplemental Results). These findings confirm that α Tm phosphorylation is increased in the atria in comparison to the ventricles, but does not vary across the LV free wall.

3.4 The phosphorylation of MLC2 does not vary in a chamber-specific or transmural manner in the heart

We next sought to assess differences in myosin light chain protein forms in the different chambers of the heart and across the LV free wall. However, while the relative molecular masses of the ventricular isoforms of MLC1 and MLC2 (MLC1v and MLC2v, respectively) from pig have previously been determined [23], the relative molecular masses of the atrial isoforms of MLC1 and MLC2 (MLC1a and MLC2a, respectively), calculated based on sequences from the NCBI database, did not match with those experimentally determined for presumed peaks corresponding to MLC1a and MLC2a. Thus, to confirm the identity of MLC1a and MLC2a, peaks presumably corresponding to these protein forms were isolated and fragmented by electron capture dissociation. Information from the tandem mass spectra was then searched against the pig database using MS-Align+ [28], which allowed for the high confidence identification of MLC1a (relative molecular mass: 21,502.94 Da) and MLC2a (relative molecular mass: 19,384.70 Da) from pig (Figs. 5). Thus, we have confirmed the expression of different MLC1 and MLC2 isoforms in the atria and ventricles of the pig heart (Figs. 6 and 7).

After confirming the identity of peaks corresponding to MLC1a and MLC2a, the phosphorylation of MLC2 was assessed in the different chambers of the heart. A total of four major MLC2 protein forms were detected by top-down MS analysis with forms

corresponding to un-phosphorylated and mono-phosphorylated MLC2v detected in the LV and RV and forms corresponding to un-phosphorylated and mono-phosphorylated MLC2a detected in the LA and RA, respectively (Fig. 7A). Additional protein forms corresponding to oxidized MLC2 and MLC2 associated with non-covalent adducts were also observed in the spectra (Fig. 7A). Quantification of MLC2 protein forms revealed that the relative abundances of un- and mono-phosphorylated (p -MLC2) MLC2 did not differ significantly between the LV, RV, LA, and RA (Fig. 7B, Supplemental Results). Likewise, although there was a minor increase in the total MLC2 phosphorylation in the RV (in comparison to the LV, LA, and RA), these differences were not significant (Fig. 7C, Supplemental Results).

Subsequent analysis of MLC2v phosphorylation across the EPI, MYO, and ENDO of the LV free wall revealed no significant differences in the relative abundances of MLC2v protein forms, or total MLC2v phosphorylation (Figs. 7D–F, Supplemental Results). Thus, these results demonstrate that MLC2 phosphorylation does not vary significantly across either the chambers of the heart or the LV free wall in pigs.

4. Discussion

Myofilament protein PTMs have emerged as a key mechanism regulating cardiac contractility, and increasing evidence has shown that the alteration of myofilament protein PTMs, particularly phosphorylation, can contribute to contractile dysfunction and heart failure [9–12, 25, 26, 29, 30]. Additionally, studies in humans, as well as large and small animals, have shown that aging and pathological cardiac conditions induce transmural changes in contractile function that correlate with differences in the modification status of myofilament proteins [1, 2, 5]. Nevertheless, whether there is basal heterogeneity in myofilament protein PTMs in the heart remains an important question—particularly given the potential usefulness of myofilament PTMs as biomarkers for heart disease [26, 31].

In the present study, we have systematically assessed regional and transmural variations in myofilament protein PTMs in the hearts of healthy pigs by top-down MS, with a focus on the phosphorylation of cTnI, cTnT, α Tm, and MLC2. Here, we have demonstrated that, while the phosphorylation of certain myofilament proteins (cTnI and α Tm) varies in a chamber-specific manner within the heart, the phosphorylation of others (cTnT and MLC2) does not. Additionally, we have shown that the phosphorylation of cTnI, cTnT, α Tm, and MLC2v do not differ significantly across the LV free wall in healthy pigs. The observed differences in cTnI and α Tm phosphorylation in the atrial and ventricular myocardium are likely due to chamber-specific variations in the expression or activity of kinases or phosphatases in the heart [32].

Previous studies have observed that there is transmural heterogeneity in the modification status of myofilament proteins across the ventricular wall in small animals [4, 13–15]. In particular, MLC2v phosphorylation has been shown to vary across the free wall of the LV in rats and mice [4, 13, 15]; however, here we found no difference in MLC2v phosphorylation (or in the phosphorylation of cTnI, cTnT, and α Tm) across the layers of the LV free wall in pigs, which is in good agreement with previous studies in pigs and humans [1, 2]. While

there is the possibility that increased workload may induce transmural differences in myofilament protein phosphorylation in large animals and humans, as it does in rodents [13], this, at least, does not appear to be the case in pigs [2]. Thus, the data presented here, as well as data from other groups [1, 2, 16], suggests that transmural heterogeneity in myofilament protein phosphorylation does not exist in large animals and humans under basal conditions. Although it is possible that the phosphorylation of other myofilament proteins, such as cardiac myosin binding protein C, may vary transmurally in the heart, it seems likely that different mechanisms regulate transmural differences in mechanical function across the LV free wall in large animals and humans. Indeed, Stelzer *et al.* have shown that a transmural gradient in the expression of α myosin heavy chain can account for the higher rates of stretch activation and force redevelopment in the EPI in comparison to the ENDO in pig hearts [16]. It should be noted that this gradient is also present in human hearts [33]. Another similarity between pig and human hearts is that α Tm phosphorylation is increased in the atria relative to the ventricles, which we show here in pigs, and in our previous study using human donor hearts [17]. Although the significance of increased α Tm phosphorylation in the atria remains unclear, consistency at the molecular level, as well as in the mechanisms regulating mechanical activity within the heart, highlight the benefits of using large animals, which more faithfully recapitulate human cardiac physiology and pathophysiology [21, 34], to model human cardiovascular diseases.

Furthermore, given that the phosphorylation of both cTnI and α Tm have been linked to altered Ca^{2+} -handling by the contractile apparatus [11, 35], chamber-specific differences in the phosphorylation of these proteins could underlie differences in myofilament Ca^{2+} sensitivity between the atria and ventricles. Analysis of skinned multicellular preparations from the atria and ventricles of pig by Locher *et al.* revealed significant differences in the rate of force redevelopment, the rate of ATP hydrolysis, the tension cost, and the calcium sensitivity between these preparations [36]. While differences in the rate of force redevelopment, rate of ATP hydrolysis, and tension cost could be explained by differences in the expression of the α and β isoforms of myosin heavy chain in the atria and ventricles, the differences in calcium sensitivity could not be explained, as no differences were found in myofilament protein phosphorylation by ProQ Diamond analysis [36]. Here, we have shown that cTnI-Ser22/23 phosphorylation, a well-known modulator of myofilament Ca^{2+} sensitivity [11], is reduced in the atria by both top-down MS analysis and Western blot, which could partly account for the differences in Ca^{2+} sensitivity between atrial and ventricular preparations observed by Locher *et al.* The reason that we were able to detect differences in cTnI phosphorylation in the atrial and ventricular myocardium, while they were not, may be due to the sensitivity of the ProQ Diamond stain to negatively charged amino acids within proteins [37]. Differences in myofilament Ca^{2+} sensitivity in the atria and ventricles may be necessary for adapting mechanical activity to differences in intracellular Ca^{2+} -handling in atrial and ventricular myocytes [38].

In summary, these results demonstrate that myofilament protein PTMs vary in a chamber- and protein-specific manner in the heart. Additionally, transmural variations in myofilament protein phosphorylation do not appear to be present in pigs under basal conditions, although the possibility that there is transmural heterogeneity in the phosphorylation of myofilament

proteins that were not analyzed in this study remains a possibility. Collectively, these findings highlight the importance of appropriate tissue sampling, particularly in studies aimed at elucidating PTM-associated disease mechanisms and biomarker studies, in order to minimize potential variations arising from basal heterogeneity in myofilament PTMs in the heart.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

PTMs	post-translational modifications
LV	left ventricle
MS	mass spectrometry
cTnI	cardiac troponin I
cTnT	cardiac troponin T
Tpm	tropomyosin
MLC2	myosin light chain 2
RV	right ventricle
LA	left atrium
RA	right atrium
MYO	mid-myocardium
ENDO	sub-endocardium
EPI	sub-epicardium
MS/MS	tandem MS
MLC1	myosin light chain 1
LC	liquid chromatography
FT-ICR	Fourier transform ion cyclotron resonance
<i>p</i>cTnI	mono-phosphorylated cTnI
<i>pp</i>cTnI	bis-phosphorylated cTnI
<i>p</i>cTnT	mono-phosphorylated cTnT
αTm	Tpm1.1

pαTm	mono-phosphorylated Tpm1.1
βTm	Tpm2.2
MLC1v	MLC1 ventricular isoform
MLC2v	MLC2 ventricular isoform
MLC1a	MLC1 atrial isoform
MLC2a	MLC2 atrial isoform
pMLC2	mono-phosphorylated MLC2
LTQ	linear ion trap

References

- Haynes P, Nava KE, Lawson BA, Chung CS, Mitov MI, Campbell SG, et al. Transmural heterogeneity of cellular level power output is reduced in human heart failure. *J Mol Cell Cardiol.* 2014 Jul;72:1–8. [PubMed: 24560668]
- van der Velden J, Merkus D, de Beer V, Hamdani N, Linke WA, Boontje NM, et al. Transmural heterogeneity of myofilament function and sarcomeric protein phosphorylation in remodeled myocardium of pigs with a recent myocardial infarction. *Front Physiol.* 2011; 2:83. [PubMed: 22131977]
- McCrossan ZA, Billeter R, White E. Transmural changes in size, contractile and electrical properties of SHR left ventricular myocytes during compensated hypertrophy. *Cardiovasc Res.* 2004 Aug 1; 63(2):283–92. [PubMed: 15249186]
- Cazorla O, Szilagyi S, Le Guennec JY, Vassort G, Lacampagne A. Transmural stretch-dependent regulation of contractile properties in rat heart and its alteration after myocardial infarction. *FASEB J.* 2005 Jan; 19(1):88–90. [PubMed: 15498894]
- Campbell SG, Haynes P, Kelsey Snapp W, Nava KE, Campbell KS. Altered ventricular torsion and transmural patterns of myocyte relaxation precede heart failure in aging F344 rats. *Am J Physiol Heart Circ Physiol.* 2013 Sep 1; 305(5):H676–86. [PubMed: 23792678]
- Lou Q, Fedorov VV, Glukhov AV, Moazami N, Fast VG, Efimov IR. Transmural heterogeneity and remodeling of ventricular excitation-contraction coupling in human heart failure. *Circulation.* 2011 May 3; 123(17):1881–90. [PubMed: 21502574]
- de Simone G, Devereux RB, Koren MJ, Mensah GA, Casale PN, Laragh JH. Midwall left ventricular mechanics. An independent predictor of cardiovascular risk in arterial hypertension. *Circulation.* 1996 Jan 15; 93(2):259–65. [PubMed: 8548897]
- Wachtell K, Gerdts E, Palmieri V, Olsen MH, Nieminen MS, Papademetriou V, et al. In-treatment midwall and endocardial fractional shortening predict cardiovascular outcome in hypertensive patients with preserved baseline systolic ventricular function: the Losartan Intervention For Endpoint reduction study. *J Hypertens.* 2010 Jul; 28(7):1541–6. [PubMed: 20410835]
- Yuan C, Solaro RJ. Myofilament proteins: From cardiac disorders to proteomic changes. *Proteomics Clin Appl.* 2008 Jun; 2(6):788–99. [PubMed: 21136879]
- Hamdani N, Kooij V, van Dijk S, Merkus D, Paulus WJ, Remedios CD, et al. Sarcomeric dysfunction in heart failure. *Cardiovasc Res.* 2008 Mar 1; 77(4):649–58. [PubMed: 18055579]
- de Tombe PP, Solaro RJ. Integration of cardiac myofilament activity and regulation with pathways signaling hypertrophy and failure. *Ann Biomed Eng.* 2000 Aug; 28(8):991–1001. [PubMed: 11144684]
- Jin W, Brown AT, Murphy AM. Cardiac myofilaments: from proteome to pathophysiology. *Proteomics Clin Appl.* 2008 Jun; 2(6):800–10. [PubMed: 21136880]

13. Hidalgo C, Wu Y, Peng J, Siems WF, Campbell KB, Granzier H. Effect of diastolic pressure on MLC2v phosphorylation in the rat left ventricle. *Arch Biochem Biophys*. 2006 Dec 15; 456(2): 216–23. [PubMed: 16949549]
14. Ait Mou Y, le Guennec JY, Mosca E, de Tombe PP, Cazorla O. Differential contribution of cardiac sarcomeric proteins in the myofibrillar force response to stretch. *Pflugers Arch*. 2008 Oct; 457(1): 25–36. [PubMed: 18449562]
15. Davis JS, Hassanzadeh S, Winitsky S, Lin H, Satorius C, Vemuri R, et al. The overall pattern of cardiac contraction depends on a spatial gradient of myosin regulatory light chain phosphorylation. *Cell*. 2001 Nov 30; 107(5):631–41. [PubMed: 11733062]
16. Stelzer JE, Norman HS, Chen PP, Patel JR, Moss RL. Transmural variation in myosin heavy chain isoform expression modulates the timing of myocardial force generation in porcine left ventricle. *J Physiol*. 2008 Nov 1; 586(Pt 21):5203–14. [PubMed: 18787035]
17. Peng Y, Yu D, Gregorich Z, Chen X, Beyer AM, Gutterman DD, et al. In-depth proteomic analysis of human tropomyosin by top-down mass spectrometry. *J Muscle Res Cell Motil*. 2013 Aug; 34(3–4):199–210. [PubMed: 23881156]
18. Siuti N, Kelleher NL. Decoding protein modifications using top-down mass spectrometry. *Nat Methods*. 2007 Oct; 4(10):817–21. [PubMed: 17901871]
19. Zhang H, Ge Y. Comprehensive analysis of protein modifications by top-down mass spectrometry. *Circ Cardiovasc Genet*. 2011 Dec; 4(6):711. [PubMed: 22187450]
20. Gregorich ZR, Ge Y. Top-down proteomics in health and disease: challenges and opportunities. *Proteomics*. 2014 May; 14(10):1195–210. [PubMed: 24723472]
21. Kooij V, Venkatraman V, Tra J, Kirk JA, Rowell J, Blice-Baum A, et al. Sizing up models of heart failure: Proteomics from flies to humans. *Proteomics Clin Appl*. 2014 Oct; 8(9–10):65–64.
22. Zhang J, Dong X, Hacker TA, Ge Y. Deciphering modifications in swine cardiac troponin I by top-down high-resolution tandem mass spectrometry. *J Am Soc Mass Spectrom*. 2010 Jun; 21(6):940–8. [PubMed: 20223681]
23. Peng Y, Gregorich ZR, Valeja SG, Zhang H, Cai W, Chen YC, et al. Top-down proteomics reveals concerted reductions in myofilament and Z-disc protein phosphorylation after acute myocardial infarction. *Mol Cell Proteomics*. 2014 Oct; 13(10):2752–64. [PubMed: 24969035]
24. Guner H, Close PL, Cai W, Zhang H, Peng Y, Gregorich ZR, et al. MASH Suite: a user-friendly and versatile software interface for high-resolution mass spectrometry data interpretation and visualization. *J Am Soc Mass Spectrom*. 2014 Mar; 25(3):464–70. [PubMed: 24385400]
25. Dong X, Sumandea CA, Chen YC, Garcia-Cazarin ML, Zhang J, Balke CW, et al. Augmented phosphorylation of cardiac troponin I in hypertensive heart failure. *J Biol Chem*. 2012 Jan 6; 287(2):848–57. [PubMed: 22052912]
26. Zhang J, Guy MJ, Norman HS, Chen YC, Xu Q, Dong X, et al. Top-down quantitative proteomics identified phosphorylation of cardiac troponin I as a candidate biomarker for chronic heart failure. *J Proteome Res*. 2011 Sep 2; 10(9):4054–65. [PubMed: 21751783]
27. Peng Y, Chen X, Zhang H, Xu Q, Hacker TA, Ge Y. Top-down targeted proteomics for deep sequencing of tropomyosin isoforms. *J Proteome Res*. 2013 Jan 4; 12(1):187–98. [PubMed: 23256820]
28. Liu X, Sirotkin Y, Shen Y, Anderson G, Tsai YS, Ting YS, et al. Protein identification using top-down. *Mol Cell Proteomics*. 2012 Jun; 11(6):M111. 008524.
29. Messer AE, Jacques AM, Marston SB. Troponin phosphorylation and regulatory function in human heart muscle: dephosphorylation of Ser23/24 on troponin I could account for the contractile defect in end-stage heart failure. *J Mol Cell Cardiol*. 2007 Jan; 42(1):247–59. [PubMed: 17081561]
30. Kooij V, Holewinski RJ, Murphy AM, Van Eyk JE. Characterization of the cardiac myosin binding protein-C phosphoproteome in healthy and failing human hearts. *J Mol Cell Cardiol*. 2013 Jul; 60:116–20. [PubMed: 23619294]
31. Zhang P, Kirk JA, Ji W, dos Remedios CG, Kass DA, Van Eyk JE, et al. Multiple reaction monitoring to identify site-specific troponin I phosphorylated residues in the failing human heart. *Circulation*. Oct 9; 126(15):1828–37. [PubMed: 22972900]

32. DeGrande ST, Little SC, Nixon DJ, Wright P, Snyder J, Dun W, et al. Molecular mechanisms underlying cardiac protein phosphatase 2A regulation in heart. *J Biol Chem*. 2013 Jan 11; 288(2): 1032–46. [PubMed: 23204520]
33. Bouvagnet P, Mairhofer H, Leger JO, Puech P, Leger JJ. Distribution pattern of alpha and beta myosin in normal and diseased human ventricular myocardium. *Basic Res Cardiol*. 1989 Jan-Feb; 84(1):91–102. [PubMed: 2647075]
34. Marston SB, de Tombe PP. Troponin phosphorylation and myofilament Ca²⁺-sensitivity in heart failure: increased or decreased? *J Mol Cell Cardiol*. 2008 Nov; 45(5):603–7. [PubMed: 18691597]
35. Nixon BR, Liu B, Scellini B, Tesi C, Piroddi N, Ogut O, et al. Tropomyosin Ser-283 pseudo-phosphorylation slows myofibril relaxation. *Arch Biochem Biophys*. 2013 Jul 1; 535(1):30–8. [PubMed: 23232082]
36. Locher MR, Razumova MV, Stelzer JE, Norman HS, Moss RL. Effects of low-level α -myosin heavy chain expression on contractile kinetics in porcine myocardium. *Am J Physiol Heart Circ Physiol*. 2011 Mar; 300(3):H869–78. [PubMed: 21217059]
37. Agnetti G, Kane LA, Guarnieri C, Caldarera CM, Van Eyk JE. Proteomic technologies in the study of kinases: novel tools for the investigation of PKC in the heart. *Pharmacol Res*. 2007 Jun; 55(6): 511–22. [PubMed: 17548206]
38. Walden AP, Dibb KM, Trafford AW. Differences in intracellular calcium homeostasis between atrial and ventricular myocytes. *J Mol Cell Cardiol*. 2009 Apr; 46(4):463–73. [PubMed: 19059414]

Figure 1. Basal cTnI phosphorylation varies in a chamber-specific but not transmural manner in the heart

(A) Representative mass spectra for cTnI from the four chambers of the heart. Circles represent the theoretical isotopic abundance distribution of the isotopomer peaks corresponding to the assigned mass. Squares and stars represent oxidized cTnI and potassium adducts, respectively. (B) Graph showing the relative abundances of cTnI, p cTnI, and pp cTnI in the four chambers of the heart. (C) Graph showing total cTnI phosphorylation in the LV, RV, LA, and RA. (D) Representative mass spectra for cTnI from the three layers of the LV free wall. Stars represent potassium adducts. (E) Graph showing the relative

abundances of cTnI, p cTnI, and pp cTnI in the EPI, MYO, and ENDO of the LV free wall.

(F) Graph showing total cTnI phosphorylation in the three layers of the LV free wall. cTnI corresponds to NCBI RefSeq accession number ABF84065. $*p < 0.05$, $**p < 0.001$.

Figure 2. cTnI-Ser22/23 phosphorylation is significantly increased in the LV in comparison to the LA and RA, but does not vary across the LV free wall

(A) Representative Western blots for cTnI phosphorylated at Ser22/23 and total cTnI, as well as the associated quantification results for the chamber comparison, are shown in the upper and lower panels, respectively (n=3). (B) Representative Western blots for cTnI phosphorylated at Ser22/23 and total cTnI, as well as the associated quantification results for the LV layer comparison, are shown in the upper and lower panels, respectively (n=3). * $p < 0.05$.

Figure 3. cTnT phosphorylation does not vary in the different chambers of the heart or across the LV free wall

(A) Representative mass spectra for cTnT from the LV, RV, LA, and RA. Circles represent the theoretical isotopic abundance distribution of the isotopomer peaks corresponding to the assigned mass. Triangles and diamonds represent sodium and phosphoric acid (+H₃PO₄) adducts, respectively. (B) Graph showing the relative abundances of cTnT and p-cTnT in the four chambers of the heart. (C) Graph showing total cTnT phosphorylation in the LV, RV, LA, and RA. (D) Representative mass spectra for cTnT from the EPI, MYO, and ENDO of the LV free wall. Triangles and diamonds represent sodium and phosphoric acid (+H₃PO₄)

adducts, respectively. (E) Graph showing the relative abundances of cTnT and p cTnT across the layers of the LV free wall. (F) Graph showing total cTnT phosphorylation in the EPI, MYO, and ENDO. cTnT corresponds to NCBI RefSeq accession number ADY80031. $*p < 0.05$, $**p < 0.001$.

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Figure 4. α Tm phosphorylation exhibits chamber-specific but not transmural heterogeneity in the heart

(A) Representative mass spectra for Tpm from the LV, RV, LA, and RA. Circles represent the theoretical isotopic abundance distribution of the isotopomer peaks corresponding to the assigned mass. Squares represent oxidized Tpm. (B) Graph showing the relative abundances of α Tm, $p\alpha$ Tm, and β Tm in the four chambers of the heart. (C) Graph showing total α Tm phosphorylation in the LV, RV, LA, and RA. (D) Representative mass spectra for Tpm from the three layers of the LV free wall. Squares represent oxidized Tpm. (E) Graph showing the relative abundances of α Tm, $p\alpha$ Tm, and β Tm in the EPI, MYO, and ENDO. (F) Graph

showing total α Tm phosphorylation in the EPI, MYO, and ENDO. α Tm and β Tm correspond to NCBI RefSeq accession numbers NP_001090952 and XP_005660265, respectively. $*p < 0.05$, $**p < 0.001$.

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Figure 5. Identification of the atrial isoforms of MLC1a and MLC2a from pig
Fragmentation maps and tandem mass spectra for (A) MLC1a and (B) MLC2a. Fragmentation maps show the fragment ions that were matched to the sequences for MLC1a (53 *c* and 44 *z*⁺ ions total) and MLC2a (39 *c* and 49 *z*⁺ ions total) from *Sus scrofa* in the NCBI database. There were minor mass discrepancies of 28.04 Da and 42.01 Da localized to the N-termini of MLC1a and MLC2a, respectively. Bracket indicates removal of the N-terminal methionine. Zoomed-in spectra for representative fragment ions are shown for each protein. The P value represents the probability that the match between the spectrum and a

random protein has a similarity score no less than the score for the assigned match. Thus, the low P values obtained for MLC1a and MLC2a ($4.3\text{E-}53$ and $3.5\text{E-}54$ for MLC1a and MLC2a, respectively) translate to high confidence in the identification of these proteins. MLC1a and MLC2a correspond to NCBI RefSeq accession numbers XP_003131354 and XP_003134933, respectively.

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Figure 6. Different MLC1 isoforms are expressed in the atria and ventricles of pig heart
 Representative mass spectra for MLC1 from the LV, RV, LA, and RA. Square, star, triangle, and diamond represent oxidation, potassium adducts, sodium adducts, and phosphoric acid adducts ($+H_3PO_4$), respectively. Star and triangle combination represents MLC1 associated with both sodium and potassium. No phosphorylation was detected for MLC1. MLC1v corresponds to NCBI RefSeq accession number NP_001265702.

Figure 7. The phosphorylation of MLC2 does not vary in a chamber-specific or transmural manner in the heart

(A) Representative mass spectra for MLC2 from the LV, RV, LA, and RA. Circles represent the theoretical isotopic abundance distribution of the isotopomer peaks corresponding to the assigned mass. Squares, triangles, stars, and crosses represent oxidation, sodium adducts, potassium adducts, and a co-eluting protein, respectively. (B) Graph showing the relative abundances of MLC2 and p MLC2 in the four chambers of the heart. (C) Graph showing total MLC2 phosphorylation in the LV, RV, LA, and RA. (D) Representative mass spectra for MLC2v from the EPI, MYO, and ENDO of the LV free wall. (E) Graph showing the

relative abundances of MLC2v and p MLC2v across the layers of the LV free wall. (F) Graph showing total MLC2v phosphorylation the EPI, MYO, and ENDO. MLC2v corresponds to NCBI RefSeq accession number AAM47004. $*p < 0.05$, $**p < 0.001$.

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