Proteomic and phosphoproteomic profiling in heart failure with preserved ejection fraction (HFPeF)

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Although the prevalence of heart failure with preserved ejection fraction (HFPeF) is increasing, evidence-based therapies for HFPeF remain limited, likely due to an incomplete understanding of this disease. This study sought to identify the cardiac-specific features of protein and phosphoprotein changes in a murine model of HFPeF using mass spectrometry. HFPeF mice demonstrated moderate hypertension, left ventricle (LV) hypertrophy, lung congestion and diastolic dysfunction. Proteomics analysis of the LV tissue showed that 897 proteins were differentially expressed between HFPeF and Sham mice. We observed abundant changes in sarcomeric proteins, mitochondrial-related proteins, and NAD-dependent protein deacetylase sirtuin-3 (SIRT3). Upregulated pathways by GSEA analysis were related to immune modulation and muscle contraction, while downregulated pathways were predominantly related to mitochondrial metabolism. Western blot analysis validated SIRT3 downregulated cardiac expression in HFPeF vs. Sham (0.8 ± 0.0 vs. 1.0 ± 0.0; P < 0.001). Phosphoproteomics analysis showed that 72 phosphosites were differentially regulated between HFPeF and Sham LV. Aberrant phosphorylation patterns mostly occurred in sarcomere proteins and nuclear-localized proteins associated with contractile dysfunction and cardiac hypertrophy. Seven aberrant phosphosites were observed at the z-disk binding region of titin. Additional agarose gel analysis showed that while total titin cardiac expression remained unaltered, its stiffer N2B isoform was significantly increased in HFPeF vs. Sham (0.144 ± 0.01 vs. 0.127 ± 0.01; P < 0.05). In summary, this study demonstrates marked
changes in proteins related to mitochondrial metabolism and the cardiac contractile apparatus in HFpEF. We propose that SIRT3 may play a role in perpetuating these changes and may be a target for drug development in HFpEF.

**KEYWORDS**

HFpEF – heart failure with preserved ejection fraction, proteomics, phosphoproteomics, titin, mitochondria, metabolism, SIRT3

### Introduction

Heart failure (HF) is a clinical syndrome caused by abnormalities in the heart that limit its ability to fill or eject blood (1). Heart failure with preserved ejection fraction (HFpEF) is asymptomatic clinical HF where left ventricular (LV) ejection fraction (EF) is preserved (LVEF ≥ 50%), and presently accounts for about 50% all HF clinical presentations. However, unlike HF with reduced EF (HFrEF), where LVEF is < 50%, there are limited evidence-based therapies for HFpEF (2–4). In addition to its escalating prevalence, HFpEF morbidity (5) and mortality (6) continues to increase. Central to HFpEF is the involvement of both cardiac and extra-cardiac abnormalities (7, 8). In contrast to HFrEF, HFpEF is highly associated with comorbidities and as such is a heterogenous multisystem disorder involving the heart, pulmonary, renal, adipose tissue, skeletal muscle, immune/inflammatory signaling and the vascular system (9, 10). Patients with HFpEF are generally older, more often female and have a predominance of comorbidities, such as hypertension, obesity, type 2 diabetes, atrial fibrillation, renal dysfunction, etc. (11, 12). However, the specific etiologies by which patients develop HFpEF are variable. Thus, a precision-based approach is needed to identify pathogenic mechanisms in HFpEF (10, 13).

Proteomic studies are powerful tools that allow for large-scale characterization of the entire protein phenotype in a biological system (14). Alterations in proteome patterns, such as global changes in protein expression and post-translational modifications (PTMs), are often indicative of marked changes in functional stages in health and disease (15). Thus, investigating the varying patterns of the proteome may provide insights into pathogenic pathways (16) and these protein signatures may facilitate rapid screening of the efficacy of novel treatments and aid in drug development (17, 18).

Previous proteomic studies have identified protein changes in dilated cardiomyopathy, atherosclerosis, and atrial fibrillation (19–23) and these types of studies likely provided a deeper mechanistic understanding of the molecular pathways in HF. For example, cardiac tissue from patients with HFrEF demonstrated protein modifications associated with cardiac metabolism, cardiac remodeling, and impaired cardiac contractility (24–27). Additionally, differentially regulated pathways by proteomic signatures were observed in HFrEF vs. HFpEF patients, which is consistent with the predominant view that the underlying pathophysiology in these two diseases are largely different, and thus the variable response to therapies. This difference is exemplified by Adamo et al., where blood samples from both HFrEF and HFpEF patients demonstrated increased growth factor signaling and increased angiogenesis markers, while proteomic signatures from only HFpEF patients showed increased humoral immunity and those from HFrEF patients showed increased extracellular matrix remodeling markers, consistent with active cardiac remodeling (28). These findings underscore the potential that high-performance proteomics, in combination with clinical assessment, may identify unique targets in specific groups of HF patients.

Although HFpEF is greatly impacted by the obesity and diabetes pandemic, hypertension remains the most prevalent and modifiable risk factor in HFpEF and is implicated in both its pathogenesis and prognosis (12, 29). Hypertensive HFpEF pathophysiology extends beyond the emphasis on LV hypertrophy development and diastolic dysfunction to impaired myocardial contractility, left atrial myopathy, cardiomyocyte remodeling, macro- and microvascular dysfunction, to systemic inflammation, fibrosis, and collagen deposition. However, despite this knowledge a paucity of therapies exists for HFpEF. Here, we applied a deep quantitative proteomics and phosphoproteomics profiling approach to identify molecular protein signatures that are altered in HFpEF in a well characterized murine model of hypertension-associated HFpEF, the SAUNA model (SAly drinking water/Unilateral Nephrectomy/Aldosterone), which recapitulates the human HFpEF phenotype (30–37) (Supplementary Figure 1). Using an unbiased and comprehensive analysis, we report...
a systematic, large-scale study of pathway, metabolic and organelle level changes that occur in the left ventricle of this HFpEF murine model.

Material and methods

All procedures related to the handling and surgery of the mice conformed to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health and were approved by the Institutional Animal Care and Use Committee at Boston University School of Medicine.

SAUNA model of HFpEF

As previously described (30–33, 35–37), eight-week-old male C57BL/6J mice (Jackson Laboratories) were anesthetized with 80–100 mg/kg ketamine and 5–10 mg/Kg xylazine intraperitoneally. Mice (20–25 g) then underwent uninephrectomy, received either a continuous infusion of saline (Sham) or d-aldosterone (0.30 µg/h, Sigma-Aldrich, St. Louis, MO, United States; HFpEF) for 4 weeks via osmotic minipumps (Alzet, Durect Corp., Cupertino, CA, United States) and were maintained on 1% sodium chloride drinking water.

Physiological measurements

Blood pressure and echocardiographic measurements were performed at the end of the 4 weeks. Systolic blood pressure was measured using a non-invasive tail-cuff blood pressure analyzer (BP-2000 Blood Pressure Analysis System; Visitech Systems Inc., Apex, NC, United States). Transthoracic echocardiography was performed using a Vevo770 High-Resolution in vivo Micro-Imaging System and a Real-Time Micro Visualization 707B Scanhead (VisualSonic Inc., Toronto, ON, Canada) as previously described (33). Briefly, interventricular septum wall thickness (IVST), left ventricle (LV) posterior wall thickness (LVPWT), LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), and LV ejection fraction (LVEF) were measured. As a measure of systolic function and cardiac contractility fractional shortening (FS) was calculated as follows (LVEDD-LVESD/LVEDD) × 100. Total wall thickness (TWT) was derived from an average of the IVST and LVPWT. Relative wall thickness (RWT) was calculated as 2 × LVPWT/LVEDD. LV mass was calculated using the formula described by Kiatchoosakun et al. (38). As diastolic function is sensitive to heart rate (HR) and loading conditions, HR was maintained at ~350 bpm during these measurements (39). Pulse wave measurements were then recorded and analyzed blinded to group.

Histopathological analyses

Paraffin-embedded sections (5 µm) of the mid-LV were stained with hematoxylin and eosin (H&E, Sigma-Aldrich) to measure LV cardiac myocyte cross-sectional area. Microscopy images (BZ-9000 BioRevo microscope, Keyence Corp. of America, Itasca, IL, United States) were analyzed blinded to group identity using ImageJ measuring software (National Institutes of Health, Bethesda, MD, United States).

Tissue sample preparation for proteomics and phosphoproteomics

Left ventricle samples from 4 mice/group were processed as previously described (22, 40–42). Briefly, freshly thawed samples were homogenized on ice in with a mixer mill MM 400 (Retsch USA Verder Scientific Inc., Newtown, PA, United States) in 10 volumes of 8 M urea, 50 mM ammonium bicarbonate, 2 mM dithiothreitol, and protease and phosphatase inhibitor cocktails (Roche Applied Science, Indianapolis, IN, United States). Tissue homogenate was then sonicated with a probe sonicator (Branson Ultrasonics Corporation, North Billerica, MA, United States) and centrifuged. After centrifugation, supernatant was decanted and total protein in each sample was determined using a modified “microtiter plate” version of the Bradford assay (Sigma-Aldrich). For phosphoproteomics experiments, aliquots containing 300 µg of protein were alkylated with 5 mM iodoacetamide for additional 45 min at room temperature in the dark. Samples were then diluted eight-fold with 50 mM ammonium bicarbonate and digested overnight with sequencing-grade trypsin (#90057, Thermo Fisher Scientific Inc., Waltham, MA, United States). Digestion was stopped by acidification to a final concentration of 1% (v/v) formic acid and the peptide solutions were desalted using disposable C18 Sep-Pak syringes (Waters Corporation, Milford, MA, United States) and lyophilized to dryness following manufacturer’s instructions.

Tandem mass tag (TMT) labeling

Peptide concentrations were determined by a colorimetric peptide assay kit (Thermo Fisher Scientific Inc., Waltham, MA, United States) and an aliquot of 100 µg was placed in 100 µl of 100 mM triethylammonium bicarbonate. Peptides were labeled with 0.4 mg of TMT label (TMT10plex™ Isobaric Label Reagent Set, Thermo Fisher Scientific Inc., Waltham, MA, United States). All samples were labeled in the same TMT-batch, representing reporter tags 126C, 127N, 127C, 128C, 129N, 129C, 130N, and 131N. Labeled samples were pooled, and 95% was set aside for phosphopeptide enrichment. The remaining 5% of
labeled peptides and the phosphopeptide enriched samples were analyzed separately by mass spectrometry.

Phosphopeptide enrichment

Phosphopeptides were selectively enriched by binding to titanium dioxide (TiO₂) beads (Titansphere Phos-TiO Bulk 10 μm, GL Sciences, Tokyo, Japan) (43). Briefly, peptides were resuspended in 200 μl 80% acetonitrile, 6% trifluoroacetic acid and incubated for 10 min with 10 μl of slurry containing TiO₂ beads. Unbound peptides and supernatant were decanted, and the beads were washed three times with a wash buffer containing 50% acetonitrile and 1% trifluoroacetic acid. After final decanting, the beads were incubated for 10 min with elution solution containing 25% ammonium hydroxide and 50% acetonitrile and the eluate was carefully removed and dried prior to mass spectrometry analysis.

Mass spectrometry analysis

Tryptic peptide mixtures and enriched phosphopeptides were analyzed by nano-scale high-performance liquid chromatography (Proxeon EASY-Nano system, Thermo Fisher Scientific Inc., Waltham, MA, United States) and online nano electrospray ionization tandem mass spectrometry (Q-Exactive HF-X mass spectrometer; Thermo Fisher Scientific Inc., Waltham, MA, United States). Briefly, samples were loaded in aqueous 0.1% (v/v) formic acid via a trap column (75 μm i.d. × 2 cm, Acclaim PepMap100 C18 3 μm, 100 Å, Thermo Fisher Scientific) and peptides were resolved over an Easy-Spray analytical column (50 cm × 75 μm ID, PepMap RSLC C18, Thermo Fisher Scientific) by an increasing mobile phase B. Mobile phase A consisted of 2% acetonitrile and 0.1% formic acid, and organic phase B contained 80% acetonitrile and 0.1% formic acid. Reverse phase separation was performed over 120 min at a flow rate of 300 nl/min. Eluted peptides were ionized directly into the mass spectrometer using a nanospray ion source. The mass spectrometer was operated in positive ion mode with a capillary temperature of 300 C, and with a potential of 2,100 V applied to the frit. Tandem mass spectrometry (MS/MS) was performed using high-energy collision-induced disassociation and 10 MS/MS data-dependent scans (45,000 resolution) were acquired in profile mode alongside each profile mode full-scan mass spectra (120,000 resolution) as reported previously (44). The automatic gain control (AGC) for MS scans was 1 × 10⁶ ions with a maximum fill time of 60 ms. The AGC for MS/MS scans was 3 × 10⁴, with 80 ms maximum injection time, 0.1 ms activation time, and 33% normalized collision energy. To avoid repeated selection of peptides for MS/MS a dynamic exclusion list was enabled to exclude all fragmented ions for 60 s.

Protein identification

Data files (RAW format) were searched using the standard workflow of MaxQuant (version 1.3.0.5) (1) under standard settings using the entire Swiss-Prot mouse database downloaded January 24, 2019, allowing for two missed tryptic cleavage sites, 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 4.5 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 datasets generated for this study have been deposited and publicly available at the PRIDE Archive, proteomics data repository (European Bioinformatics Institute, European Molecular Biology Laboratory) with the data set identifier PXD033501.

Bioinformatics analysis

The searched intensity data were filtered, normalized, and clustered using Omics Notebook (45). Filtering was performed to remove any proteins or phosphopeptides not quantified in at least 70 percent of samples, with 2,905 and 281 proteins and phosphopeptides passing the filter, respectively. After filtering, both datasets showed low levels of sparsity and no missing value imputation was performed. The LIMMA R package was used for LOESS normalization and differential expression analysis (46). A combined ranked list for both sets was generated where duplicate gene entries were removed to keep the entry with the highest absolute rank value.

GSEA analysis

Gene Set Enrichment analysis (GSEA) software from the fgsea R package was used to compute gene set enrichment after ranking proteins by differential expression in HFpEF vs. Sham (45, 47, 48). Briefly, GSEA was used in rank mode along with gene sets downloaded from the Bader Lab (Mouse_GOBP_AllPathways_no_GO_iea_October_01_2018.gmt) (49, 50). GSEA results were visualized using the Enrichment Map app (Version 3.1) in Cytoscape (Version 3.6.1) and highly related pathways were grouped into a theme and labeled by AutoAnnotate (version 1.2). For the merged gene set analyses, we applied an enrichment P < 0.01 and FDR ≤ 0.1 cutoffs and calculated overlap between gene set annotations.
using a combination of Jaccard and overlap coefficients with a cutoff of 0.375.

**Titin isoform analysis**

Additional studies were performed to investigate changes in titin isoforms in HFpEF. Briefly, LV protein lysates from Sham (N = 7) and HFpEF (N = 11) mice were extracted and electrophoresed in 1% agarose gels using a SE600X vertical gel system (Hoefler Inc., Holliston, MA, United States) as previously described (51). Gels were run at 15 mA constant current, stained with Neuhoff’s Coomassie (52), and then scanned using Epson Perfection V750 PRO scanner (Epson America Inc., Los Alamitos, CA, United States) and analyzed using One-D scan EX analysis software (Scanalytics Inc., Rockville, MD, United States). The integrated optical density of titin and total myosin heavy chain (MHC) was determined as a function of the slope of the linear range between integrated optical density and loaded volume (53). The expression of compliant N2BA titin, stiffer N2B titin and total titin (TT) was normalized to the expression of total MHC. The expression of titin degraded product (T2) was normalized to the TT expression.

**SIRT3 immunoblotting analysis**

Protein lysates were extracted from LV tissue using in-ice-cold RIPA buffer as previously described (54). Equal amounts of protein were then subjected to electrophoresis in SDS-polyacrylamide gel under reducing conditions and blotted to polyvinylidene difluoride (PVDF) membranes using the Bio-Rad Transblot Turbo Transfer System (Hercules, CA, United States). The membranes were blocked in 5% BSA, 0.1% Tween-20 in tris-buffered saline for 1 h at room temperature and then incubated overnight at 4°C BSA, 0.1% Tween-20 in tris-buffered saline for 1 h at 37°C in room temperature: anti-rabbit antibody and respective horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h in room temperature: anti-rabbit antibody (R&D system, HAF008; 1:5,000). Immune complexes were detected with the enhanced chemiluminescence ECL detection system (Bio-Rad, #1705060) in the ImageQuant LAS 4000 biomolecular imaging system (GE Healthcare, Pittsburgh, PA, United States). The intensity of bands for each protein was normalized to the loading control mouse anti-GAPDH (Abcam, Ab8245; 1:10,000).

**Statistical analysis**

Proteomics and phosphoproteomics differential analysis were based on a moderated t-test and performed using R. A language and environment for statistical computing (R Foundation for Statistical Computing, Vienna, Austria) (45, 55). For histology analysis, titin isoform studies and SIRT3 expression, data are shown as mean ± SEM and statistical significance of differences was assessed using the Student’s t-test (two sided). In those cases when data were not sampled as a normal distribution, non-parametric Mann–Whitney U test was used. P ≤ 0.05 values were considered significant. These statistical tests were performed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, United States).

**Results**

**Mouse model of HFpEF**

As previously described (30–33, 35–37), salty drinking water, unilateral nephrectomy, and chronic exposure to aldosterone (SAUNA) induced hypertension associated HFpEF in mice. Compared to Sham, HFpEF mice demonstrated a moderate increase in systolic blood pressure (137.8 ± 7.0 mmHg vs. 115.4 ± 6.0 mmHg; P < 0.05), lung congestion (4.5 ± 0.1 vs. 4.0 ± 0.1 P < 0.01), and LV hypertrophy, measured by the LV weight-to-total body weight ratio (3.7 ± 0.1 mg/g vs. 3.3 ± 0.1 mg/g; P < 0.05). Additionally, cardiomyocyte size was increased 1.2-fold in HFpEF mice vs. Sham; P < 0.05 (Supplementary Figure 2).

Echocardiography demonstrated preserved LVEF and increased LV mass (107.5 ± 4.9 mg vs. 78.2 ± 7.9 mg in Sham; Table 1).

| TABLE 1 Characteristics and echocardiographic parameters of HFpEF (SAUNA) mice 4 weeks after d-Aldosterone or saline (Sham) infusion. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | HFpEF            | Sham            |
| Systolic blood pressure (mmHg) | 137.8 ± 7.0*    | 115.4 ± 6.0     |
| Wet-to-dry lung ratio | 4.5 ± 0.1**     | 4.0 ± 0.1       |
| Heart weight-to-body weight (mg/g) | 3.7 ± 0.1*    | 3.3 ± 0.1       |
| **Left ventricle structure and function** |                |                |
| LV mass (mg)        | 107.5 ± 4.9*    | 78.6 ± 7.9      |
| Total wall thickness (mm) | 1.0 ± 0.0***   | 0.8 ± 0.1       |
| Posterior wall thickness (mm) | 1.0 ± 0.1*    | 0.8 ± 0.1       |
| Relative wall thickness | 0.7 ± 0.1***   | 0.5 ± 0.0       |
| LV end-systolic diameter (mm) | 1.1 ± 0.2*    | 1.6 ± 0.1       |
| LV end-diastolic diameter (mm) | 3.0 ± 0.2      | 3.3 ± 0.1       |
| LV ejection fraction (%) | 91.1 ± 1.3     | 83.1 ± 3.0      |
| LV fractional shortening | 62.1 ± 2.3    | 52.0 ± 3.5      |
| E/A               | 1.9 ± 0.2       | 1.7 ± 0.2       |
| Early filling deceleration time (ms) | 21.0 ± 3.0    | 17.6 ± 2.6      |
| Isovolumetric relaxation time (ms) | 24.3 ± 2.6*   | 14.4 ± 1.6      |

Data are expressed as mean ± SEM. A, peak late transmital flow velocity; E, peak early transmital flow velocity; LV, left ventricular (N = 5 mice/group). *P < 0.05 vs. Sham, **P < 0.01 vs. Sham; ***P < 0.005 vs. Sham. Statistical analysis by two-tailed Student’s t-test.
Wall thickness was significantly increased in HFpEF and there was evidence of concentric hypertrophy, as demonstrated by the increased relative wall thickness (0.7 ± 0.1 vs. 0.5 ± 0.0 in Sham; \( P < 0.005 \)). As previously shown (33), LV end-systolic dimensions and end-diastolic dimensions were also decreased in HFpEF (Table 1). HFpEF mice had impaired diastolic function, characterized by an increase in isovolumetric relaxation time (24.3 ± 2.6 ms vs. 14.4 ± 1.6 ms in Sham; \( P < 0.05 \)).

**Comparison to human HFpEF:** Recently, two clinical scores (HFA-PEFF and H2FPEF) were developed to standardize the clinical diagnosis of human HFpEF. However, a discrepancy exists between these scores (56). The H2FPEF score largely includes clinical parameters whereas the HFA-PEFF score includes predominantly echocardiographic measures and natriuretic peptides. The HFA-PEFF score can rule in human HFpEF with high specificity (93%) and positive predictive value (98%) when the score is high (5–6 points) (57). As such, the translational utility of the HFpEF SAUNA mouse model was demonstrated in the context of this HFpEF score with a HFA-PEFF score of \( \geq 6 \) as described by Withaar et al. (58), where a score of \( \geq 5 \) is a high probability of clinical HFpEF.

**Proteome profile of the left ventricle in HFpEF**

To achieve comprehensive evaluation of the cardiac signaling that is seen in HFpEF, a global quantitative proteome and phosphoproteome profile was performed in LV cardiac tissue obtained from HFpEF mice and their respective Shams (\( N = 4 \) mice/group; Figure 1).

Proteomics analysis found a total of 2,905 identified proteins that were then used for comparative analysis (Supplementary Table 1). Among them, 897 proteins were differentially expressed between HFpEF and Sham LV, with 19% of these...
being predominantly higher in HFpEF than in Sham \( (P < 0.05; \text{Figures 2A,B}). \)

Systematic evaluation of the datasets revealed abundant changes in sarcomeric proteins, namely skeletal alpha (\( \alpha \))-actin (ACTA1; \( P = 0.000039 \)), beta (\( \beta \))-myosin heavy chain (MYH7; \( P = 0.006963 \)), myosin heavy chain 9 (MYH9; \( P = 0.000408 \)), troponym alpha (\( \alpha \))-1 chain (TPM1; \( P = 0.048698 \)); the mitochondria-related proteins mitofusin 1 (MFN1; \( P = 0.001059 \)), mitochondrial dynamin like GTPase (aka optic atrophy protein 1, OPA1; \( P = 0.046441 \)) and transcription factor A mitochondrial (TFAM; \( P = 0.005837 \)); and the NAD-dependent protein deacetylase sirtuin-3 (SIRT3; \( P = 0.005837 \)), recently implicated in cardiac function and cardiac stress responsiveness in HFpEF \((59, 60)\) (\textbf{Figure 2B} and \textit{Supplementary Table 1}).

**Impaired mitochondrial function and oxidative metabolism of energy substrates in HFpEF**

There was an extensive reduction in the abundance of proteins involved in cardiac metabolism in the LV of HFpEF mice, including the oxidation of free fatty acid (FFA), pyruvate, and ketone bodies. Significant changes are summarized in \textbf{Figure 3}. These include:

(I) \( \beta \)-oxidation related enzymes, implicated in FFA metabolism to acetyl-CoA, such as acyl-CoA dehydrogenase (ACAD) family member 11 (ACAD11; \( P = 0.00002 \)), long-chain specific ACAD (ACADL; \( P = 0.00449 \)), short-chain specific ACAD (ACADS; \( P = 0.00896 \)), short-branched chain specific ACAD (ACADSB; \( P = 0.011317 \)), 3-ketoacyl-CoA thiolase (ACAA2, \( P = 0.00609 \)), enoyl-CoA hydratase (ECHS1, \( P = 0.02647 \)), hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex (HADH) beta (\( \beta \))-subunit (HADHB, \( P = 0.00729 \) and HADH alpha (\( \alpha \))-subunit (HADHA, \( P = 0.03235 \)).

(II) the pyruvate oxidation enzyme pyruvate dehydrogenase X component (PDHX, \( P = 0.00961 \)), which is part of the pyruvate dehydrogenase complex that catalyzes pyruvate to acetyl-CoA; and

(III) the ketone metabolism enzyme succinyl-CoA:3-keto-acid coenzyme A transferase 1 (OXCT1, \( P = 0.01237 \)), which catalyzes ketone bodies and produces acetyl-CoA for the tricarboxylic acid (TCA) cycle.

These cumulative results suggest that the energy substrates for mitochondrial oxidative metabolism may be inefficient in HFpEF. Interestingly, although there were no significant alterations in the protein signature of fatty acid and glucose transporters (CD36 and GLUT1 and 4, respectively) in HFpEF, there was an upregulation of the ketone bodies transporter monocarboxylate transporter 1 (SLC16A1, \( P = 0.00376 \)) in the LV of HFpEF.

Additional analysis revealed that the mitochondrial proteins involved in the TCA cycle were also significantly decreased in the LV of HFpEF mice. These mitochondrial enzymes, namely citrate synthase (CS, \( P = 0.008068 \)),...
succinyl-CoA ligase beta subunit (SUCLA2, $P = 0.02523$), isocitrate dehydrogenase (IDH1, $P = 0.00105$) and pyruvate carboxylase (PC, $P = 0.00206$), are required to catalyze acetyl-CoA and produce essential intermediates for the biosynthesis process, and most importantly, high energy molecules such as nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH$_2$) for the electron transport chain (ETC). Subsequent analysis then showed that 27 proteins involved in the ETC (namely the respiratory complex I, III, IV, and V) were also differentially expressed between HFpEF and Sham. Of these 27 proteins, 19 proteins were significantly reduced in the HFpEF, suggesting impaired ETC, which was consistent with an additional reduction of the uncoupling protein 3 (UCP3, $P = 0.02765$).

Lastly, additional proteins involved in mitochondrial biogenesis (transcription factor A, TFAM, $P = 0.00584$) and fusion (mitofusin-1, MFN1, $P = 0.001056$ and dynamin-like 120 kDa protein, OPA1, $P = 0.04644$) were similarly decreased in the LV tissue from HFpEF mice.

These findings (Figure 3) suggest that mitochondrial dysfunction may lead to inefficient metabolism of energy substrates, possibly contributing to an energy deficit and thus affecting cardiac function in HFpEF.

**Pathway enrichment analysis**

Pathway enrichment analyses of the proteomics and phosphoproteomics combined datasets were performed by means of GSEA, which detects biology-driven gene sets of canonical pathways from databases of molecular signatures (61). These analyses revealed that the most relevant and over-represented (enriched) biological annotations in the LV from HFpEF to be: (I) processes involving immune system modulation, (II) cardiac muscle cell development and differentiation, and (III) muscle contraction (Table 2). These processes included positive regulation of cytokine production (GO:0001819; $P = 0.0000$), striated muscle contraction (Wikipathway; $P = 0.0000$), positive regulation of adaptive immune response (GO:0002821; $P = 0.00578$), cardiac muscle cell development (GO:0055013; $P = 0.03158$) and cardiac muscle cell differentiation (GO: 0055007; $P = 0.03571$). In contrast, the downregulated pathways were related to a multitude of GO terms associated with cellular metabolism (Table 3). This is consistent with the earlier data from Figure 3, where pathways and processes involving acetyl-CoA metabolic process (GO:0006084, $P = 0.0000$), fatty acid metabolic process (GO:0006631, $P = 0.0000$),

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**Figure 3**
Schematic overview of identified metabolic and mitochondrial related targets in HFpEF mice in the proteome dataset. Blue and red represent significant ($P < 0.05$) up-regulation and down-regulation in HFpEF vs. Sham, respectively. Black targets are unchanged.
TABLE 2 Biological annotations terms enriched in significantly up-regulated proteins of the proteome dataset.

| Name                                                                 | Group                        | P-value  | Size | ES        |
|----------------------------------------------------------------------|------------------------------|----------|------|-----------|
| Positive regulation of cytokine production                           | GO:0001819                   | 0.00000  | 57   | −0.34     |
| Pallium development                                                  | GO:0021543                   | 0.00000  | 32   | −0.38     |
| Platelet degranulation                                               | Reactome pathway              | 0.00000  | 56   | −0.33     |
| Response to elevated platelet cytosolic cal2⁺                         | Reactome pathway              | 0.00000  | 58   | −0.33     |
| Signaling by ROBO receptors                                          | Reactome pathway              | 0.00000  | 98   | −0.26     |
| Striated muscle contraction                                          | Wikipathway                  | 0.00000  | 30   | −0.42     |
| Positive regulation of adaptive immune response                      | GO:0002821                   | 0.00578  | 18   | −0.47     |
| Positive regulation of wound healing                                 | GO:0009303                   | 0.00595  | 16   | −0.51     |
| Intrinsic pathway for apoptosis                                      | Reactome pathway              | 0.00633  | 15   | −0.54     |
| Positive regulation of response to wounding                          | GO:1903036                   | 0.00671  | 20   | −0.44     |
| Integrin pathway                                                     | Biocarta pathway              | 0.00690  | 23   | −0.39     |
| Nucleus organization                                                 | GO:0006997                   | 0.00893  | 31   | −0.36     |
| Fosco pathway                                                        | PID pathway                  | 0.01500  | 16   | −0.48     |
| G2 m checkpoints                                                     | Reactome pathway              | 0.01754  | 52   | −0.28     |
| Coagulation                                                          | Hallmark Pathway             | 0.01818  | 54   | −0.29     |
| Complement and coagulation cascades                                  | Wikipathway                  | 0.01829  | 21   | −0.45     |
| Cerebral cortex development                                          | GO:0012187                   | 0.02143  | 27   | −0.39     |
| Rho GTPases activate PKNs                                            | Reactome pathway              | 0.02158  | 25   | −0.40     |
| Positive regulation of adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains | GO:0002824                   | 0.02222  | 17   | −0.47     |

ES, enrichment score. Results are sorted by the nominal P-value in an ascending order.

acyl-CoA biosynthesis process (GO:0071616, \(P = 0.0000\)), fatty acid oxidation (GO:0019395, \(P = 0.0011\)), coenzyme metabolic process (GO:0006732, \(P = 0.0101\)) were significantly reduced in HFpEF.

Phospho-proteome profile of the left ventricle in HFpEF

We next investigated the phosphoproteomics dataset. Phosphoproteomics analysis profiled 281 mouse reference protein sequences, of which 240 mapped to serine, 37 mapped to threonine and 3 mapped to tyrosine residues, consistent with the expected 90:9:1 cellular distribution ratio (22). The abundance of 72 phosphosites was differentially altered (elevated or reduced) between HFpEF and Sham (\(P < 0.05\); Figures 4A,B).

Aberrant phosphorylation patterns occurred on proteins linked to disparate subcellular compartments, ranging from sarcomeric proteins (LIM domain-binding protein 3, LDB3; myozenin 2, MYOZ2; titin, TTN), to nuclear-localized proteins (BAG family molecular chaperone regulator 3, BAG3; high mobility group protein HMG-I/HMG-Y, HMGA1) with established links to cardiac contractile function, cardiac hypertrophy and/or cardiomyopathy (Figure 4B and Supplementary Table 2).

Left ventricular titin expression and phosphorylation in HFpEF

Despite global proteomics not showing a significant change in total titin in the LV between HFpEF and Sham mice,
### Table 3: Biological annotations terms significantly enriched in down-regulated proteins of the proteome dataset.

| Name                                                   | Group                                | P-value | Size | ES  |
|---------------------------------------------------------|--------------------------------------|---------|------|-----|
| Regulation of tp53 activity                            | Reactome pathway                     | 0.00000 | 23   | 0.60|
| Purine nucleoside bisphosphate metabolic process       | GO:0034032                           | 0.00000 | 43   | 0.51|
| Acetyl-CoA metabolic process                           | GO:0006084                           | 0.00000 | 16   | 0.64|
| Negative regulation of lipid metabolic process         | GO:0045833                           | 0.00000 | 17   | 0.63|
| Ribonucleoside bisphosphate metabolic process          | GO:0033875                           | 0.00000 | 43   | 0.51|
| Monocarboxylic acid catabolic process                   | GO:0072329                           | 0.00000 | 46   | 0.53|
| Nucleoside bisphosphate metabolic process              | GO:0033865                           | 0.00000 | 43   | 0.51|
| **Fatty acid metabolic process**                       | GO:0006631                           | 0.00000 | 91   | 0.48|
| Carboxylic acid catabolic process                       | GO:0046395                           | 0.00000 | 78   | 0.46|
| Monocarboxylic acid metabolic process                   | GO:0032787                           | 0.00000 | 138  | 0.45|
| Organic acid catabolic process                          | GO:0016054                           | 0.00000 | 78   | 0.46|
| **Acyl-CoA biosynthetic process**                      | GO:0071616                           | 0.00000 | 15   | 0.67|
| Thioester biosynthetic process                         | GO:0035384                           | 0.00000 | 15   | 0.67|
| Sulfur compound metabolic process                       | GO:0006790                           | 0.00000 | 99   | 0.44|
| Carboxylic acid metabolic process                       | GO:0019752                           | 0.00000 | 259  | 0.41|
| Oxalic acid metabolic process                           | GO:0043436                           | 0.00000 | 264  | 0.41|
| Organic acid metabolic process                          | GO:006082                            | 0.00000 | 268  | 0.41|
| Cellular monovalent inorganic cation homeostasis        | GO:0030004                           | 0.00000 | 17   | 0.66|
| Response to nitrogen compound                           | GO:1901698                           | 0.00000 | 196  | 0.39|
| Small molecule metabolic process                        | GO:0044281                           | 0.00100 | 463  | 0.34|
| Thioester metabolic process                             | GO:0035383                           | 0.00109 | 39   | 0.53|
| **Fatty acid oxidation**                                | GO:0019395                           | 0.00111 | 38   | 0.52|
| Positive regulation of ion transmembrane transporter activity | GO:0032414                           | 0.00111 | 34   | 0.51|
| Cilium assembly                                          | GO:0060271                           | 0.00115 | 22   | 0.59|
| Protein trimerization                                   | GO:0007026                           | 0.00121 | 17   | 0.66|
| Sulfur compound biosynthetic process                    | GO:0044272                           | 0.00229 | 26   | 0.58|
| Protein dephosphorylation                               | GO:0096470                           | 0.00231 | 25   | 0.59|
| Protein localization                                    | Reactome pathway                     | 0.00310 | 84   | 0.42|
| **Acyl-CoA metabolic process**                          | GO:0006637                           | 0.00327 | 39   | 0.53|
| **Fatty acid catabolic process**                        | GO:0090682                           | 0.00328 | 39   | 0.51|
| Activation of GTPase activity                           | GO:0090630                           | 0.00362 | 16   | 0.61|
| Response to oxygen-containing compound                  | GO:1901700                           | 0.00400 | 256  | 0.35|
| **Fatty acid beta-oxidation**                           | GO:0086635                           | 0.00439 | 31   | 0.54|
| **Lipid oxidation**                                     | GO:0034440                           | 0.00443 | 38   | 0.52|
| Monovalent inorganic cation homeostasis                 | GO:0055967                           | 0.00473 | 20   | 0.62|
| Laminin interactions                                    | Reactome pathway                     | 0.00486 | 15   | 0.63|
| Small molecule catabolic process                        | GO:0044282                           | 0.00509 | 110  | 0.40|
| **Lipid modification**                                  | GO:0030258                           | 0.00536 | 45   | 0.50|
| Dephosphorylation                                       | GO:0016311                           | 0.00553 | 34   | 0.52|
| Ion channel transport                                   | Reactome pathway                     | 0.00553 | 34   | 0.51|
| Metabolism of water-soluble vitamins and cofactors      | Reactome pathway                     | 0.00559 | 32   | 0.54|
| Response to organonitrogen compound                     | GO:0010243                           | 0.00604 | 178  | 0.38|
| Cellular amino acid metabolic process                   | GO:006520                            | 0.00609 | 98   | 0.41|
| Positive regulation of transporter activity             | GO:0032411                           | 0.00661 | 37   | 0.51|
| Cilium organization                                     | GO:0044782                           | 0.00685 | 23   | 0.58|
| Nucleoside bisphosphate biosynthetic process            | GO:0033866                           | 0.00823 | 19   | 0.56|
| Neurotransmitter transport                              | GO:0006836                           | 0.00894 | 27   | 0.54|
| Positive regulation of ion transmembrane transport      | GO:0034767                           | 0.00966 | 49   | 0.46|

(Continued)
| Name                                                                 | Group                                                                 | P-value  | Size  | ES  |
|----------------------------------------------------------------------|----------------------------------------------------------------------|----------|-------|-----|
| Cell projection assembly                                             | GO:0030031                                                          | 0.00968  | 49    | 0.46|
| Long-chain fatty acid metabolic process                              | GO:0001676                                                          | 0.00980  | 17    | 0.60|
| Response to drug                                                      | GO:0042493                                                          | 0.01006  | 137   | 0.38|
| Cell projection organization                                          | GO:0030030                                                          | 0.01006  | 155   | 0.37|
| **Coenzyme metabolic process**                                       | GO:0006732                                                          | 0.01015  | 126   | 0.38|
| **Fatty acid metabolism**                                            | Reactome pathway                                                   | 0.01053  | 72    | 0.42|
| Mitochondrial fatty acid beta-oxidation                              | Reactome pathway                                                   | 0.01114  | 28    | 0.52|
| Cellular response to hormone stimulus                                | GO:0032870                                                          | 0.01148  | 75    | 0.43|
| Pyrimidine-containing compound metabolic process                     | GO:0072527                                                          | 0.01214  | 17    | 0.59|
| Cellular response to oxygen levels                                   | GO:0071453                                                          | 0.01350  | 26    | 0.52|
| Plasma membrane bounded cell projection assembly                     | GO:0120031                                                          | 0.01609  | 47    | 0.46|
| Purine nucleoside bisphosphate biosynthetic process                  | GO:0034033                                                          | 0.01667  | 19    | 0.56|
| Negative regulation of cellular response to TGFbeta stimulus         | GO:1903845                                                          | 0.01914  | 15    | 0.58|
| Ribonucleoside bisphosphate biosynthetic process                     | GO:0034030                                                          | 0.01932  | 19    | 0.56|
| Transmission across chemical synapses                                | Reactome pathway                                                   | 0.01967  | 45    | 0.45|
| Neprhon development                                                  | GO:0072006                                                          | 0.01975  | 15    | 0.59|
| Cellular response to endogenous stimulus                             | GO:0071495                                                          | 0.02018  | 163   | 0.35|
| Positive regulation of transmembrane transport                       | GO:0034764                                                          | 0.02030  | 62    | 0.42|
| Plasma membrane bounded cell projection organization                  | GO:0120036                                                          | 0.02113  | 149   | 0.36|
| Cellular response to organic substance                               | GO:0071310                                                          | 0.02200  | 323   | 0.33|
| Response to organic substance                                        | GO:0010033                                                          | 0.02200  | 448   | 0.32|
| Branched-chain amino acid catabolism                                 | Reactome pathway                                                   | 0.02241  | 21    | 0.54|
| Regulation of coenzyme metabolic process                            | GO:0051196                                                          | 0.02281  | 18    | 0.56|
| Neuronal system                                                      | Reactome pathway                                                   | 0.02318  | 59    | 0.42|
| Cellular response to inorganic substance                             | GO:0071241                                                          | 0.02341  | 34    | 0.48|
| Negative regulation of transmembrane receptor protein serine/threonine kinase signaling pathway | GO:0090101 | 0.02392 | 19 | 0.55 |
| FCgamma receptor dependent phagocytosis                             | Reactome pathway                                                   | 0.02540  | 25    | 0.51|
| Fatty acid biosynthetic process                                      | GO:0086633                                                          | 0.02549  | 23    | 0.52|
| Cellular response to nitrogen compound                               | GO:1901699                                                          | 0.02554  | 108   | 0.38|
| Negative regulation of TGFbeta receptor signaling pathway           | GO:0030512                                                          | 0.02599  | 15    | 0.58|
| Positive regulation of cation transmembrane transport                | GO:1904064                                                          | 0.02612  | 46    | 0.45|
| Negative regulation of organelle organization                        | GO:0010639                                                          | 0.02764  | 87    | 0.39|
| Regulation of muscle organ development                               | GO:0048634                                                          | 0.02772  | 34    | 0.46|
| Dicarboxylic acid metabolic process                                 | GO:0043648                                                          | 0.02793  | 42    | 0.46|
| Positive regulation of striated muscle tissue development            | GO:0045844                                                          | 0.02818  | 21    | 0.52|
| Positive regulation of muscle tissue development                     | GO:1901863                                                          | 0.02904  | 21    | 0.52|
| Cellular amino acid biosynthetic process                             | GO:008652                                                           | 0.03012  | 17    | 0.56|
| Positive regulation of muscle organ development                      | GO:0048636                                                          | 0.03030  | 21    | 0.52|
| Signal release                                                      | GO:0023061                                                          | 0.03111  | 26    | 0.49|
| Heterotrimeric G protein signaling pathway-GI alpha and G6 alpha mediated pathway | Panther pathway | 0.03222 | 22 | 0.50 |
| Response to endogenous stimulus                                      | GO:0009719                                                          | 0.03307  | 215   | 0.33|
| Protein complex oligomerization                                      | GO:0051259                                                          | 0.03313  | 163   | 0.36|
| Neurotransmitter secretion                                           | GO:0007269                                                          | 0.03410  | 16    | 0.57|
| Signal release from synapse                                          | GO:0099643                                                          | 0.03431  | 16    | 0.57|
| Regulation of transporter activity                                   | GO:0032409                                                          | 0.03434  | 71    | 0.40|
| Regulation of transmembrane transporter activity                     | GO:0022988                                                          | 0.03441  | 68    | 0.40|
| Cellular response to lipid                                           | GO:0071396                                                          | 0.03470  | 67    | 0.40|
| Regulation of TGFbeta receptor signaling pathway                     | GO:0017015                                                          | 0.03477  | 21    | 0.52|

(Continued)
### TABLE 3 (Continued)

| Name                                                                 | Group                          | P-value | Size | ES  |
|---------------------------------------------------------------------|--------------------------------|---------|------|-----|
| Regulation of transmembrane receptor protein serine/threonine kinase signaling pathway | GO:0090092                     | 0.03528 | 35   | 0.46|
| NABA basement membranes                                             | MSIGDB                         | 0.03534 | 19   | 0.53|
| Coenzyme biosynthetic process                                       | GO:0009108                     | 0.03560 | 67   | 0.40|
| Cellular response to chemical stimulus                              | GO:0070887                     | 0.03600 | 417  | 0.31|
| Response to organic cyclic compound                                 | GO:0014070                     | 0.03858 | 123  | 0.36|
| Transition metal ion transport                                      | GO:0000041                     | 0.03943 | 19   | 0.54|
| Regulation of striated muscle tissue development                    | GO:0016202                     | 0.04013 | 34   | 0.46|
| Response to ammonium ion                                            | GO:0060359                     | 0.04152 | 23   | 0.49|
| Positive regulation of ion transport                               | GO:0043270                     | 0.04280 | 73   | 0.39|
| Opioid signaling                                                    | Reactome pathway               | 0.04282 | 22   | 0.52|
| Cell-cell adhesion                                                  | GO:0098609                     | 0.04366 | 66   | 0.40|
| Positive regulation of sodium ion transport                         | GO:0010765                     | 0.04380 | 16   | 0.55|
| Regulation of muscle tissue development                             | GO:1901861                     | 0.04402 | 34   | 0.46|
| Blood vessel morphogenesis                                          | GO:0048514                     | 0.04516 | 48   | 0.42|
| Regulation of NIK/NF-kappaB signaling                               | GO:1901222                     | 0.04535 | 19   | 0.52|
| Regulation of response to drug                                      | GO:2001023                     | 0.04642 | 15   | 0.55|
| Alpha-amino acid metabolic process                                  | GO:1901605                     | 0.04674 | 55   | 0.41|
| Negative regulation of cell proliferation                           | GO:0008285                     | 0.04689 | 84   | 0.37|
| Activation of cysteine-type endopeptidase activity involved in apoptotic process | GO:0006919                     | 0.04711 | 20   | 0.50|
| Integrin signaling pathway                                          | MSIGDB                         | 0.04718 | 26   | 0.48|
| Cooperation of PDCL (PHLP1) and TRIC CCT in G-protein beta folding | Reactome pathway               | 0.04785 | 15   | 0.54|
| Response to peptide                                                 | GO:1901652                     | 0.04876 | 84   | 0.37|
| Glutamine family amino acid metabolic process                       | GO:0009164                     | 0.04901 | 20   | 0.50|

ES, enrichment score. Results are sorted by the nominal P-value in an ascending order.

---

**FIGURE 4**

Phosphoproteomics analysis. (A) Heatmap of the differentially expressed phosphosites. (B) Volcano plot presenting log-transformed p-values (t-test) associated with individual significantly altered phosphosites plotted against log-transformed fold change in abundance between the left ventricles in Sham and HFpEF mice. Blue and red dots represent up-regulation and down-regulation in HFpEF (N = 4) vs. Sham (N = 4), respectively.
extensive phosphorylation changes across titin were observed in HFP EF vs. Sham. We identified 22 titin phosphosites (including 76% serines, 19% threonines, and 4% tyrosines) in the phospho-dataset, and among them, seven phosphosites were \( P < 0.05 \), all considered class 1 (localization probability 0.75–1.00) (Table 4). Interestingly, five of these were located at the z disk binding region (S262, S264, T266, S1411, and S1415) while the remainder were residues in the C-terminal region (S34464, T34467), suggesting changes in the mechano-sensing activity of titin. These regions are known to act as titin “hotspots,” which respond to mechanical stress and regulate specific actions such as activating the hypertrophic gene program or interacting with the protein quality control machinery.

Additionally, high-resolution gel electrophoresis was performed to further examine other potential switches in titin isoform expression which may affect titin stiffness, i.e., to quantitatively detect changes in the stiffer N2B or the compliant N2BA isoforms of titin (Figure 5A). As expected, there were no changes in total titin (TT) expression between HFP EF and Sham mice. However, as previously described (64), N2B expression was significantly increased in the LV from HFP EF mice compared to Sham mice (0.144 ± 0.010 vs. 0.127 ± 0.010; \( P < 0.05 \)). Neither N2BA expression, N2BA/N2B ratio nor titin degradation were differentially altered between HFP EF and Sham mice (Figure 5B).

Left ventricular expression of SIRT3 in HFP EF mice

Accumulating evidence suggests that SIRT3 plays a critical role in the development of HF (65), particularly in HFP EF (60, 66, 67). As the global proteomics dataset showed a decreased in SIRT3 in the LV from HFP EF mice vs. Sham (\( P = 0.000914 \)), we thus performed additional immunoblot analysis to validate these findings. Indeed, SIRT3 expression was significantly decreased in the LV from HFP EF mice vs. Sham (0.8 ± 0.0 vs. 1.0 ± 0.0; \( P < 0.001 \); Figure 6A).

Discussion

Heart failure with preserved ejection fraction is a complex disease involving several sub-phenotypes within a heterogeneous HFP EF syndrome (10, 13, 68). Of all the comorbidities in HFP EF, hypertension remains the most common, and is implicated in both the pathogenesis and the prognosis of the disease (12, 29). However, the exact biological mechanisms that underlie hypertension associated HFP EF remain largely unclear. In this study, we investigated the proteomic and phosphoproteomics profile underlying HFP EF in a clinically relevant murine model of hypertension associated HFP EF. The SAUNA model of HFP EF model fulfils the

| Feature   | Log FC (Sham/HFP EF) | \( P \) value | Position | Site probability (%) | Peptide sequence |
|-----------|----------------------|---------------|----------|----------------------|-----------------|
| Ttn_A2ASS6.55 | −1.01327            | 0.00203       | S262     | 0.99876              | QLPHKTPPRIPPKRSRSTTPPSIALAAKQAQLA |
| Ttn_A2ASS6.56 | −1.01327            | 0.00203       | S264     | 0.99836              | PHKTPPRIPPKRSRSTTPPSIALAAKQALARQ |
| Ttn_A2ASS6.60 | −1.01327            | 0.00203       | T266     | 0.99033              | KTTPPPRIPPKRSRSTTPSIALAAKQALRQ |
| Ttn_A2ASS6.36 | −0.96294            | 0.03670       | S34464   | 0.96352              | VTSPRPKRVSPEPVTSPKRVSPEP|
| Ttn_A2ASS6.40 | −0.96294            | 0.03670       | T34467   | 0.81504              | PVPKPVPKRVSPEPVTSPKRVSPEPVT |
| Ttn_A2ASS6.29 | −0.54438            | 0.04350       | S1411    | 0.79632              | PTPEAVSRISVSSRLSRPRMPSAMSPA |
| Ttn_A2ASS6.30 | −0.54438            | 0.04350       | S1415    | 0.99695              | AVSRISVSSRLSRPRMPSAMSPA |
| Ttn_A2ASS6.27 | −0.41168            | 0.05240       | S283     | 0.89160              | PSLAAKQAQLAQQPSIPSRPSVRHVRAP |
| Ttn_A2ASS6.28 | −0.41168            | 0.05240       | S290     | 0.95908              | QRPSVRPSIPSRPSVRHVRAP |
| Ttn_A2ASS6.23 | 0.42746              | 0.06498       | S34451   | 0.82907              | TLTQVQARVEKAVTPKRVSPEPVRKSP |
| Ttn_A2ASS6.24 | 0.42746              | 0.06498       | S34451   | 0.98108              | ARVIEKATPSVRKSPKVRKVSK |
| Ttn_A2ASS6.38 | 0.31502              | 0.10499       | T33859   | 0.99883              | LTQDDLEMVVRPARRTPSPDYDDLRRRRR |
| Ttn_A2ASS6.31 | −0.33131             | 0.19004       | S34107   | 0.99068              | DAEERSPTPERRPSSVRPSSRLSRFSA |
| Ttn_A2ASS6.32 | −0.33175             | 0.22516       | S34109   | 0.76541              | ERRPSPTERRPSPSVSSRLSRFSA |
| Ttn_A2ASS6.25 | 1.12758              | 0.27026       | S34106   | 0.00000              | APTYMPTPEAVSRISVSSRLSRPRMSAP |
| Ttn_A2ASS6.26 | 1.12758              | 0.27026       | S34108   | 0.00000              | TYMPTPEAVSRISVSSRLSRPRMSAP |
| Ttn_A2ASS6.41 | 0.46224              | 0.28856       | Y33864   | 0.89320              | LEMVRPARRTPSPDYDDLRRRRRSGDM |
| Ttn_A2ASS6.33 | −0.27602             | 0.31791       | S34112   | 0.88295              | SPTPTERRPSPSVSSRLSRFSA |
| Ttn_A2ASS6.22 | 0.19164              | 0.43120       | S307     | 0.93434              | VRHVRAPTPSPVRVSAGRISTSPRSVKS |
| Ttn_A2ASS6.42 | −0.06749             | 0.79212       | S307     | 0.99417              | RSHVVRAPTPSPVRVSAGRISTSPRSVKS |
| Ttn_A2ASS6.43 | −0.06749             | 0.79212       | S307     | 0.93434              | VRHVRAPTPSPVRVSAGRISTSPRSVKS |
| Ttn_A2ASS6.58 | −0.06749             | 0.79212       | T299     | 0.99713              | PVRHVRAPTPSPVRVSAGRISTSPRSVKS |

Results are sorted by the nominal \( P \)-value in an ascending order. Highlighted values indicate significantly regulated phosphorylation between Sham and HFP EF.

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criteria for a “high probability of HFpEF” based on HFA-PEFF diagnostic algorithm for human HFpEF (58, 69).

In the present study, extensive proteomics and phosphoproteomics analysis permitted in-depth screening of the changes in protein expression, post-translational modifications (i.e., phosphorylation), and pathway alterations in HFpEF. These included but were not limited to: (I) changes in cardiac metabolism, where the predominant components were the mitochondrial metabolic processes and mitochondrial dysfunction; (II) alteration in cardiac contractile function-related proteins; (III) overexpression of pathways related to immune modulation; and (IV) a significant decrease in SIRT3 expression, that was validated by immunoblotting.

We found marked changes in signatures of protein expression related to mitochondrial function and oxidative metabolism of energy substrates in HFpEF. There was a significant decrease in targets related to mitochondrial substrate oxidation, suggesting that cardiac mitochondrial metabolic function is impaired in HFpEF. Interestingly, there was an upregulation of the ketone bodies transporter SLC16A1 in the LV of HFpEF, but this was not accompanied by comparable changes in ketone metabolism enzymes. Although not investigated in this study, these findings may contribute to the metabolic impairment seen in HFpEF by increasing the transport of ketone bodies into the mitochondria, but without a compensatory catabolic response. We hypothesize that this mismatch in mitochondrial substrate intake and utilization results in mitochondrial ketone bodies accumulation which may detrimentally affect cardiac function (70). Ketone bodies are thought to be a relevant energy source in both preclinical HFpEF models (71) and advance HFrEF patients (72). Additionally, it has been shown that HFpEF patients have significantly higher circulating ketone levels than HFrEF patients (73) suggesting that some of the beneficial effects of SGLT2 inhibitors in HFpEF may be due to enhanced ketone bodies availability and cardiac utilization (74–76), a process known as “thrifty substrate/fuel hypothesis” (77). We also observed decreased OXCT1 (aka SCOT, succinyl-CoA:3-ketoacid CoA transferase) expression in HFpEF hearts (Figure 3). OXCT1 allows cells to utilize energy stored in ketone bodies thus its decrease in HFpEF hearts supports a role for ketone body cardiac metabolism. Similarly, others have shown worse HF in pre-clinical models with cardio-specific deletion of OXCT1 (78).

Proteomic evaluation of PTMs is essential to understand the function of many proteins in physiological and pathophysiological settings. PTMs are regulators of protein structure and function, and in the heart the predominant PTM is phosphorylation, followed by acetylation (79), and it is also recognized that many proteins are regulated by phosphorylation independently of their expression (80).

Titin is a major cardiac protein regulated by phosphorylation and facilitates myocardial passive tension by conditioning cardiomyocyte-derived stiffness (81). Titin regulates cardiomyocyte stiffness both at the transcriptional and post-transcriptional level. At the transcriptional level, titin shifts from its compliant isoform N2BA toward its stiff isoform N2B, which contributes to the impaired diastolic function that is seen in HFpEF (33, 64, 82). In the present study,
translational and PTMs in titin are apparent in the LV of HFP EF hearts. The stiffer N2B isoform was significantly increased in HFP EF mice. However, it is notably that the N2B isoform is also the predominant isoform expressed in the LV of rodents (83). At the post-transcriptional level, despite comparable global proteomics expression between HFP EF mice and Sham, phosphoproteomics analysis showed that titin was one of the proteins with the greatest alterations in phosphorylation in HFP EF mice. Similar to a Dahl salt-sensitive rat study (84), in these SAUNA HFP EF mice most of the significantly hyper-phosphorylated titin residues were located at the Z-disk binding region of the titin protein (Figure 5 C). Interestingly, it has been suggested that titin may be part of a Z-disk macromolecular machinery acting as a node for hypertrophic signaling (85). As such, our findings that the myofilament and myofilament-associated proteins viz. ACTA1, MYH7, MHY9, TPM1, and MYOZ2 were differentially expressed in both global and phosphoproteomics dataset, support the premise that alterations in sarcomeric and myofilament regulating proteins play a central role in HFP EF. Of these proteins, MYH7, TPM1 and MYOZ2 are known to be important in hypertrophic cardiomyopathy (86ñ88), and may play a similar role in HFP EF. However, although their function in muscle contraction is well known (89, 90), their role in cardiac hypertrophy and adverse cardiac remodeling remains elusive. It has been hypothesized that changes in cardiac architecture may be a compensatory response that eventually fails, resulting in a re-induction of fetal genes, fibrosis replacing necrotic and apoptotic cardiac cells, and a shift in metabolic substrates (91). However, additional studies are warranted to identify the precise role these myofilament-associated proteins play in HFP EF.

Because of its size, titin has more phosphorylation sites than other smaller proteins and hundreds of phosphorylation sites have been predicted based on proteomic analysis (83), Z-disk Similarly, multiple kinases are also involved in titin phosphorylation (92), representing more opportunities for the regulation of the cardiomyocyte structure and function. However, the effect that a specific phosphorylation pattern has on the function of titin is largely dependent on the specific structural domain which is modified within the protein (93). For example several studies have focused on the "spring-like" I-domain, including the N2bus and PEVK regions, likely due to the mechanically active nature of this specific domain, where phosphorylation may modulate passive and active tension of the sarcomere (85, 92ñ94). Conversely, the proline-directed kinases, including extracellular signal-regulated kinase-1/-2 (ERK1/2) and cyclin-dependent protein kinase-2 (Cdc2) were able to regulate the phosphorylation status of non-extensible Z-disk (95, 96) and C-terminal (M-band) (97) regions (98). Although additional studies using site-specific methods are needed (92), it has been suggested that changes in the phosphorylation status of these regions may have an important function, not only during developmental stages, but also regulating the binding of titin to other and M-band proteins, as well as the assembly and turnover of these binding partners (99, 100).

In addition to phosphorylation, HFP EF is also associated with hyperacetylation of mitochondrial proteins in the myocardium (101, 102). In the mitochondria, the acetylation state of key enzymes involved in mitochondrial metabolism, oxidative stress defense and mitochondrial dynamics is regulated by the mitochondrial, NAD-dependent protein deacetylase SIRT3 (103ñ106). SIRT3 interacts with at least 84 mitochondrial proteins involved in many aspects of mitochondrial biology, such as maintaining mitochondrial integrity and function (67, 106). In the present study, the global proteomics data set showed decreased expression of SIRT3 in the LV of HFP EF mice, which was also confirmed by immunoblotting. Others have shown that reduced SIRT3

![Figure 6](https://example.com/figure6.png)
expression is related to reduced NAD+ bioavailability in HFpEF, and that cardiomyocyte specific SIRT3 knockout mice developed worse diastolic dysfunction in HFpEF (60). Additional studies using whole-body knockout or transgenic mice similarly showed that SIRT3 is required to maintain cardiac contractile function under pro-hypertrophic or ischemic stress (107–110). A recent study showed that a deficit of cardiac NAD+ exists not only in pre-clinical HFpEF models but also in patients with HFpEF, and that increasing NAD+ levels with nicotinamide improved diastolic dysfunction (111). The authors hypothesized the beneficial effects were mediated, partly by increasing deacetylation of proteins that regulate the mechanico-elastic properties of cardiac myocytes such as titin and sarco/endoplasmic reticulum Ca2+ ATPase 2a (SERCA2a). Although not investigated in the present study, SIRT3 may also play a role in cardiomyocyte stiffness and impaired diastolic function in HFpEF, possibly by titin acetylation (59). SIRT3 may be a future target since, compared to younger subjects, exercise increases SIRT3 protein expression in muscle, which is decreased in older sedentary individuals (112). Interestingly, of the 7 mammalian sirtuins described, SIRT3 is the only analog whose increased expression associates with longevity in humans (113–115). Since HFpEF is highly associated with aging, and exercise training is effective in improving the quality of life in HFpEF patients (116), future studies are warranted to explore the role of SIRT3 expression in muscle in patients with HFpEF.

In conclusion, untargeted proteomics have demonstrated a key role of protein PTMs in metabolism, cell preservation and sarcomere function in the heart (117). In the present study marked proteomics and phosphoproteomics changes occurred in the heart in HFpEF mice which were related to altered mitochondrial metabolism and sarcomere contractility. It is possible that SIRT3 plays a pivotal role in HFpEF, by regulating mitochondrial metabolism and titin stiffness but this requires further study (Figure 6B).

Data availability statement

The datasets presented in this study can be found online at the PRIDE Archive, proteomics data repository (European Bioinformatics Institute, European Molecular Biology Laboratory) with the data set identifier PXD033501. Raw unedited gels images are shown in Supplementary Figure 3.

Ethics statement

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee at Boston University School of Medicine.

Author contributions

MV-M and FS contributed to the conception and design of the study. MV-M and ES performed the surgeries and physiological measurements. MV-M, ES, and ZH performed the molecular analysis. RH and BB performed the proteomic sample preparation and carried out the mass spectrometry and bioinformatics analysis. MV-M, ES, and FS wrote the first draft of the manuscript. RH wrote sections of the manuscript. All authors contributed to the manuscript revision, read, and approved the submitted version.

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Conflict of interest

FS was a full-time employee of Eli Lilly and Co, Indianapolis, IN and holds a joint, academic appointment at Boston University School of Medicine. All the work in this publication is from The Sam Lab, Whitaker Cardiovascular Institute at Boston University School of Medicine, Boston, MA. This work was funded by NIH RO1HL145985 awarded to FS. None of the work was funded nor supported by Eli Lilly and Co. FS has no other conflicts to disclose regarding the work in this manuscript.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2022.966968/full#supplementary-material
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