



Exercise-responsive phosphoproteins in the heart



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ABSTRACT

Endurance exercise improves cardiac performance and affords protection against cardiovascular diseases but the signalling events that mediate these benefits are largely unexplored. Phosphorylation is a widely studied post-translational modification involved in intracellular signalling, and to discover novel phosphorylation events associated with exercise we have profiled the cardiac phosphoproteome response to a standardised exercise test to peak oxygen uptake (VO₂peak).

Male Wistar rats (346 ± 18 g) were assigned to 3 independent groups (n = 6, in each) that were familiarised with running on a motorised treadmill within a metabolic chamber. Animals performed a graded exercise test and were killed either immediately (0 h) after or 3 h after terminating the test at a standardised physiological end point (i.e. peak oxygen uptake; VO₂peak). Control rats were killed at a similar time of day to the exercised animals, to minimise possible circadian effects. Cardiac proteins were digested with trypsin and phosphopeptides were enriched by selective binding to titanium dioxide (TiO₂). Phosphopeptides were analysed by liquid chromatography and high-resolution tandem mass spectrometry, and phosphopeptides were quantified by MS1 intensities and identified against the UniProt knowledgebase using MaxQuant (data are available via ProteomeXchange, ID PXD006646).

The VO₂peak of rats in the 0 h and 3 h groups was 66 ± 5 ml kg⁻¹ min⁻¹ and 69.8 ± 5 ml kg⁻¹ min⁻¹, respectively. Proteome profiling detected 1169 phosphopeptides and one-way ANOVA found 141 significant (P < 0.05 with a false discovery rate of 10%) differences. Almost all (97%) of the phosphosites that were responsive to exercise are annotated in the PhosphoSitePlus database but, importantly, the majority of these have not previously been associated with the cardiac response to exercise. More than two-thirds of the exercise-responsive phosphosites were different from those identified in previous phosphoproteome profiling of the cardiac response to β₁-adrenergic receptor stimulation. Moreover, we report entirely new phosphorylation sites on 4 cardiac proteins, including S81 of muscle LIM protein, and identified 7 exercise-responsive kinases, including myofibrillar protein kinases such as obscurin, titin and the striated-muscle-specific serine/threonine kinase (SPEG) that may be worthwhile targets for future investigation.

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1. Introduction

Exercise has an irrefutable role in preventing heart failure and cardiac diseases, for example acute exercise has cardio-protective effects similar to ischaemic preconditioning [1] and chronic exercise training

Abbreviations: AR, adrenergic receptor; VCO₂, carbon dioxide production; ESI, electrospray ionization; FDR, false discovery rate; HCD, High-energy collision-induced dissociation; MS, mass spectrometry; VO₂, oxygen uptake; VO₂peak, peak oxygen uptake; MS/MS, tandem mass spectrometry; S, serine; TiO₂, titanium dioxide; T, threonine; Y, tyrosine.

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results in physiological cardiac hypertrophy [2] and a heart phenotype that affords protection against pathological insults such as ischaemia/reperfusion injury [3]. Although the physiological benefits of exercise are clear, less is known about the molecular mechanisms that underlie these effects. Yet greater molecular understanding could enable the benefits of exercise to be further optimised or personalised and could suggest new targets for more effective modes of diagnosis, prevention or rehabilitation of debilitating cardiac diseases.

Previous work has investigated discrete signalling events activated in response to exercise, for example in the context of acute cardiac preconditioning [1] or adaptive versus maladaptive cardiac hypertrophy [2]. The IGF-1 receptor/PI3K (p110α)/Akt1 pathway is perhaps the most well-explored regulatory pathway associated with exercise-induced cardiac hypertrophy but it is unlikely that a biological phenomenon as complex as cardiac growth is entirely mediated by a single pathway and more often integrated networks of molecules across

multiple pathways are required to achieve physiological adaptations to environmental stimuli [4]. Therefore, events outside of the canonical IGF-1R/PI3K (p110 α)/Akt1 pathway are likely to also contribute to exercise-induced cardiac adaptations and remain to be discovered.

Vigorous exercise is associated with significant elevations in cardiac work and myocardial contractility which are driven by the chronotropic and inotropic effects of beta-adrenergic receptor (AR) signalling (sympathetic drive) as well as local metabolic responses and mechanical strain. In addition to driving acute increases in cardiac output, the molecular events associated with exercise also instigate adaptive processes that alter the cardiac proteome [5] and increase the capacity for work (i.e. VO₂peak). Phosphorylation networks are recognised widely in the literature and are known to transduce signals involved in the skeletal muscle response to exercise in humans [6] but until now the cardiac phosphoproteome response to exercise has not been reported. Phosphoproteome profiling is a useful approach to discover the pathways and signalling events involved in physiological processes, and a key advantage of this technique is its non-targeted approach that it is not biased by preconceptions about which pathways or events may be of greatest importance.

Due to the implausibility of sampling human cardiac tissue in the context of exercise physiology, models are required that simulate exercise prescription in humans while allowing access to the heart for molecular investigation. The exercise stimulus is a composite of 3 inter-related variables, i.e. exercise intensity, duration and frequency, and the cardio-protective of exercise is intensity-dependent [1]. Therefore, to control and standardise exercise intensity we [5] have used indirect calorimetry and an incremental protocol of exercise on a motorised treadmill to measure peak oxygen uptake (VO₂peak) of rats in a manner that is equivalent to best practice in human studies (e.g. [7]). During the VO₂peak test the animal's respiratory gases are monitored and the test is terminated when the animal reaches its peak aerobic capacity (this intensity of exercise is attainable even by previously sedentary animals). By using this physiological end-point we minimise the influence of acute stress induced by an unrealistic exercise load. Such, standardisation is important because differences in exercise capacity exist even within a colony of animals exposed to identical environmental conditions. Therefore, exposure to the same relative exercise stimulus represents an optimised model with the best chances of successfully identifying the key regulatory networks that mediate exercise-induced adaptation.

2. Methods

2.1. Graded treadmill test of peak oxygen uptake

Experiments were conducted under the British Home Office Animals (Scientific Procedures) Act 1986 and according to UK Home Office guidelines. Male Wistar rats were bred in-house in a conventional colony and the environmental conditions controlled at 20 \pm 2 °C, 45–50% humidity with a 12-h light (1800–0600) and dark cycle. Water and food (containing 18.5% protein) were available ad libitum.

Exercise sessions were conducted during the animals' dark period. All rats (n = 18) completed a 14-day familiarization procedure encompassing daily bouts (15 min duration) at various belt speeds and inclines on a motorised treadmill within a metabolic chamber (Columbus Instruments, OH). On the 15th day the VO₂peak of animals (n = 12) assigned to the exercise groups was measured using an incremental test, as described previously [5,8]. Briefly, a warm-up (5 min running at 6 m min⁻¹, 0° incline) was completed followed by a series of 3 min stages of alternating increases in speed (increments of 2 m min⁻¹) and incline (increments of 5°; maximum incline 25°). Air pumped (2.5 l min⁻¹) through the chamber was analysed for concentrations of oxygen and carbon dioxide (Oxymax system; Columbus Instruments, OH; calibrated to an external standard) and a metal grid at the rear of the treadmill belt, which delivered a maximum of 3 electric

stimuli (0.1 mA, 0.3 s duration), was used to encourage the animals to achieve their VO₂peak. Independent groups (n = 6, in each) of animals were killed by cervical dislocation either immediately (0 h) after cessation of the exercise test or 3 h after completing the exercise test. Hearts were isolated from the exercised animals and from control rats (n = 6) that completed the familiarization training but did not perform an incremental exercise test. Hearts were rapidly isolated, cleaned and weighed before being stored at –80 °C. To minimise the influence of circadian differences, control rats were killed at a time of day coinciding with the incremental exercise test.

2.2. Sample preparation

Left ventricles were pulverized in liquid nitrogen and an accurately weighed portion (100 mg) homogenized on ice in 10 volumes of 8 M urea, 4% w/v CHAPS, 40 mM Tris base including protease and phosphatase inhibitor cocktails (Roche Diagnostics, Lewes, UK) at 4 °C. After centrifugation at 20,000g, 4 °C for 45 min the supernatant was decanted and the protein concentration measured using a modified 'microtitre plate' version of the Bradford assay (Sigma, Poole, Dorset, UK).

Aliquots containing 2 mg protein were reduced with 2.5 mM dithiothreitol for 1 h at room temperature then alkylated with 5 mM iodoacetamide for 45 min in the dark at room temperature. Samples were diluted with 50 mM ammonium bicarbonate to bring the concentration of urea to 1 M and sequencing-grade trypsin (Promega) was added at a substrate to enzyme ratio of 50:1. After 4 h, samples were diluted threefold with 50 mM ammonium bicarbonate containing additional trypsin, and the digestion was allowed to proceed overnight. After acidification to a final concentration of 1% (v/v) formic acid, the peptide solutions were desalted using disposable Tiptip C18 columns (Glygen) and lyophilized to dryness. Phosphopeptides were selectively enriched by binding to titanium dioxide (TiO₂)-coated magnetic beads (Pierce) according to the manufacturer's instructions, as described in previously [9]. Briefly, peptides were resuspended in 200 μ l 80% acetonitrile, 2% formic acid and incubated for 1 min with 10 μ l of slurry containing TiO₂ magnetic beads. Unbound peptides and supernatant were decanted and the beads were washed three times with 200 μ l binding buffer (supplied with the kit). After final decanting, the beads were incubated for 10 min with 30 μ l elution buffer and the eluate was carefully removed and dried prior to mass spectrometry analysis.

2.3. Mass spectrometry analysis

Tryptic peptide mixtures were analysed by nano-scale high-performance liquid chromatography (Proxeon EASY-Nano system) and online nano electrospray ionization (ESI) tandem mass spectrometry (LTQ-Orbitrap Velos mass spectrometer; Thermo Fisher Scientific). Samples were loaded in aqueous 0.1% (v/v) formic acid via a trap column constructed from 25 mm of 75 μ m i.d. silica capillary packed with 5 μ m Luna C18 stationary phase (Phenomenex). The analytical column was constructed in a 100 mm \times 75 μ m i.d. silica capillary packed with 3 μ m Luna C18 stationary phase. Mobile phase A, consisted of 5% acetonitrile and 0.1% formic acid, and organic phase B contained 95% acetonitrile and 0.1% formic acid. Reverse phase separation was performed over 120 min at a flow rate of 300 nl/min, rising to 6% B in 1 min then from 6% to 24% B over 89 min followed by a 16 min gradient to 100% B, which was held for 5 min prior to re-equilibration to 0% B over 9 min. Eluted peptides were sprayed directly in to an LTQ-Orbitrap Velos mass spectrometer using a nanospray ion source (Proxeon). Tandem mass spectrometry (MS/MS) was performed using high-energy collision-induced dissociation (HCD) and 10 MS/MS data-dependent scans (7500 resolution) were acquired in centroid mode alongside each profile mode full-scan mass spectra (30,000 resolution), as reported previously [9]. The automatic gain control (AGC) for MS scans was 1 \times 10⁶ ions with a maximum fill time of 250 ms. The AGC for MS/MS scans was 3 \times 10⁴, with 150 ms maximum injection time, 0.1 ms

activation time, and 40% normalized collision energy. To avoid repeated selection of peptides for MS/MS a dynamic exclusion list was enabled to exclude a maximum of 500 ions over 30 s.

2.4. Protein identification

Data files (RAW format) were searched using the standard workflow of MaxQuant (version 1.3.0.5; <http://maxquant.org/>) against a non-redundant rat protein sequence FASTA file from the UniProt/SwissProt database modified to contain porcine trypsin sequences. The search parameters allowed 2 missed cleavages, carbamidomethylation of cysteine (fixed) and variable oxidation of methionine, protein N-terminal acetylation and phosphorylation of STY residues. Precursor ion tolerances were 20 ppm for first search and 6 ppm for a second search. The MS/MS peaks were de-isotoped and searched using a 20 ppm mass tolerance. A stringent false discovery rate threshold of 1% was used to filter candidate peptide, protein, and phosphosite identifications. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [10] partner repository with the dataset identifier PXD006646.

2.5. Bioinformatic analysis

Raw intensities were extracted from the MaxQuant evidence files using an in-house Perl script. Briefly, the intensities from each biological replicate were collapsed to a specific phosphorylation site as opposed to a specific peptide. The residue number (e.g. S224 – phosphorylation on the 224th residue (serine) of the protein) was extracted from the FASTA file used for the original MaxQuant protein search and in any given biological replicate every intensity that can attributed to S224 is summed. If multiple phosphorylations exist on a peptide then the intensities are counted only for the multi-phosphorylation, i.e. single, double and multi phosphorylation become different entities and are scored accordingly. Phospho expression sets were normalized in R using quantile normalization in the limma package. Each modification was scored for differential expression using one-way analysis of variance (ANOVA) across the 3 different time points (control, 0 h and 3 h) complemented by independent t-tests of each pairwise comparison (i.e. 0 h vs control, and 3 h vs control). The false discovery rate (FDR) was assessed by calculating q values [11] from the P value distribution of the ANOVA outputs. Protein identifiers associated with statistically significant ($P < 0.05$, $FDR < 10\%$) exercise-responsive phosphopeptides were uploaded to David GO (<https://david.ncifcrf.gov>) [12,13] for functional annotation and association to KEGG pathways. Hierarchical clustering was performed using the Graphical Proteomics data Explorer (GProX) [14] and protein interactions were investigated using bibliometric mining in the search tool for the retrieval of interacting genes/proteins (STRING; <http://string-db.org/>) [15].

2.6. Western blot analyses

Immuno-detection of selected targets was performed using previously described [16] methods. Briefly, samples containing 50 µg protein were resolved by denaturing gel electrophoresis and transferred on to polyvinylidene difluoride membranes. Non-specific protein interactions were blocked by incubating the membranes with 5% non-fat dry milk in 20 mM Tris, 150 mM NaCl, and 0.1% Tween 20, pH 7.6 (TBS-T) for 1 h at room temperature. Membranes were then washed in TBS-T and incubated overnight with TBS-T containing 5% BSA and primary antibodies specific for: p38 mitogen activated protein kinase (p38 MAPK; 9212 Cell Signalling Technology; 1:1000 dilution) and phosphorylated (T180/Y182) p38 MAPK (9211 Cell Signalling Technology; 1:1000 dilution) or alpha B crystallin (CRYAB; ab13497 Abcam; 1:10,000 dilution) and phosphorylated (S59) CRYAB (ab5577 Abcam; 1:5000 dilution). Serial washes in TBS-T were per-formed prior to and after incubation with secondary antibodies (goat anti-rabbit IgG; ab205718 Abcam; 1:20,000

dilution) in 5% BSA in TBS-T for 2 h followed by enhanced chemiluminescence (ECL Prime; GE Healthcare) and digitization (Gel Doc XRS; Bio-Rad, Hercules, CA) of immuno-reactive protein bands. Image analysis (Quantity One, version 4; Bio-Rad) was used to measure the relative abundances of target proteins. Analysis of phosphorylated and non-phosphorylated species was achieved by stripping (incubation in 62.5 mM Tris, 70 mM SDS, 50 mM β-mercaptoethanol, pH 6.8 at 50 °C for 30 min) and re-probing of membranes.

3. Results

Three independent groups ($n = 6$, in each) of rats were used to investigate the time course of changes in the heart phosphoproteome in response to a standardised bout of endurance exercise. The body weight or heart weight of rats assigned to the control, 0 h and 3 h groups was similar and rats that performed the incremental exercise test (i.e. 0 h and 3 h groups) had equivalent peak exercise capacities (Table 1). An example of VO₂ VCO₂ traces recorded during an incremental exercise test is illustrated in Fig. 1. The average time to complete the incremental exercise test was 21 min and the average VO₂peak of animals in the 0 h and 3 h groups was $66 \pm 5 \text{ ml kg}^{-1} \text{ min}^{-1}$ and $69.8 \pm 5 \text{ ml kg}^{-1} \text{ min}^{-1}$, respectively.

LC-MS/MS profiled 1169 phosphopeptides and 841 singly phosphorylated peptides were detected and of these 11 were pY, 90 were pT and 840 were pS. There were also 289 doubly phosphorylated peptides, 30 triply phosphorylated and 10 peptides that had between 4 and 6 phosphorylated residues. One-way ANOVA found 141 peptide differences at $P < 0.05$, the false discovery rate (FDR) calculated from q values [11] was estimated to be 10%. Volcano plots are illustrated in Fig. 2 to highlight post-hoc analysis of phosphopeptides that differed between the control and 0 h group (Fig. 2A) or between control and 3 h group (Fig. 2B). Immediately after cessation of exercise similar numbers of phosphopeptides were increased and decreased in abundance compared to control. After 3 h recovery (Fig. 2B) the majority of phosphopeptides were more abundant in exercised hearts compared to control.

The 141 peptides that significantly differed in response to acute exercise mapped to 97 proteins, i.e. some proteins had more than one phosphopeptide. Examples of proteins that had multiple phosphorylated peptides include titin (10 peptides), tensin (5 peptides), Bcl2-interacting death suppressor (5 peptides), alpha-2-HS-glycoprotein (4 peptides), pyruvate dehydrogenase E1 component subunit alpha (4 peptides) and isoform 2 of NDRG2 protein (3 peptides).

Exercise-responsive phosphopeptides were uploaded to David GO for functional annotation and the top ranking significant ($P < 0.05$; Fischer with BH correction) KEGG pathways were arrhythmogenic right ventricular cardiomyopathy, cardiac muscle contraction and adrenergic signalling in cardiomyocytes.

Mapping to PhosphoSitePlus (<http://www.phosphosite.org>) found all but 4 (97%) of the identified phosphopeptides had previously been reported. The most commonly reported phosphosites matching to published high-throughput (MS2) data were pyruvate dehydrogenase E1 component subunit alpha S232 & S239, gap junction alpha-1 protein S325 & S328, septin-2 S218 and heat shock protein beta-1 S15. Approximately 28% (39 of 141) of the exercise-responsive phosphorylation

Table 1
Physical and physiological characteristics.

	Control	0 h	3 h
Body weight (g)	338 ± 16	350 ± 27	351 ± 9
Heart weight (mg)	1071 ± 44	1005 ± 76	1060 ± 40
VO ₂ peak (ml kg ⁻¹ min ⁻¹)		66 ± 5	69.8 ± 5
Peak RER		1.046 ± 0.03	1.021 ± 0.03
Time to completion (min)		21.3 ± 3.6	21.3 ± 3.1

Data are presented as mean ± SD ($n = 6$, in each group). There were no statistically significant ($P < 0.05$) differences between the groups for any of the variables measured.

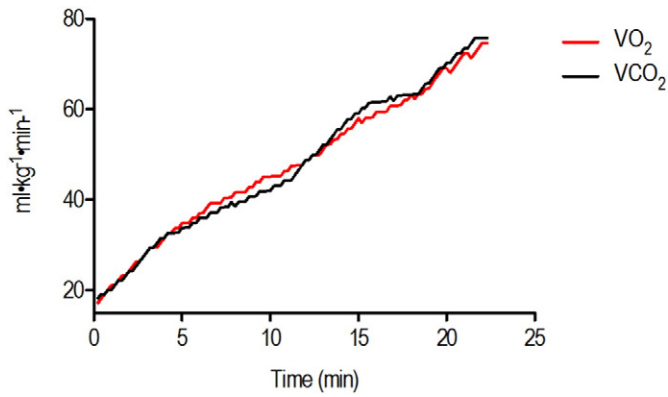


Fig. 1. Measurement of VO₂peak. Example oxygen uptake (VO₂) and carbon dioxide production (VCO₂) traces during an incremental exercise test designed to elicit peak oxygen uptake (VO₂peak).

sites were also associated with low-throughput experimental evidence in PhosphoSitePlus, including p38 mitogen-activated protein kinase Y182, cardiac phospholamban S16, alpha B-crystallin S59 and cardiac troponin I S23. Western blot analysis of phosphorylated and non-phosphorylated forms of p38 MAPK and alpha B crystallin (Fig. 3) verified statistically significant differences in the phosphorylation status of these proteins discovered by LC-MS/MS phosphopeptide profiling.

The time-series experimental design was used to provide further associational evidence between phosphorylation events and the cardiac exercise response. Hierarchical cluster analysis was performed in GProX to find similarities in the temporal patterns of exercise responsive phosphopeptides ($n = 141$, $P < 0.05$). The temporal responses in phosphopeptide abundance organised in to 3 prominent clusters (Fig. 4). Gene identifiers of exercise responsive phosphoproteins from each cluster were uploaded to STRING and Panels A, B, and C of Fig. 4 illustrate interaction networks within each cluster based on literature and database information, including co-expression, protein-protein interaction and literature mining.

4. Discussion

The mediators of exercise-induced cardiac adaptation have been less thoroughly investigated than the mechanisms of pathological cardiac maladaptation, but greater knowledge regarding the physiological responses of the heart could provide a valuable contrast to data from pathological models. To address this need, we performed phosphoproteomic profiling to generate new knowledge regarding the cardiac phosphoproteome response to exercise. To minimise potential misidentification of phosphorylation events that may be associated with a supra-physiological cardiac stress rather than the response to physiological exercise, the oxygen uptake (Fig. 1) of each animal was monitored and the exercise test was terminated at a standardised physiological end point (VO₂peak). We discovered entirely new phosphorylation sites on 4 cardiac proteins (Table 2), including S81 of muscle LIM protein, and identified 7 exercise-responsive kinases (Table 3). Almost all (97%) of the phosphosites that responded significantly to exercise (Supplementary Table S1) were annotated in the PhosphoSitePlus database but, importantly, the majority of these had not previously been associated with the cardiac response to exercise. Therefore the current data provides a rich source of new information relating to the potential mediators of exercise-induced cardiac protection.

Muscle LIM protein (MLP; also known as cysteine and glycine-rich protein 3) is an essential component of myogenic differentiation [17] and contains 2 LIM domains which facilitate protein-protein interactions. LIM domain containing proteins are important mediators of signals between the cytoskeleton and nucleus [18] and we discovered a new phosphorylation of S81 (significantly greater 3 h after exercise)

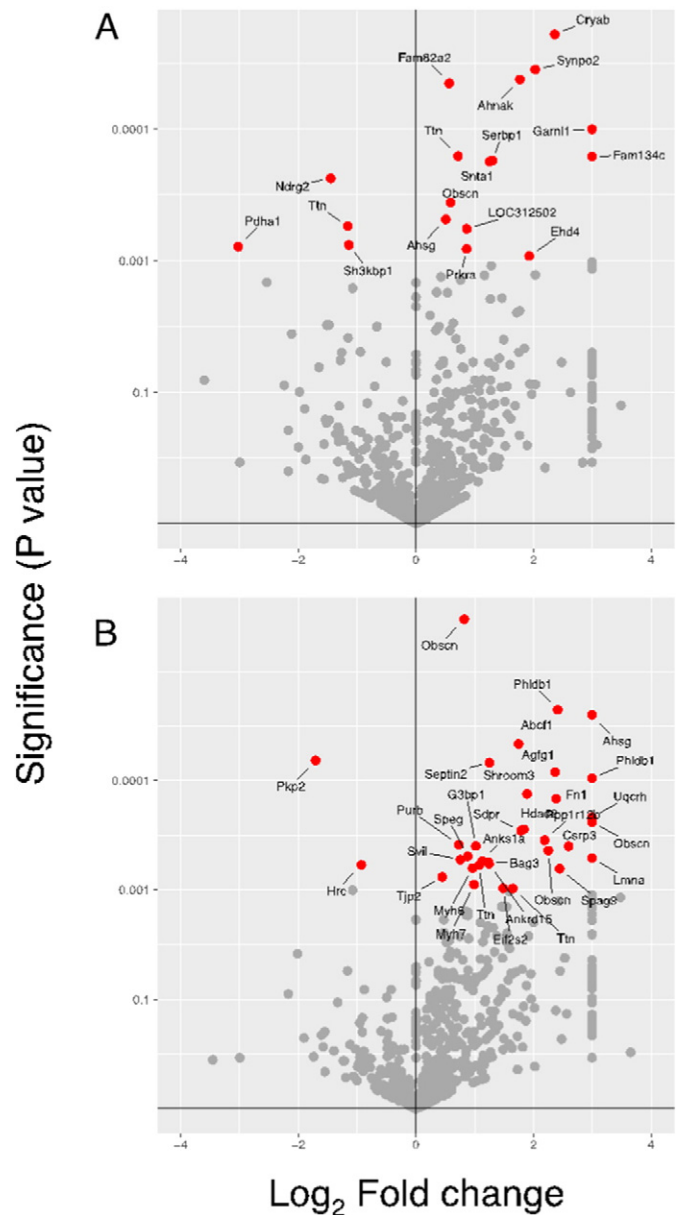


Fig. 2. Changes in the abundance of exercise responsive phosphopeptides. Volcano plots presenting the distribution of the fold-change (\log_2) in abundance and statistical significance of phosphorylated peptides. Post-hoc comparisons are shown for (A) non-exercised control hearts vs hearts isolated immediately (0 h) after cessation of the graded exercise test, or (B) non-exercised control hearts vs hearts isolated 3 h after cessation of the graded exercise test.

which lies within a flexible region between LIM domain 1 (residues 10–61) and LIM domain 2 (residues 120–171) of MLP and is close to a previously reported site (S95) that is phosphorylated during beta-1 AR stimulation [19]. Other phosphorylation sites of rat MLP include S111 and S153 but phosphorylation/de-phosphorylation of these sites has not yet been linked to environmental stimuli or cell signalling processes. MLP can interact with a number of myogenic factors [20] and also proteins at the myofibril z-disc, including alpha-actinin [21], beta-spectrin [22] and the titin capping protein, telethonin/TCAP [23]. Translocation of MLP from the sarcomere to the nucleus is facilitated by a nuclear localisation signal (residues 64–69) and inhibition of MLP nuclear translocation prevents the protein synthetic response to cyclic strain in cardiomyocytes [24].

We speculate MLP may also be involved in transducing signals in response to exercise *in vivo* and the novel S81 phosphorylation reported

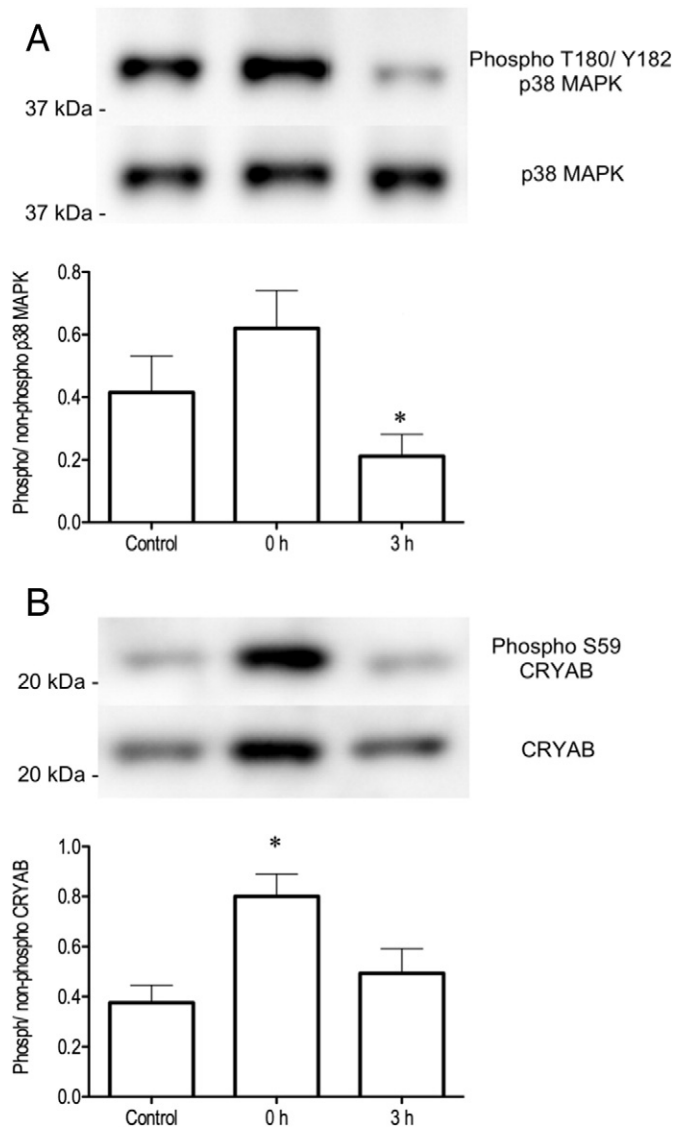


Fig. 3. Exercise responsive phosphorylation of cardiac p38 MAPK and CRYAB. Western blot analysis of the ratio of phosphorylated: non-phosphorylated p38 mitogen activated kinase (p38 MAPK; A) and alpha B crystallin (CRYAB; B). Cropped images of 3 representative lanes from a single animal from the control, 0 h and 3 h groups are shown. Data are presented as mean \pm SEM ($n = 6$, per group) and statistical significance (* $P < 0.05$ different from control group) was determined by one-way analysis of variance and Tukey HSD post-hoc analysis.

here may influence the protein-protein interactions and subcellular localisation of MLP. The amino acid sequence flanking S81 of MLP (Table 1) does not match the linear motifs recognised by well-defined protein kinases, but our phosphoproteome profiling identified a selection of exercise-responsive myofibrillar protein kinases (Tables 2 and 3) that could be potential mediators of MLP S81 phosphorylation at the z-disc. Two novel exercise-induced phosphorylation events (Table 2) were discovered on myofibrillar protein kinases (myosin light chain kinase 3 and obscurin) and may be involved in the transduction of mechanical signals within the exercised heart. Myosin light chain kinase 3 is responsible for the phosphorylation of ventricular regulatory myosin light chain, which contributes to the enhancement of myocardial contractility [25] and we report novel S444 phosphorylation of myosin light chain kinase 3 occurs during vigorous exercise (Cluster 1).

Obscurin is also a member of the myosin light chain kinase family along with striated muscle-specific serine/threonine kinase (SPEG; Table 3) and these kinases are predicted to target similar conserved sites [26] and may be involved in the hypertrophic response of the

heart [27]. In exercised hearts, we discovered greater phosphorylation of obscurin S2974, which has not previously been reported, and phosphorylation of SPEG S2410 & S2414 that was reported [19] in phosphoproteome profiling of the cardiac response to β_1 -adrenergic receptor (AR) stimulation. Phosphorylation of SPEG has also recently been reported [28] in phosphoproteome analysis of mouse skeletal muscle submitted to a bout of maximal isometric contractions. These independent discoveries of SPEG phosphorylation using non-targeted techniques provide reciprocal verification and further highlight SPEG as an exercise-responsive phosphoprotein/kinase of interest for future mechanistic study. Phosphorylation of the giant myofibrillar protein kinase, titin, was also detected after exercise (Table 3) and each of the titin phosphorylation sites reported here (Supplementary Table S1) is also known to be responsive to β_1 -AR stimulation. Taken together, our data describe a collection of myofibrillar protein kinases and phosphorylation events associated with the z-disc region that are responsive to exercise and warrant further investigation as mediators of exercise-induced cardiac adaptation.

Exercise training has protective effects against cardiomyocyte death and proteins that interact with Bcl-2 family members involved in the regulation of apoptosis and autophagy were enriched amongst the exercise-responsive phosphoproteome. We discovered new phosphorylation sites (T93 and Y94; Table 1) on Bcl-2 interacting killer-like protein (Bik) which became significantly more phosphorylated 3 h after cessation of exercise. These sites are different to the previously reported ERK1/2 mediated phosphorylation of Bik at T124 that is associated with ubiquitination and subsequent degradation of Bik [29] and represent new targets for further exploration. Phosphorylation of BCL2/adenovirus E1B 19 kDa-interacting protein 3 (BNIP3) was increased after exercise and this protein has been implicated in the regulation of both apoptosis and mitophagy [30] in a manner similar to the better characterised protein Beclin-1 [31]. In addition, exercise was associated with phosphorylation of Bcl-2-interacting death suppressor (Bag3) on sites (S176, S277, S278, S377, S387) previously reported in response to beta-adrenergic receptor stimulation [19]. Bag3 is a co-chaperone of heat shock cognate 70 (hsc70), interacts with heat shock protein 22 and regulates the interaction with poly-glutamate (Poly-Q) proteins which are prone to aggregation. Phosphorylation of S397 of Bcl-2 associated transcription factor 1 (BCLAF1) increased after cessation of the exercise (Cluster 3) and this protein is required for efficient DNA repair and genome stability [32]. Together our findings describe an unappreciated network of responses in proteins that regulate apoptosis and autophagy processes, beyond the more widely reported effector proteins such as Bcl-2 and Bax.

During exercise myocardial contractility increases to meet the greater demand for cardiac output and this response is in part driven by β -AR signalling. Approximately one-third (41 of 141 phosphopeptides) of the exercise-responsive phosphopeptides were previously identified in similar phosphoproteome profiling [19] of the cardiac response to β_1 -AR stimulation, including PKA and archetypal proteins involved in myocardial contractility/ Ca^{2+} -handling and metabolism. For example, ryanodine receptor phosphorylation increased during exercise (Fig. 4, Cluster 1) and this has previously been associated with augmentation of intracellular calcium release and enhanced myocardial contractility [33]. The SERCA inhibitor, phospholamban, was phosphorylated at S16, which is noted to be sufficient for a maximal cardiac response to β -AR stimulation [34], and in addition, we report phosphorylation of lesser-known proteins such as histidine-rich calcium binding protein that also regulates SR calcium release [35]. With regard to metabolism, exercise increased S694 phosphorylation of phosphorylase kinase beta (Table 3) which is responsible for phosphorylation of glycogen phosphorylase and therefore acceleration of glycogenolysis. The monocarboxylate transporter 1 (Slc161a) was also phosphorylated at β_1 -AR responsive site immediately after exercise and this may be associated the transport lactate or ketones in to cardiac muscle cells. Conversely, phosphorylation of the pyruvate dehydrogenase E1 complex subunit

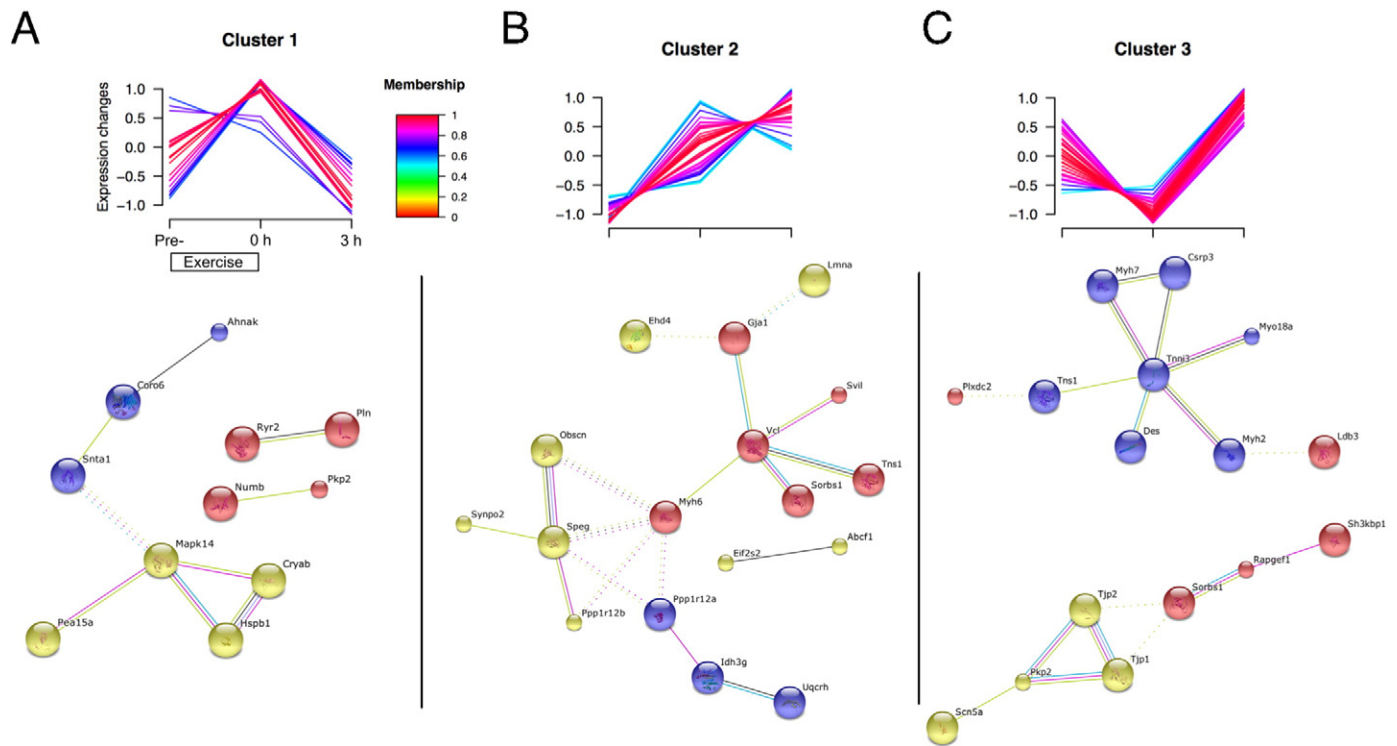


Fig. 4. Hierarchical clustering of exercise responsive phosphopeptides. Unsupervised hierarchical clustering was performed on 141 phosphopeptides that exhibited statistically significant ($P < 0.05$) differences across time by one-way ANOVA. Known and predicted interactions between proteins within each cluster were then investigated using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING). (A) Cluster 1 contains phosphopeptides whose abundance significantly increased immediately after exercise and then returned to basal levels within 3 h after cessation of the exercise test; this cluster included phosphorylation of phospholamban (Pln) and a network of p38 α (MAPK14) stress-responsive proteins including alpha B-crystallin (Cryab) and heat shock 27 kDa protein (Hspb1). (B) Cluster 2 contains phosphopeptides whose abundance increased immediately after exercise and further increased 3 h after cessation of the exercise test; this cluster included phosphorylation of costamere and gap junction proteins such as vinculin (Vcl) and connexin 43 (Gja1). In addition, ribosomal proteins, such as eukaryotic initiation factor 2 (eIF2s2) and ATP binding cassette sub-family F member 1 (Abcf1), which regulate both cap-dependent and independent translation were phosphorylated in response to exercise. (C) Cluster 3 contains phosphopeptides whose abundance decreased immediately after exercise and then returned to basal levels within 3 h after cessation of the exercise test; this cluster included phosphorylation of myofibrillar proteins, including muscle LIM protein (Csrp3).

alpha (Pdha1) is associated with inhibition of pyruvate entry to the TCA cycle and was increased 3 h after the cessation of exercise (Fig. 4, Cluster 3) and may be more associated with restoration of cardiac glycogen stores. Notably, phosphorylation sites reported here in response to exercise and by Lundby et al. [19] in response to β_1 -AR stimulation do not entirely overlap, and even after taking in to account potential technical differences between the 2 studies, it is evident that the cardiac exercise response is not entirely driven by β_1 -AR stimulation.

Cardiac β_1 -AR stimulation is associated with the activation of p38 MAP kinase [19] and this was also detected in response to exercise (Table 3 and Fig. 3A). Previous [36] targeted (western blot) analysis of signalling proteins in hearts of high- and low-capacity runner rats isolated 10 min after performing a ramped treadmill test measured a 1.6-fold increase in p38 MAPK (T180/Y182) phosphorylation, which is corroborated by our data (Fig. 3A). We further show Y182-specific phosphorylation of p38 MAPK (measured by LC-MS; Supplementary Table S1) is transient and was not significantly different from control 3 h after exercise. Moreover the change in p38 MAPK phosphorylation clusters with the phosphorylation of proteins including alpha B-crystallin,

Table 2
New site-specific phosphorylation sites discovered in cardiac proteins.

Cluster	Protein name	UniProt	Residue (\pm 7)	Sequence
1	Myosin light chain kinase 3	E9PT87	S444	TEAGR RVSpSAAEAAI
2	Obscurin	A0A0G2K8N1	S2974	LGLTSKASpLKDSGEY
3	Cysteine and glycine-rich protein 3	P50463	S81	GQGAGCLSpTDTGEHL
3	Bcl2-interacting killer-like protein	Q925D2	T93 & Y94	MHRLAATpYpSQTGVR

heat shock protein 27 and astrocytic phosphoprotein PEA-15 (Fig. 4; Cluster 1). Astrocytic phosphoprotein PEA-15 modulates the localisation and activity of ERK 1/2 MAP Kinases (MAPK1 and MAPK3), phosphorylation of PEA-15 at both S104 and S106 is necessary and sufficient to prevent its interaction with ERK 1/2 whereas non-phosphorylated PEA-15 blocks the nuclear translocation and transcriptional capacity of ERK 1/2 [37]. In the current work PEA-15 was phosphorylated at S104 only, but nonetheless the exercise-responsive phosphoproteome was enriched for proteins involved in ERK1/2 mitogen-activated protein kinases pathway and approximately 18% (25 of 141) of the cardiac phosphorylation sites reported here have previously

Table 3
Phosphorylated kinase enzymes.

Cluster	Protein name	UniProt	Residue
1	Myosin light chain kinase 3	E9PT87	S444
1	p38 mitogen-activated protein kinase	Q56A33	Y182
1	Phosphorylase kinase beta	Q5RKH5	S694
1	Titin	Q9JHQ1	S402
1	Titin	Q9JHQ1	S1990
2	cAMP-dependent protein kinase	P09456	S77 & S83
2	Obscurin	A0A0G2K8N1	S2974
2	Striated muscle specific serine/threonine kinase	Q63638	S2410 & S2414
2	Titin	Q9JHQ1	S256 & T267
2	Titin	Q9JHQ1	S32863
3	cAMP-dependent protein kinase	P09456	S83
3	Titin	Q9JHQ1	T300 & S302
3	Titin	Q9JHQ1	S1332 & S1336

been identified as ERK1/2 targets by phosphoproteomic analysis of epithelia cells [38].

MEK1-ERK1/2 signalling can inhibit Calcineurin-NFAT signalling which is strongly implicated in pathological cardiac hypertrophy [39]. Given the large degree of cross-talk between these pathways more intricate studies are needed to decipher the networks of interactions associated with pathological versus physiological cardiac adaptations. For example, the role of currently lesser known components such as Cyma5 costamere protein, which was phosphorylated in response to exercise and is a negative regulator of calcineurin-NFAT signalling cascade [39], will need to be integrated with the existing canonical pathways.

The IGF-1 receptor/PI3K (p110 α)/Akt1 pathway is the most thoroughly studied signalling pathway associated with exercise-induced cardiac adaptation and is associated with Akt S473 phosphorylation [40]. We found no significant change in Akt S473 phosphorylation after an acute bout of treadmill running which is consistent with previous [36] findings and suggests a single exercise bout is not sufficient to instigate the IGF-1 receptor signalling in the heart. Nonetheless, acute exercise was associated with phosphorylation of direct regulators of ribosomal translation such as eukaryotic initiation factors eIF2 and eIF5. The interaction between eIF-5B and eIF2 β is essential for GTP hydrolysis and release of eIF2-GDP from the 40 S initiation complex and the formation of the 80 S ribosome. Phosphorylation of eIF2 clustered with ATP-binding cassette sub-family F member 1 (ABCF1) and this interaction (including S109 phosphorylation of ABCF1) has previously been reported to be necessary in both cap-dependent and independent translation [41]. Therefore our findings draw attention to regulators of ribosomal translation initiation that have largely been ignored in previous exercise-related studies.

A single bout of exercise can precondition the heart against I/R damage [1] and gap junction proteins could be a key mechanism underlying this protective effect [42]. Gap junction alpha-1 protein (Cx43) is the main component of gap junctions in the ventricular myocardium and phosphorylation of S325, S328 and T326 of Cx43 increased 3 h after exercise. Cx43 has a short (<5 h) half-life and phosphorylation is required for gap junction formation whereas de-phosphorylation is associated with the disassembly of the gap junction and Cx43 degradation [43]. Phosphorylation at 325, 328 and 330 reported here may be mediated by casein kinase 1 [44] or fibroblast growth factor [45] and regulate gap junction assembly [46]. In contrast, Cx43 S262 phosphorylation has more commonly been associated with cardiac preconditioning mediated via PKC [47] and was not responsive to exercise. Therefore the current findings highlight a novel exercise-induced mechanism involving gap-junction assembly/turnover separate from those involved in ischaemic preconditioning. In addition, phosphorylation of CX43 co-occurred with the phosphorylation of tight junction protein 2, Palkophilin-2 and the alpha subunit of the voltage-gated sodium channel (Fig. 4, Cluster 3), which have previously been reported as interaction partners.

5. Summary

Signal transduction is a dynamic process and we used a time-series design to dissect immediate/early events such as phospholamban phosphorylation (Fig. 4; Cluster 1), which may be more associated with myocardial contractility, from sustained (Fig. 4; Cluster 2) or latter (Fig. 4; Cluster 3) phosphorylation events that may be more associated with the adaptive response to exercise or the restoration of cardiac homeostasis. Non-targeted analysis detected well established phosphorylation events associated with myocardial contractility while simultaneously detecting new site-specific phosphorylation events on proteins that are not shared with the cardiac response to β_1 -AR stimulation and have not previously been associated with the cardiac exercise response. In particular, we discovered new phosphorylation sites on 4 cardiac proteins (Table 2), including S81 of muscle LIM protein, and identified a

selection of myofibrillar protein kinases that were also responsive to exercise and may constitute a putative network of signal transduction for the adaptation to mechanical work in the heart.

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Disclosures

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References

- [1] C.R. Frasier, R.L. Moore, D.A. Brown, Exercise-induced cardiac preconditioning: how exercise protects your achy-breaky heart, *J. Appl. Physiol.* 111 (2011) 905–915.
- [2] B.C. Bernardo, J.R. McMullen, Molecular aspects of exercise-induced cardiac remodeling, *Cardiol. Clin.* 34 (2016) 515–530.
- [3] S.K. Powers, J.C. Quindry, A.N. Kavazis, Exercise-induced cardioprotection against myocardial ischemia-reperfusion injury, *Free Radic. Biol. Med.* 44 (2008) 193–201.
- [4] U.S. Bhalla, R. Iyengar, Emergent properties of networks of biological signaling pathways, *Science* 283 (1999) 381–387.
- [5] J.G. Burniston, Adaptation of the rat cardiac proteome in response to intensity-controlled endurance exercise, *Proteomics* 9 (2009) 106–115.
- [6] N.J. Hoffman, B.L. Parker, R. Chaudhuri, K.H. Fisher-Wellman, M. Kleinert, S.J. Humphrey, et al., Global phosphoproteomic analysis of human skeletal muscle reveals a network of exercise-regulated kinases and AMPK substrates, *Cell Metab.* 22 (2015) 922–935.
- [7] K.V. Holloway, M. O’Gorman, P. Woods, J.P. Morton, L. Evans, N.T. Cable, et al., Proteomic investigation of changes in human vastus lateralis muscle in response to interval-exercise training, *Proteomics* 9 (2009) 5155–5174.
- [8] J.G. Burniston, Changes in the rat skeletal muscle proteome induced by moderate-intensity endurance exercise, *Biochim. Biophys. Acta* 1784 (2008) 1077–1086.
- [9] H. Guo, R. Isserlin, X. Chen, W. Wang, S. Phanse, P.W. Zandstra, et al., Integrative network analysis of signaling in human CD34+ hematopoietic progenitor cells by global phosphoproteomic profiling using tio2 enrichment combined with 2D LC-MS/MS and pathway mapping, *Proteomics* 13 (2013) 1325–1333.
- [10] J.A. Vizcaino, A. Csordas, N. del Toro, J.A. Dianes, J. Griss, I. Lavidas, et al., Update of the PRIDE database and its related tools, *Nucleic Acids Res.* 44 (2016) (2016) D447–56.
- [11] J.D. Storey, R. Tibshirani, Statistical significance for genome-wide studies, *Proc. Natl. Acad. Sci. U. S. A.* 100 (2003) 9440–9445.
- [12] D.W. Huang, B.T. Sherman, R.A. Lempicki, Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources, *Nat. Protoc.* 4 (2009) 44–57.
- [13] D.W. Huang, B.T. Sherman, R.A. Lempicki, Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists, *Nucleic Acids Res.* 37 (2009) 1–13.
- [14] K.T. Rigbolt, J.T. Vanselow, B. Blagoev, GProX, a user-friendly platform for bioinformatics analysis and visualization of quantitative proteomics data, *Mol. Cell. Proteomics* 10 (2011) O110.007450.
- [15] A. Franceschini, D. Szklarczyk, S. Frankild, M. Kuhn, M. Simonovic, A. Roth, et al., STRING v9.1: protein-protein interaction networks, with increased coverage and integration, *Nucleic Acids Res.* 41 (2013) D808–15.
- [16] J.G. Burniston, J. Kenyani, D. Gray, E. Guadagnin, L.H. Jarman, J.N. Cobley, et al., Conditional independence mapping of DIGE data reveals PDIA3 protein species as key nodes associated with muscle aerobic capacity, *J. Proteome* 106C (2014) 230–245.
- [17] S. Arber, G. Halder, P. Caroni, Muscle LIM protein, a novel essential regulator of myogenesis, promotes myogenic differentiation, *Cell* 79 (1994) 221–231.
- [18] J.L. Kadmas, M.C. Beckerle, The LIM domain: from the cytoskeleton to the nucleus, *Nat. Rev. Mol. Cell Biol.* 5 (2004) 920–931.
- [19] A. Lundby, M.N. Andersen, A.B. Steffensen, H. Horn, C.D. Kelstrup, C. Francavilla, et al., In vivo phosphoproteomics analysis reveals the cardiac targets of β -adrenergic receptor signaling, *Sci. Signal.* 6 (2013) rs11.
- [20] Y. Kong, M.J. Flick, A.J. Kudla, S.F. Konieczny, Muscle LIM protein promotes myogenesis by enhancing the activity of myod, *Mol. Cell. Biol.* 17 (1997) 4750–4760.
- [21] C. Geier, A. Perrot, C. Özcelik, P. Binner, D. Counsell, K. Hoffmann, et al., Mutations in the human muscle LIM protein gene in families with hypertrophic cardiomyopathy, *Circulation* 107 (2003) 1390–1395.
- [22] M.J. Flick, S.F. Konieczny, The muscle regulatory and structural protein MLP is a cytoskeletal binding partner of beta1-spectrin, *J. Cell Sci.* 113 (Pt. 9) (2000) 1553–1564.
- [23] R. Knöll, M. Hoshijima, H.M. Hoffman, V. Person, I. Lorenzen-Schmidt, M.L. Bang, et al., The cardiac mechanical stretch sensor machinery involves a Z disc complex that is defective in a subset of human dilated cardiomyopathy, *Cell* 111 (2002) 943–955.
- [24] S.Y. Boateng, S.E. Senyo, L. Qi, P.H. Goldspink, B. Russell, Myocyte remodeling in response to hypertrophic stimuli requires nucleocytoplasmic shuttling of muscle LIM protein, *J. Mol. Cell. Cardiol.* 47 (2009) 426–435.

- [25] T. Kampourakis, Y.B. Sun, M. Irving, Myosin light chain phosphorylation enhances contraction of heart muscle via structural changes in both thick and thin filaments, *Proc. Natl. Acad. Sci. U. S. A.* 113 (2016) E3039–47.
- [26] S.B. Sutter, M.O. Raeker, A.B. Borisov, M.W. Russell, Orthologous relationship of obscurin and unc-89: phylogeny of a novel family of tandem myosin light chain kinases, *Dev. Genes Evol.* 214 (2004) 352–359.
- [27] A.B. Borisov, S.B. Sutter, A. Kontrogianni-Konstantopoulos, R.J. Bloch, M.V. Westfall, M.W. Russell, Essential role of obscurin in cardiac myofibrillogenesis and hypertrophic response: evidence from small interfering rna-mediated gene silencing, *Histochem. Cell Biol.* 125 (2006) 227–238.
- [28] G. Potts, R. McNally, R. Blanco, J. You, A. Herbert, M. Westphal, et al., A map of the phosphoproteomic alterations that occur after a bout of maximal-intensity contractions, *J. Physiol.* 595 (15) (2017) 5209–5226.
- [29] J. Lopez, C. Hesling, J. Prudent, N. Popgeorgiev, R. Gadet, I. Mikaelian, et al., Src tyrosine kinase inhibits apoptosis through the erk1/2-dependent degradation of the death accelerator bik, *Cell Death Differ.* 19 (2012) 1459–1469.
- [30] S.C. Choe, A. Hamacher-Brady, N.R. Brady, Autophagy capacity and sub-mitochondrial heterogeneity shape bnip3-induced mitophagy regulation of apoptosis, *Cell Commun. Signal* 13 (2015) 37.
- [31] Y. Maejima, M. Isobe, J. Sadoshima, Regulation of autophagy by beclin 1 in the heart, *J. Mol. Cell. Cardiol.* 95 (2016) 19–25.
- [32] K.I. Savage, J.J. Gorski, E.M. Barros, G.W. Irwin, L. Manti, A.J. Powell, et al., Identification of a brca1-mrna splicing complex required for efficient DNA repair and maintenance of genomic stability, *Mol. Cell* 54 (2014) 445–459.
- [33] S.O. Marx, S. Reiken, Y. Hisamatsu, T. Jayaraman, D. Burkhoff, N. Rosemblit, A.R. Marks, PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): defective regulation in failing hearts, *Cell* 101 (2000) 365–376.
- [34] G. Chu, J.W. Lester, K.B. Young, W. Luo, J. Zhai, E.G. Kranias, A single site (ser16) phosphorylation in phospholamban is sufficient in mediating its maximal cardiac responses to β -agonists, *J. Biol. Chem.* 275 (2000) 38938–38943.
- [35] D.A. Arvanitis, E. Vafiadaki, D. Sanoudou, E.G. Kranias, Histidine-rich calcium binding protein: the new regulator of sarcoplasmic reticulum calcium cycling, *J. Mol. Cell. Cardiol.* 50 (2011) 43–49.
- [36] C.J. Hunter, L.G. Koch, S.L. Britton, M.O. Boluyt, Initial signaling response to acute exercise bout is similar in hearts of rats bred for divergent exercise capacities, *Front. Biosci.* 13 (2008) 347–355.
- [37] J. Krueger, F.L. Chou, A. Glading, E. Schaefer, M.H. Ginsberg, Phosphorylation of phosphoprotein enriched in astrocytes (PEA-15) regulates extracellular signal-regulated kinase-dependent transcription and cell proliferation, *Mol. Biol. Cell* 16 (2005) 3552–3561.
- [38] M. Courcelles, C. Frémin, L. Voisin, S. Lemieux, S. Meloche, P. Thibault, Phosphoproteome dynamics reveal novel ERK1/2 MAP kinase substrates with broad spectrum of functions, *Mol. Syst. Biol.* 9 (2013) 669.
- [39] J.D. Molkenint, Calcineurin-NFAT signaling regulates the cardiac hypertrophic response in coordination with the mapks, *Cardiovasc. Res.* 63 (2004) 467–475.
- [40] K.L. Weeks, X. Gao, X.-J. Du, E.J. Boey, A. Matsumoto, B.C. Bernardo, et al., PI3K(p110 α) is a master regulator of exercise-induced cardioprotection and PI3K gene therapy rescues cardiac dysfunction, *Circ. Heart Fail.* 5 (2012) 523–534.
- [41] S. Paytubi, X. Wang, Y.W. Lam, L. Izquierdo, M.J. Hunter, E. Jan, et al., ABC50 promotes translation initiation in mammalian cells, *J. Biol. Chem.* 284 (2009) 24061–24073.
- [42] M.M. Jeyaraman, W. Srisakuldee, B.E. Nickel, E. Kardami, Connexin43 phosphorylation and cytoprotection in the heart, *Biochim. Biophys. Acta Biomembr.* 1818 (2012) 2009–2013.
- [43] J.L. Solan, P.D. Lampe, Key connexin 43 phosphorylation events regulate the gap junction life cycle, *J. Membr. Biol.* 217 (2007) 35–41.
- [44] C.D. Cooper, P.D. Lampe, Casein kinase 1 regulates connexin-43 gap junction assembly, *J. Biol. Chem.* 277 (2002) 44962–44968.
- [45] T. Sakurai, M. Tsuchida, P.D. Lampe, M. Murakami, Cardiomyocyte FGF signaling is required for cx43 phosphorylation and cardiac gap junction maintenance, *Exp. Cell Res.* 319 (2013) 2152–2165.
- [46] P.D. Lampe, C.D. Cooper, T.J. King, J.M. Burt, Analysis of connexin43 phosphorylated at S325, S328 and S330 in normoxic and ischemic heart, *J. Cell Sci.* 119 (2006) 3435–3442.
- [47] A.A. Waza, K. Andrabi, M.U. Hussain, Protein kinase C (PKC) mediated interaction between connexin43 (cx43) and K⁺(ATP) channel subunit (kir6.1) in cardiomyocyte mitochondria: implications in cytoprotection against hypoxia induced cell apoptosis, *Cell. Signal.* 26 (2014) 1909–1917.