

Proteomics and β -hydroxybutyrylation Modification Characterization in the Hearts of Naturally Senescent Mice

Authors

Xuechun Yang, Xuehui Li, Na Yu, Rong Yan, Yan Sun, Congmin Tang, Wenjing Ding, Mingying Ling, Yiping Song, Haiqing Gao, Wenjuan Gao, Junchao Feng, Shaopeng Wang, Zhen Zhang, and Yanqiu Xing

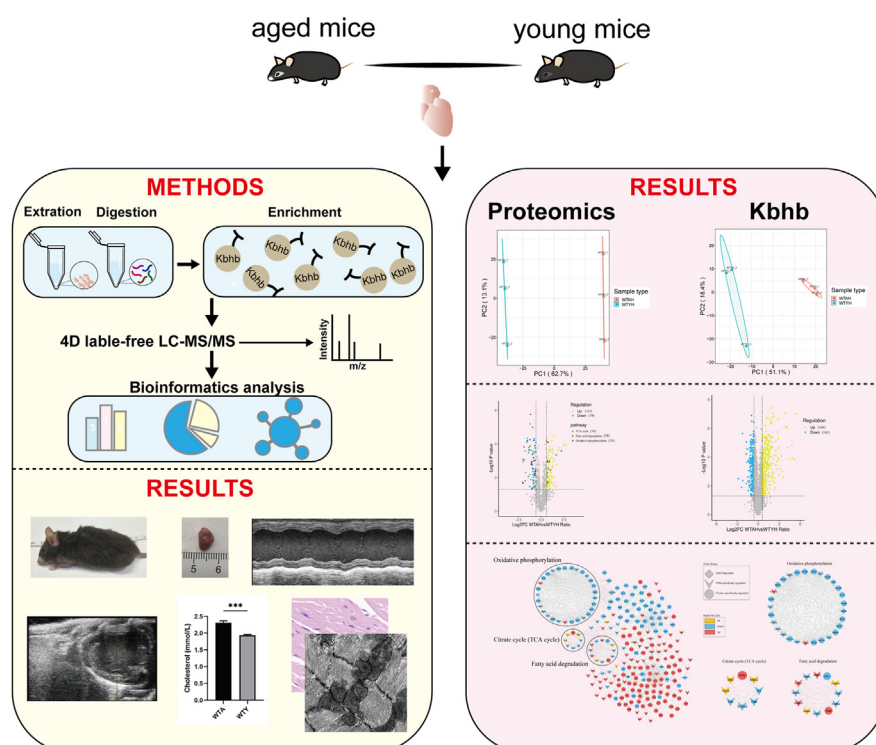
Correspondence

18560088221@163.com;
xingyanqiu@sina.com

Graphical Abstract

In Brief

In our study, we conducted quantitative proteomics and Kbhb PTM omics to comprehensively elucidate the alterations of global proteome and Kbhb modification in the hearts of aged mice. The results revealed a decline in grip strength and cardiac diastolic function in the hearts of aged mice. 183 Kbhb sites identified in 134 proteins exhibited significant differential modification in aged hearts. These Kbhb-modified proteins were predominantly localized in the mitochondria and were primarily detected in energy metabolism pathways.



Highlights

- Cardiac diastolic function were significantly declined in the hearts of aged mice.
- Proteomics and Kbhb modification omics were performed in the hearts of aged and young mice.
- Kbhb-modified proteins were primarily detected in energy metabolism pathways.

Proteomics and β -hydroxybutyrylation Modification Characterization in the Hearts of Naturally Senescent Mice

Xuechun Yang^{1,‡}, Xuehui Li^{1,‡}, Na Yu^{2,3,‡}, Rong Yan¹, Yan Sun¹, Congmin Tang¹, Wenjing Ding¹, Mingying Ling¹, Yiping Song¹, Haiqing Gao¹, Wenjuan Gao⁴, Junchao Feng², Shaopeng Wang², Zhen Zhang^{1,*}, and Yanqiu Xing^{1,*}

Aging is widely accepted as an independent risk factor for cardiovascular disease (CVD), which contributes to increasing morbidity and mortality in the elderly population. Lysine β -hydroxybutyrylation (Kbhb) is a novel post-translational modification (PTM), wherein β -hydroxybutyrate is covalently attached to lysine ϵ -amino groups. Recent studies have revealed that histone Kbhb contributes to tumor progression, diabetic cardiomyopathy progression, and postnatal heart development. However, no studies have yet reported a global analysis of Kbhb proteins in aging hearts or elucidated the mechanisms underlying this modification in the process. Herein, we conducted quantitative proteomics and Kbhb PTM omics to comprehensively elucidate the alterations of global proteome and Kbhb modification in the hearts of aged mice. The results revealed a decline in grip strength and cardiac diastolic function in 22-month-old aged mice compared to 3-month-old young mice. High-throughput liquid chromatogram-mass spectrometry analysis identified 1710 β -hydroxybutyrylated lysine sites in 641 proteins in the cardiac tissue of young and aged mice. Additionally, 183 Kbhb sites identified in 134 proteins exhibited significant differential modification in aged hearts (fold change (FC) > 1.5 or < 1/1.5, $p < 0.05$). Notably, the Kbhb-modified proteins were primarily detected in energy metabolism pathways, such as fatty acid elongation, glyoxylate and dicarboxylate metabolism, tricarboxylic acid cycle, and oxidative phosphorylation. Furthermore, these Kbhb-modified proteins were predominantly localized in the mitochondria. The present study, for the first time, provides a global proteomic profile and Kbhb modification landscape of cardiomyocytes in aged hearts. These findings put forth novel possibilities for treating cardiac aging and aging-related CVDs by reversing abnormal Kbhb modifications.

Aging is widely acknowledged as an independent risk factor for cardiovascular disease (CVD) (1). It encompasses various conditions, including coronary atherosclerotic cardiopathy, hypertension, arrhythmias, and heart failure, all of which significantly contribute to increased morbidity and mortality in the elderly population (2). While aging, cardiomyocytes undergo hypertrophic growth, accompanied by various cellular aging progresses, such as metabolic dysregulation, mitochondrial malfunction, and loss of proteostasis, which ultimately reduce the cardiac diastolic and/or conduction functions (3). However, to date, there is no available treatment to prevent or delay age-associated cardiac deterioration. A thorough elucidation of the molecular mechanisms involved in cardiac aging seems promising to decipher the regulatory mechanism of cardiac aging which in turn would facilitate the prognosis and diagnosis of aging-related CVDs.

Post-translational modification (PTM) is one of the major regulatory mechanisms that enhance the functional diversity of the proteome, which encompasses chemical modifications occurring at specific amino acid residues (4). PTMs, including phosphorylation, ubiquitination, SUMOylation, acetylation, and carbonylation, are implicated in cardiac aging and various CVDs that are affected *via* protein activity, stability, subcellular locations, and interactions (3, 5). Recently, lysine β -hydroxybutyrylation (Kbhb) has emerged as a novel PTM in which β -hydroxybutyrate (β HB) is covalently attached to lysine ϵ -amino groups (6). Histone Kbhb contributes to tumor progression, diabetic cardiomyopathy progression, and postnatal heart development by regulating energy metabolism and apoptosis (6–9). In a previous study, we established a correlation between Kbhb modification and AMP-activated protein kinase alpha 2 (AMPK α 2)-mediated energy metabolism in the

From the ¹Department of Geriatric Medicine, Key Laboratory of Cardiovascular Proteomics of Shandong Province, Qilu Hospital of Shandong University, Jinan, Shandong, China; ²Shandong Precision Medicine Engineering Laboratory of Bacterial Anti-tumor Drugs of Shandong Xinchuang Biotechnology Co., LTD, Jinan, Shandong, China; ³College of Clinical Medicine, Shandong University, Jinan, Shandong, China; ⁴Laboratory of Basic Medical Sciences, Qilu Hospital of Shandong University, Jinan, Shandong, China

[‡]These authors contributed equally to this work.

*For correspondence: Yanqiu Xing, xyngyanqiu@sina.com; Zhen Zhang, 18560088221@163.com.

hearts of AMPK α 2 knockout mice. However, no studies have yet reported a global analysis of Kbbh proteins in aging hearts or elucidated the mechanisms underlying this modification in the aging process.

Considering the paramount significance of Kbbh and the research gap within the field of cardiac aging, we performed quantitative proteomics and Kbbh PTM omics in the hearts of young and aged mice. Herein, we established the first proteome atlas and Kbbh PTM omics for hearts from both young and aged healthy mice to comprehensively illustrate the alterations of global proteome and Kbbh modification in aged hearts. Finally, the Kbbh proteome profile during cardiac aging was established; a significant reduction in Kbbh modification level was observed in aged hearts. Quantitative PTM omics analysis identified 1710 Kbbh-modified sites in 641 proteins, among which 183 Kbbh-modified sites in 134 proteins displayed significant alterations in aged hearts ($FC > 1.5$ or $< 1/1.5$, $p < 0.05$). Notably, a subset of the differential Kbbh sites was located on key proteins involved in energy metabolism, such as fatty acid degradation and tricarboxylic acid cycle (TCA), suggesting that the Kbbh modification on these key protein sites plays a pivotal role in the progression of cardiac aging. These findings contribute to the development of novel therapeutic strategies for cardiac aging and age-associated CVDs.

EXPERIMENTAL PROCEDURES

Experimental Design and statistical Rationale

Two groups of C57BL/6J mice were set up: 3- and 22-months-old comprised the young controls (WTY, $n = 10$) and aged models (WTA, $n = 10$), respectively. The various parameters measured during the experiment were body weight, heart weight, grip strength, echocardiography, serum parameters, and myocardial morphology (Fig. 1A). Three mice from each group were used as biological replicates for proteomics and Kbbh modification omics analyses. Three semi-quantitative metrics of reproducibility, Pearson's correlation coefficient, principal component analysis, and relative standard deviation, were analyzed (supplemental Fig. S1). These assessments confirmed the statistical power of the proteomic study. Physiological data were presented as mean $\pm$ standard deviation (SD). Student's t tests were conducted to calculate the p -value, and $p < 0.05$ was considered statistically significant.

Animals

All experimental procedures were approved by the Animal Ethics Committee of Shandong University (Approval No. 20157). All mice were housed in the specific pathogen-free animal chamber of Shandong University Laboratory Animal Center, with controlled temperature (20–25 °C) and humidity (55 $\pm$ 5%) under an artificial light cycle; food and water were provided ad libitum. The mice's body weights were recorded weekly throughout the study. At the end of the experiment, all animals were anesthetized with sodium pentobarbital.

Grip Strength

The grip strength of the mice's front paws was measured using a grip strength meter (Jiangsu Science Biological Technology Co., Ltd, SANS, SA417). The measurements were recorded at 3 and 22 months,

as described previously (10). Each mouse underwent five trials, and the final grip strength value per mouse was recorded as the average of the top three trials.

Serum Analysis

Blood samples were collected *via* cardiac puncture, and serum was obtained after clotting and centrifugation (3000 rpm for 20 min at 4 °C). Enzyme-linked immunosorbent assay (ELISA) was performed to detect the levels of triglycerides (TGs), cholesterol (CHO), free fatty acids (FFAs), and β HB. The following serum assays were conducted using kits, according to the manufacturer instructions: triglycerides (RayTo, Shenzhen, S03027), cholesterol (RayTo, Shenzhen, S03042), FFA (Jiancheng, Nanjing, A042-2-1), and β HB (USCN Kit Inc., CEB022Ge).

Histological Examination

The mice cardiac tissue was fixed in a 4% paraformaldehyde for 3 to 5 days and dehydrated in ethanol from Kelong Chemical Reagent Factory. Subsequently, the dehydrated tissues were embedded in paraffin and sectioned into 4- μ m-thick slices using a LEICA RM2235 microtome. These slices were stained with hematoxylin-eosin (H&E) for histological examination under a light microscope (OLYMPUS, DX45).

Transmission Electron Microscopy

For ultrastructural evaluation, small sections of the cardiac tissue were sliced into 1-mm³ blocks on ice. These blocks were fixed in a 3% glutaraldehyde solution in cacodylate buffer at 4 °C for 2 h, followed by post-fixation in 1% osmium-tetroxide phosphate buffer for 2 h. Subsequently, the samples were dehydrated using a graded series of ethanol and acetone and then permeated and embedded in an epoxy resin. Semithin slices were prepared from the embedded samples, and approximately 70-nm ultrathin sections were sliced using a Marada-G2 type CCD photo ultrastructure of pictures. Finally, after dyeing with uranyl acetate and lead citrate, the ultrathin sections were observed under a JEM-1200EX (JEOL, Japan) transmission electron microscope.

Western Blot (WB)

Proteins extracted from isolated cardiac tissue of young and aged mice were separated using SDS-PAGE and transferred onto polyvinylidene difluoride membranes (0.22 μ m, New York, USA). Then, the membranes were blocked with 5% nonfat milk in Tris-buffered saline Tween 20 at room temperature for 1 h. Subsequently, the membrane was probed with the primary antibody at 4 °C overnight and then with the secondary antibody at room temperature for 1 h before detection with an enhanced chemiluminescence (ECL) system (apparatus: Chemiluminescent Imaging Shanghai, China). The antibodies were as follows: anti-succinylated lysine (PTM419 1:1000; PTM Biolab), anti-malonylated lysine (PTM901 1:1000; PTM Biolab), anti-crotonylated lysine (PTM-502 1:1000; PTM Biolab), anti- β -hydroxybutyrylated lysine (PTM-1201RM 1:1000; PTM Biolab), mouse IgG (H + L) secondary antibody (Pierce), and rabbit IgG (H + L) secondary antibody (Pierce).

Proteome and Kbbh PTM Omics Procedures

Protein Extraction—The heart samples from both WTA and WTY mice were ground into powder in liquid nitrogen. To extract the proteins, the powder was homogenized in a lysis buffer containing 8 M urea, 1% protease inhibitors, 3 μ M trichostatin A, and 50 mM nicotinamide and sonicated using a high-intensity ultrasonic processor (Scientz) on ice. The supernatant was collected by centrifugation at 12,000g, 4 °C for 10 min.

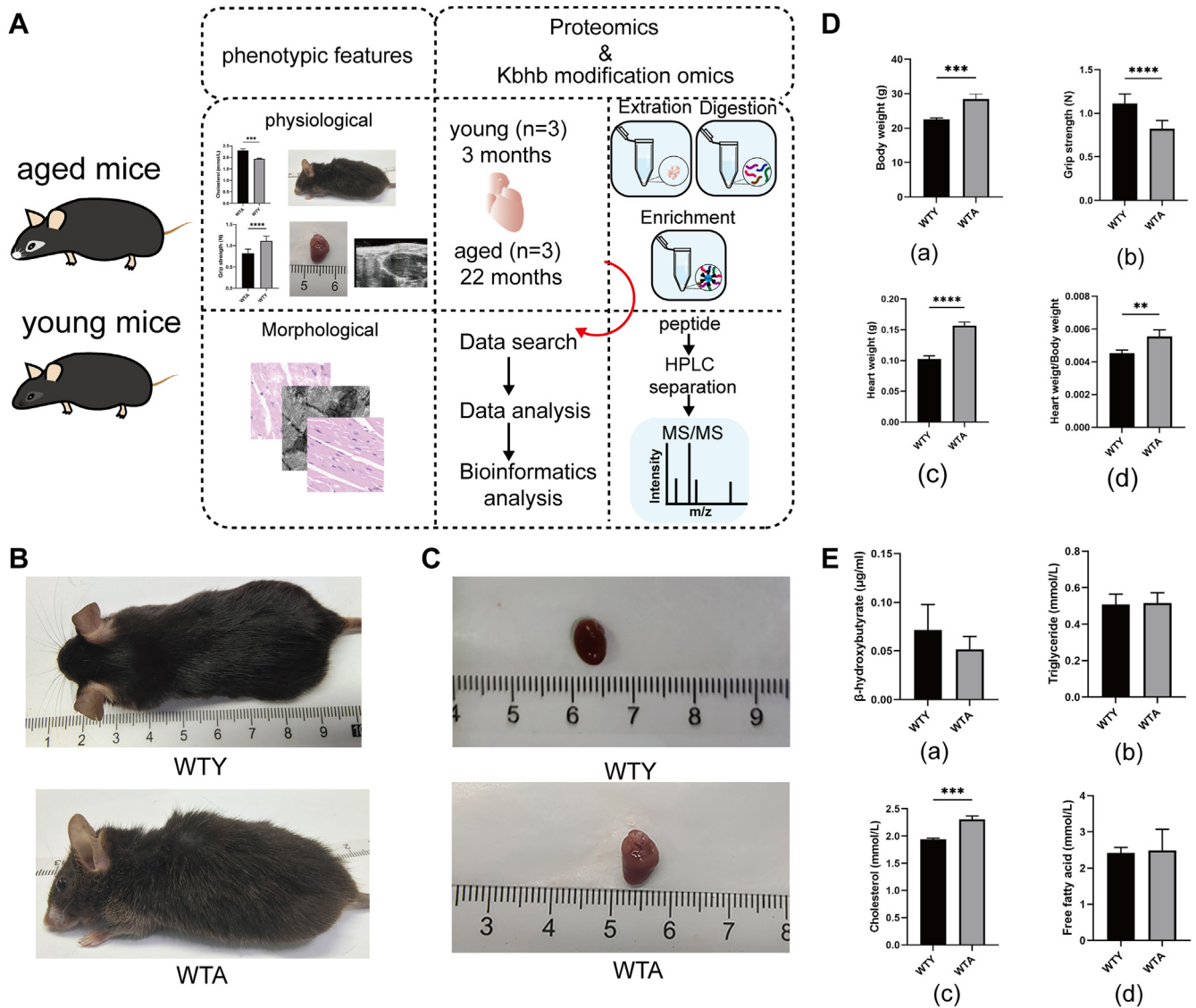


FIG. 1. Characterization of aging-associated physiological changes in mice. A, flowchart of our whole study. B, appearance of WTA and WTY mice. C, comparison of heart size of WTA and WTY mice. D, difference in body weight (a), grip strength (b), heart weight (c), ratio of heart weight to body weight (d) between WTA and WTY mice. E, levels of β HB (a), TG (b), CHO (c), FFA (d) between WTA and WTY mice. β HB, β -hydroxybutyrate; CHO, cholesterol; FFA, free fatty acid; TG, triglyceride; WTA, wild-type aged; WTY, wild-type young. Data are presented as the mean $\pm$ SD. $n = 10$ mice per group. $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

Trypsin Digestion—An equivalent volume of 40% tricarboxylic acid was added to the above supernatant, followed by centrifugation at 4500g, 4 °C for 5 min. The resulting pellet was washed with precooled acetone and resuspended in a buffer containing 20% tetraethylammonium bromide. This lysate was dispersed using a sonicator. The protein extract was reduced with 5 mM dithiothreitol at 56 °C for 30 min, alkylated with 11 mM iodoacetamide, and incubated at room temperature for 15 min in the dark. The protein samples were diluted with 100 mM NH_4HCO_3 to make sure the urea concentration was less than 2 M. Then, trypsin was added to the samples at a mass ratio of 1:50 (trypsin to protein) and incubated overnight at 37 °C, followed by a second digestion step at a mass ratio of 1:100 (trypsin to protein) for 4 h. Finally, the digested peptides were desalted on a C18 SPE column.

Kbbhb-Modified Affinity Enrichment—The peptides were dissolved in NETN buffer (100 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, 0.5%

NP-40, pH 8.0) and incubated with 20 μ l of agarose beads conjugated with anti-Kbbhb antibodies on a gentle shaker at 4 °C overnight. Following incubation, the beads were washed four times with NETN buffer to remove nonspecific enrichment and washed twice with ddH_2O . The enriched peptides were eluted with 0.1% trifluoroacetic acid (TFA), vacuum-dried, and desalted for mass spectrometry analysis.

LC-MS/MS

The peptides were solubilized in solvent A (0.1% formic acid and 2% acetonitrile), loaded onto a reversed-phase analytical column (25 cm $\times$ 75 μ m), and separated using a gradient of 6 to 24% solvent B (0.1% formic acid in acetonitrile) over 70 min, 24 to 35% in 14 min, up to 80% in 3 min, and holding at 80% for the last 3 min, at a flow rate of

450 nL/min on a nanoElite HPLC system (Bruker Daltonics). The separated peptides were ionized and analyzed on a timsTOF Pro mass spectrometry system (Bruker Daltonics), operated with an electrospray voltage of 1.60 kV. The precursors and fragments were analyzed by the TOF detector, with an MS/MS scan range of 100 to 1700 m/z. The timsTOF Pro was operated in parallel accumulation serial fragmentation (PASEF) mode. The precursors with a charge state of 0 to 5 were selected for fragmentation. A total of 10 PASEF-MS/MS scans were acquired per cycle. The dynamic exclusion was set to 30 s.

Data Search

The MS/MS raw data were processed using MaxQuant software (v.1.6.15.0). The data were searched against *Mus musculus* SwissProt database (updated on 21/07/21, 17,089 entries) along with a reverse decoy database. Trypsin/P was specified as the cleavage enzyme, and four missed cleavages were allowed (proteomics allowed two missed cleavages). The mass tolerance for precursor ions was set at 20 ppm in the first search and 5 ppm in the main search, while the mass tolerance for fragment ions was set at 0.02 Da. Carbamidomethyl on Cys was set as a fixed modification, and acetylation on protein N-terminal, β -hydroxybutyrylation on Lys, and oxidation on Met were set as variable modifications. The false discovery rate (FDR) was adjusted to <1%. Peptides with a minimum length of seven amino acid residues were considered, and a localization probability of 0.75 was set for β -hydroxybutyrylation sites.

Data Analysis

The FC was calculated as the ratio of the mean relative quantitative values of the modified sites in the WTA (aged) group to the WTY (young) group. To select different modified sites, $FC > 1.5$ or $< 1/1.5$ was set, indicating upregulated and downregulated proteins/sites, respectively. The quantitative data were utilized to compare protein abundance between the WTA and WTY groups. A Student's *t* test was performed at log₂-transformed values, and a *p*-value < 0.05 was considered statistically significant.

Bioinformatics Analysis

The proteome was annotated with gene ontology (GO) terms using the UniProt-GOA database: <http://www.ebi.ac.uk/GOA/>. Proteins were classified based on their involvement in biological processes (BPs), cellular components (CCs), and molecular functions (MFs). The subcellular localization of proteins was predicted using Wolfpsort software. The MOMO software, based on motif-x (11), was employed to derive the sequence model around each modified site in all protein sequences (10 amino acids upstream and downstream of the β -hydroxybutyrylated site). When the number of peptide segments of a characteristic sequence form was >20 and the statistical *p*-value was <0.000001, the characteristic sequence form was considered a motif of the modified peptide segment. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed using the DAVID database. The protein-protein interaction (PPI) network of Kbbh-modified proteins was constructed using the STRING database (v11, <http://www.string-db.org/>) and visualized using Cytoscape (v.3.7.2).

Statistical Analysis

Proteomic and Kbbh modification omics analyses were performed on heart samples from three mice per group. The reproducibility of the experiments was evaluated using relative standard deviation, principal component analysis, and Pearson's correlation coefficient (supplemental Fig. S1). For physiological data, quantitative values are presented as mean $\pm$ SD, and an independent Student's *t* test was employed to assess the differences between WTA mice and WTY

mice using SPSS 26.0 software. Statistical significance was defined as *p* < 0.05. All the graphs were generated using GraphPad Prism 8 software.

RESULTS

Physiological Phenotypes in Aged Mice

To investigate the physiological and morphological changes associated with cardiac aging, we obtained cardiac tissues from 10 young (3-months-old) and 10 aged (22-months-old) C57BL/6J mice, which are equivalent to young (20-years-old) and aged (70-years-old) humans, respectively. The experimental flowchart is illustrated in Figure 1A. The aged mice exhibited reduced activity, disheveled hair, and white whiskers, indicating signs of aging, compared to young mice (Fig. 1B). Furthermore, the cardiac size of the aged mice was significantly larger than that of the young mice (Fig. 1C). Also, the body weight, heart weight, and the ratio of heart weight to body weight were significantly higher in aged mice compared to the young mice. Additionally, the grip strength of the WTA group was significantly lower than the WTY group (Fig. 1D), and the level of β HB in aged mice showed a slight decrease compared to the young mice, although not statistically significant. However, the levels of TG and FFA in aged mice were slightly increased, while CHO was significantly higher than that of the WTY group (Fig. 1E).

To assess age-related alterations in cardiac function, we conducted an echocardiographic evaluation of the left ventricle (LV). The results revealed a slight decline in global left ventricle systolic function, as indicated by reduced left ventricle ejection fraction (LVEF) and left ventricle fractional shortening (LVFS) in the 22-month-old mice compared to the 3-month-old mice. Moreover, LV diastolic function was significantly compromised during aging, as indicated by a decreased ratio of the early to late diastolic mitral flow velocity (E/A) and the declined ratio of the early to late diastolic mitral annular velocity (E'/A') (Fig. 2).

Morphological Phenotypical Features in Aged Mice

In the H&E-stained sections, the young hearts exhibited regularly arranged and intact cardiomyocytes with uniform and well-organized myocardial nuclei (Fig. 3A). In contrast, the aged hearts showed disorganized cardiomyocytes with lightly stained cytoplasm and unclear nuclei (Fig. 3A, indicated by red arrow). Additionally, some cardiomyocytes appeared fragmented or distorted.

Since mitochondria play a crucial role in energy metabolism and myocardial cell homeostasis, we specifically examined the alterations in mitochondria within cardiomyocytes of aging mice. The ultrastructure of the mitochondria in cardiomyocytes is presented in Figure 3B. In the cardiomyocytes of young mice, mitochondria were well-arranged and distributed, appearing circular or oval in shape, with clear and uniform mitochondrial cristae (Fig. 3B). However, the mitochondria

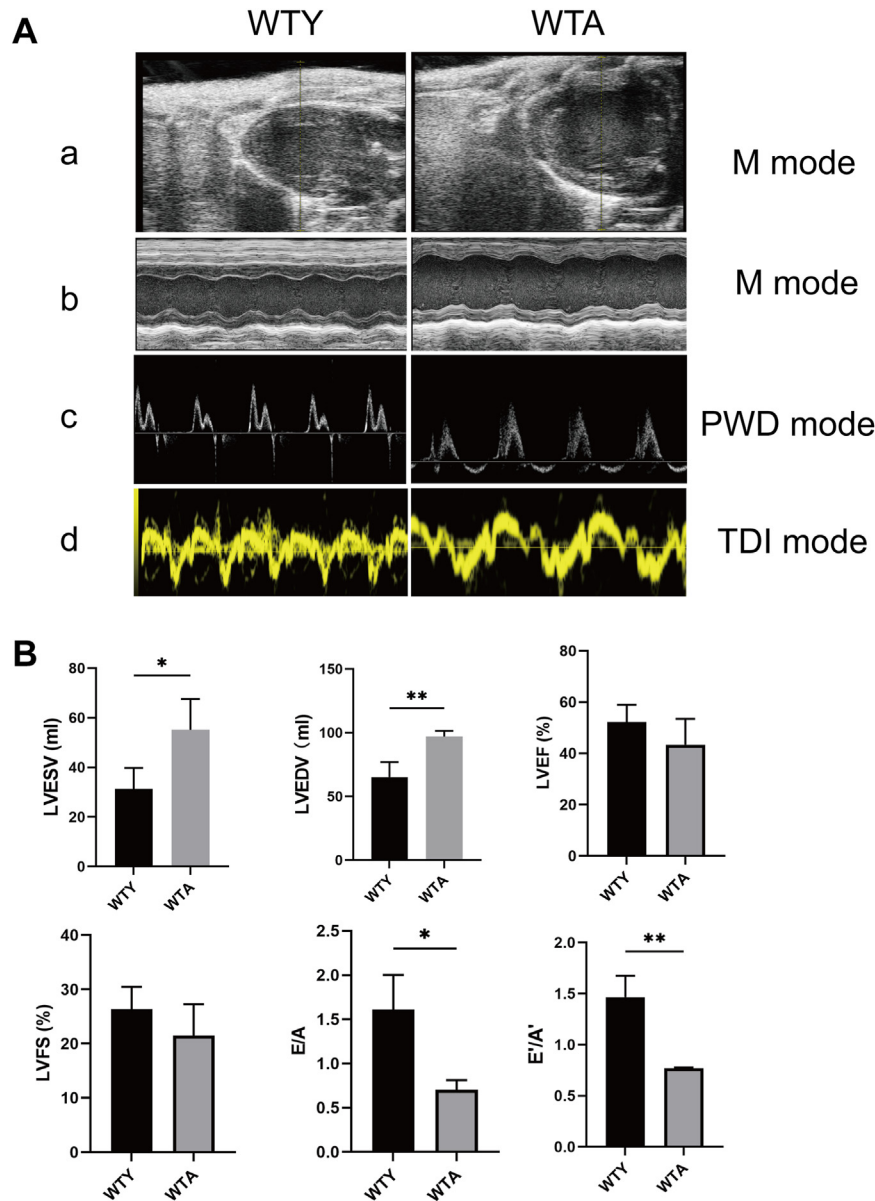


FIG. 2. Echocardiogram of WTA and WTY mice. A, representative image of echocardiography. Left ventricular long-axis views on M-echocardiography (a), size of left ventricular on M-echocardiography (b), pulse wave Doppler (PWD) measurements of mitral inflow (c), tissue Doppler imaging (TDI) of lateral wall motion at the septal annulus (d). B, effects of aging on the left ventricular systolic and diastolic function in mice. LVESV: left ventricular end-systolic volume; LVEDV: left ventricular end-diastolic volume; LVEF: left ventricular ejection fraction; LVFS: left ventricular fraction shortening. Data are presented as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$.

exhibited uneven distribution and showed shortened and flattened cristae in aged mice (Fig. 3B).

Quantification and Identification of the Cardiac Global Proteome by Data-Dependent Acquisition Proteomics

In order to identify proteins associated with cardiac aging, we conducted high-throughput and quantitative data-dependent acquisition (DDA) proteomics analysis on cardiac tissue from 3- and 22-month-old mice ($n = 3$ per group). A total of 5113 proteins were quantified in aged and young mice, and

590 significantly differentially expressed proteins (DEPs) were obtained by defining the truncation value as 1.5-fold and $p < 0.05$. Among these DEPs, 348 proteins were upregulated and 242 proteins were downregulated in the hearts of aged mice (Fig. 4A). The details of DEPs, including protein name, molecular weight, subcellular localization, and main functions, are provided in [Supplementary Data S1](#).

To gain insights into the proteome alterations during cardiac aging, the DEPs were categorized based on subcellular localization, BPs, CCs, and MFs using GO analysis, Clusters

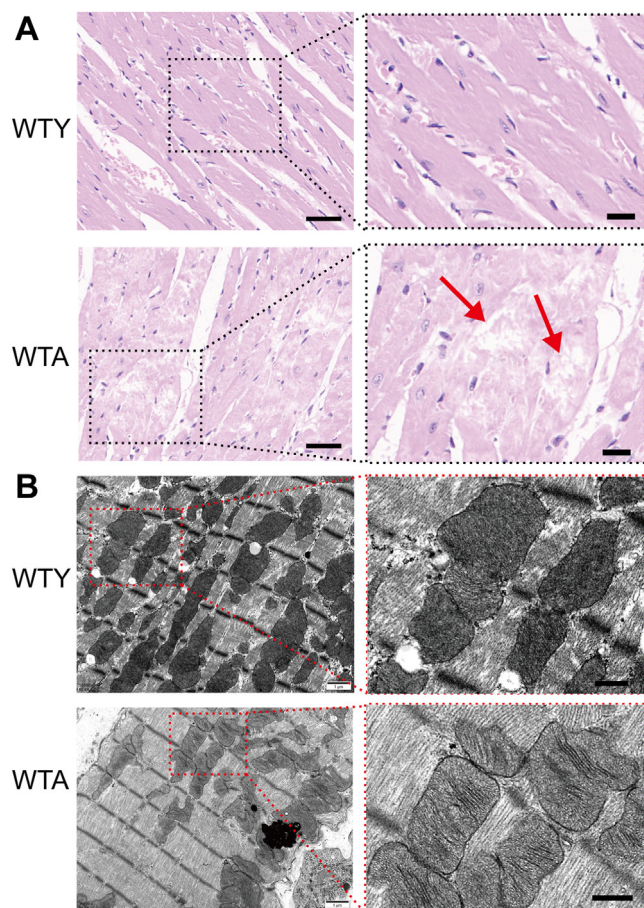


FIG. 3. **Morphological analysis of WTA and WTY mice.** A, H&E staining of hearts from the WTA and WTY mice [scale bar: 100 μ m (left), 50 μ m (right)]. B, mitochondrial ultrastructures in the cardiomyocytes of each group under transmission electron microscope [scale bar: 1 μ m (left), 0.5 μ m (right)].

of Orthologous Groups (COG/KOG) category analysis, and KEGG analysis. The subcellular localization of these DEPs is shown in Figure 4B. The largest proportion of DEPs was detected in the cytoplasm (29.32%), followed by the extracellular matrix (20%) and nucleus (17.46%). Notably, 16.1% of the DEPs were localized in the mitochondrion (Fig. 4B). GO analysis revealed that in the BP category, the proteins related to the regulation of biological process and organic substance metabolic process occupied the top two positions, while CC consisted of intracellular, cytoplasm, and organelle. In the MF classification, the majority of the proteins were categorized into binding and catalytic activity (Fig. 4C). COG analysis revealed that a large proportion (47.06%) of the DEPs are involved in the cellular processes and signaling. Additionally, 29.22% of proteins were associated with metabolism processes, specifically energy production and conversion (42/527), lipid transport and metabolism (29/527), and carbohydrate transport and metabolism (24/527) (Fig. 4D).

In addition to classifying the MF of DEPs by GO analysis, the prominent pathways involving these DEPs were confirmed by KEGG analysis. The KEGG category analysis showed that pathways associated with hypertrophic cardiomyopathy, dilated cardiomyopathy, and collecting duct acid secretion were significantly downregulated in the hearts of aged mice ($FC < 0.5$). Additionally, oxidative phosphorylation and longevity regulation pathways were also downregulated in the hearts of aged mice ($0.5 < FC < 1/1.5$). On the other hand, DEPs involved in fatty acid biosynthesis and pentose phosphate pathway were significantly upregulated in the hearts of aged mice ($FC > 2.0$); also, complement and coagulation cascades were upregulated to higher >2.0 -fold in the hearts of aged mice. The DEPs related to lipid/amino acid/carbohydrate metabolism pathways were also significantly upregulated in the hearts of aged mice ($1.5 < FC < 2.0$, Fig. 4E).

Quantitative and Functional Characteristics of Differential Modified Kbhbs Proteins in Aged Mice

Post-translational modifications (PTMs) are crucial and ubiquitous regulatory mechanisms by which specific amino acid residues of proteins are chemically modified. Moreover, these modifications play a critical role in maintaining the activity and stability of protein function and are closely related to cardiac aging and various CVDs (4, 5). In the present study, five forms of PTMs, including lysine succinylation, crotonylation, malonylation, β -hydroxybutyrylation (Kbhbs), and lactylation were analyzed in the hearts of both young and aged mice based on WB analysis. Among these, the abundance of lysine Kbhbs proteins was discovered as the most significantly reduced PTM in aged mice (Fig. 5), suggesting that the level of Kbhbs in aged hearts was significantly inhibited.

To identify and quantify specific β -hydroxybutyrylated lysine sites in the proteins in aged heart tissue, we used antibody-based enrichment of β -hydroxybutyrylated peptides followed by the DDA proteomics technique as described previously (10). Subsequently, we identified 1710 β -hydroxybutyrylated lysine sites in 641 proteins in the cardiac tissue from young and aged mice. Applying a 1.5-fold cutoff, we identified 183 differentially modified sites in 134 proteins. Among these, 104 sites in 85 proteins were upregulated (yellow), while 79 sites in 49 proteins were downregulated (blue) in aged hearts (Fig. 6A).

Next, we analyzed the subcellular localization and conducted GO analysis to categorize the differentially Kbhbs-modified proteins based on BPs, CCs, and MFs. The subcellular localization analysis revealed that a significant proportion of these proteins was localized in the mitochondria (34.4%), indicating that the organelle might be the primary site for Kbhbs (Fig. 6B). The upregulated Kbhbs-modified proteins were primarily located in the cytoplasm (40.48%), while the downregulated modified proteins were distributed in the mitochondria (60.42%) (supplemental Fig. S2).

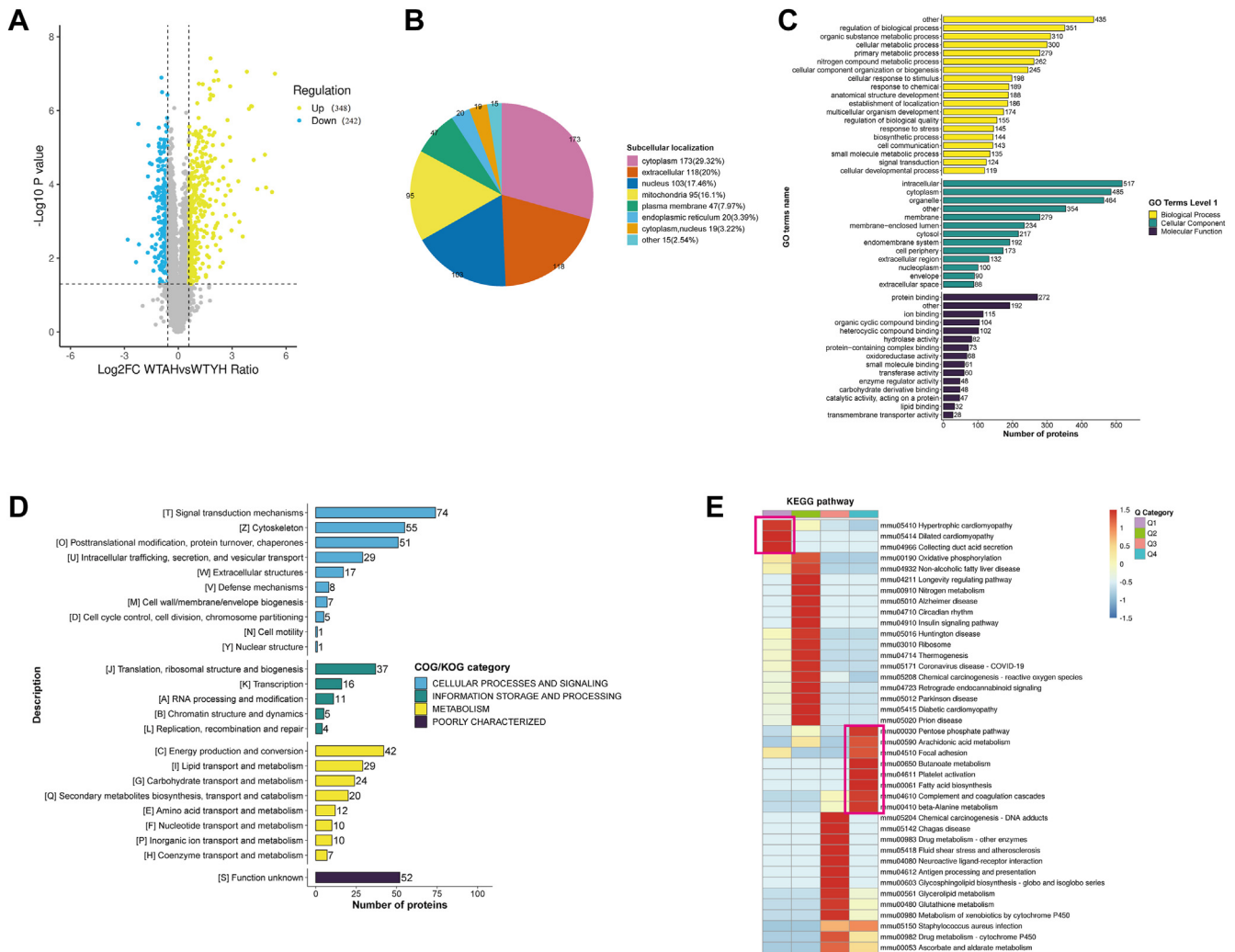


FIG. 4. Whole proteomics analysis of WTA and WTY mice. A, DEPs in WTA and WTY mice; Yellow represents upregulated proteins, and blue represents downregulated proteins. B, subcellular location of these proteins. C, GO classification of DEPs. (yellow: biological process; green: cellular component; purple: molecular function). D, COG/KOG classification of DEPs. (blue: cellular processes and signaling, green: information storage and processing, yellow: metabolism, purple: poorly characterized). E, KEGG pathways of DEPs depended on four categories. (Q1: FC < 0.5; Q2: 0.5 < FC < 1/1.5; Q3: 1.5 < FC < 2; Q4: FC > 2). COG/KOG, clusters of orthologous groups/eukaryotic ortholog groups; DEPs, differentially expressed proteins; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; WTA, wild-type aged; WTY, wild-type young.

In terms of BPs, the Kbh-modified proteins participated in cellular processes (117/134) and metabolic processes (91/134), as shown in Figure 6C. In MF analysis, the top two functions of Kbh-modified proteins were binding and catalysis (Fig. 6C) and were located in the cell based on the CC analysis (Fig. 6C). Further functional analysis based on COG/KOG categories revealed that about 28 of the 134 Kbh-modified proteins were categorized into energy production and conversion under metabolism (Fig. 6D). The GO and COG/KOG analysis for upregulated and downregulated Kbh-modified proteins are shown in supplemental Figs. S3 and S4. The results indicated that Kbh-modified proteins are mostly localized in the mitochondria of aged cardiomyocytes

and closely related to metabolism. Detailed information on the Kbh can be found in Supplementary Data S2.

Functional Enrichment Analysis of Differential Kbh Proteins in Aged Hearts

To gain further insights into the preferred protein types that undergo Kbh modification, we conducted a functional enrichment analysis of the Kbh-modified proteins using GO analysis. In terms of BPs, the upregulated Kbh-modified proteins were enriched in cardiac muscle hypertrophy and adaptation, cell differentiation, and secretion. On the other hand, the downregulated Kbh-modified proteins were

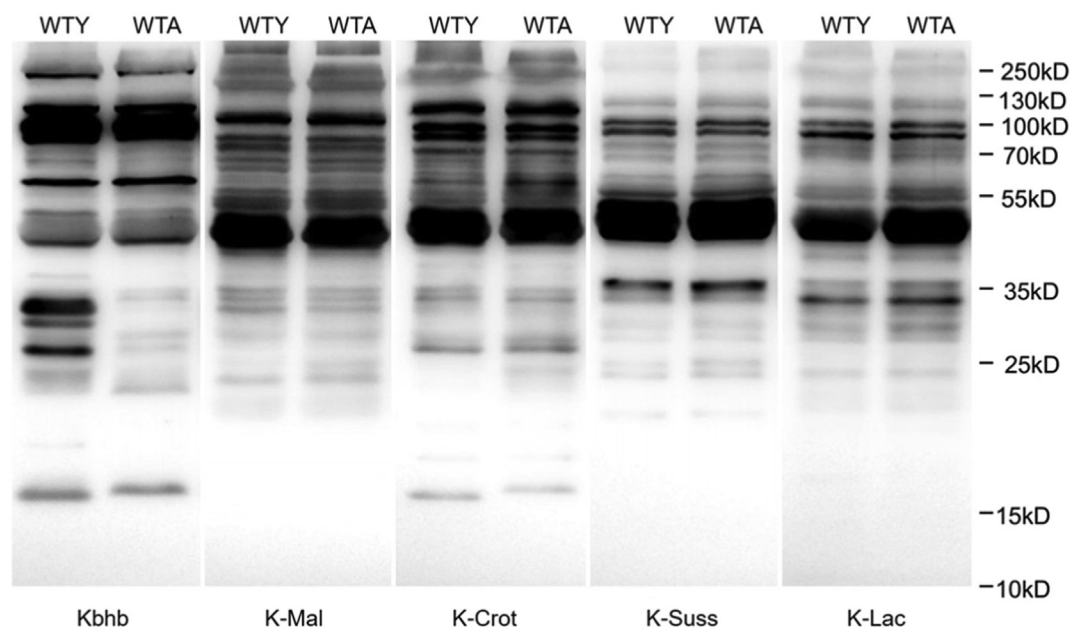


FIG. 5. **Five modifications of the hearts of WTA and WTY (Kbhb, K-mal, K-crot, K-succ, and K-lac).** Kbhb, Lysine β -hydroxybutyrylation; K-mal, Lysine malonylation; K-crot, Lysine crotonylation; K-suss, Lysine succinylation; K-lac, Lysine lactylation.

enriched in lipid metabolism and oxidation, TCA metabolic process, and carboxylic acid catabolic process (Fig. 7A).

Regarding MF, the upregulated Kbhb-modified proteins were primarily enriched in binding activity, while the down-regulated proteins were highly enriched in catalytic enzymes and transferase activity involved in energy metabolism, such as C-acyltransferase and acetyl-CoA C-acyltransferase activities (Fig. 7B).

In terms of CC enrichment, the upregulated Kbhb-modified proteins were mainly localized in the cytoplasm, including the endoplasmic reticulum and endocytic vesicles. Conversely, the downregulated Kbhb-modified proteins were predominantly associated with the mitochondrion (Fig. 7C). This finding suggested that the mitochondria are crucial for the Kbhb modification of proteins involved in energy metabolism.

Furthermore, KEGG pathway enrichment analysis revealed that the upregulated Kbhb-modified proteins are involved in pathways such as antigen processing and presentation, protein processing in the endoplasmic reticulum, and dilated cardiomyopathy (Fig. 7D). In contrast, the downregulated Kbhb-modified proteins are associated with fatty acid elongation, glyoxylate and dicarboxylate metabolism, the TCA cycle, and oxidative phosphorylation pathways (Fig. 7E). These findings indicated that the downregulated Kbhb-modified proteins are closely related to energy metabolism in cardiac aging.

To comprehensively understand the effects of Kbhb modification in cardiac aging, the differentially Kbhb-modified proteins were divided into four clusters based on differential expression (Q1: <0.5 , Q2: $0.5\text{--}1/1.5$, Q3: $1.5\text{--}2.0$, Q4: >2.0). The subsequent KEGG pathway analysis for each cluster

revealed that hypertrophic cardiomyopathy, dilated cardiomyopathy, and the mTOR signaling pathway were enriched with an FC ratio of >2.0 . In contrast, pathways related to oxidative phosphorylation, fat digestion and absorption, TCA cycle, and synthesis and the degradation of ketone bodies, are significantly downregulated with an FC < 0.5 (Fig. 7F).

Quantitation and Identification Characteristics of Kbhb Sites in Aged Hearts

In our present study, a total of 1710 β -hydroxybutyrylated lysine sites in 641 proteins were identified in the cardiac tissue of young and aged mice. Among these, a total of 347 proteins (54.13%, 347/641) had only one Kbhb site. The proportion of proteins with more than two lysine β -hydroxybutyrylated residues was 45.87% (294/641) (Fig. 8A).

Notably, several specific proteins exhibited significant Kbhb modifications (Fig. 8B). Most of these proteins were the regulators of myocardial function and energy metabolism. The giant sarcomere protein Titin (Ttn) heavily modified with 184 lysine residues regulates cardiomyocyte muscle and cardiac stiffness and function (12). Isocitrate dehydrogenase 2 (IDH2), a key enzyme in the TCA cycle, consists of 17 Kbhb sites. Trifunctional enzyme subunit alpha (HADHA), a mitochondrial β -oxidation enzyme that regulates hepatic glucagon response by promoting β -hydroxybutyrate production, displayed 16 Kbhb sites (13). Additionally, the K34 site of cytochrome C oxidase, the rate-limiting enzyme of mitochondrial respiration, was downregulated in aged hearts. Aspartate aminotransferase (got2) involved in ATP energy synthesis during cellular energy deficiency harbored 4 Kbhb sites (14). These findings suggested that specific Kbhb modifications of key enzymes

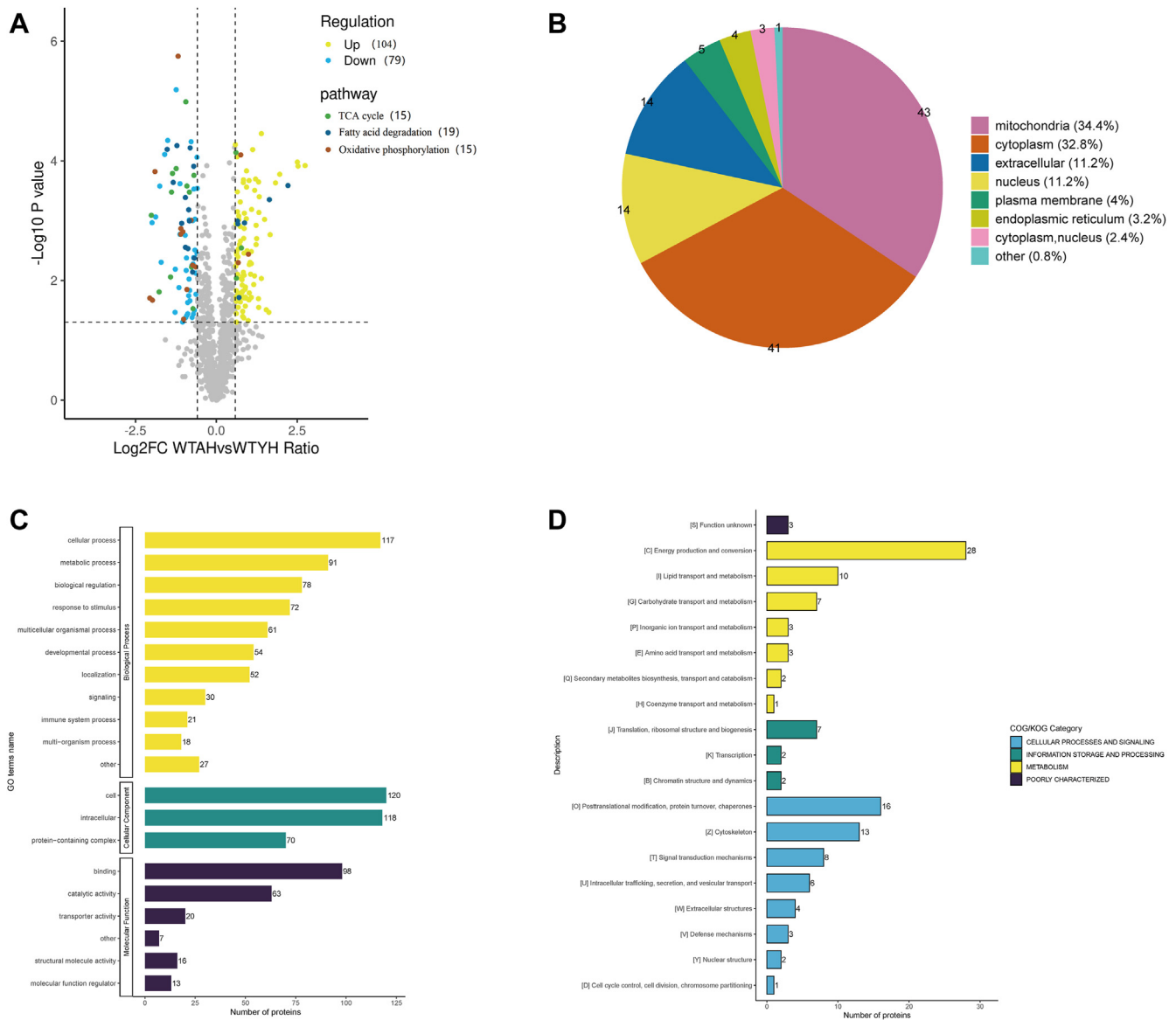


FIG. 6. Characteristics of differential modified Kbh proteins in aged mice. A, Volcano plot of significant identified sites. (FC > 1.5 or < 1/1.5). B, subcellular location of differential modified Kbh proteins. C, GO classification of differential modified Kbh proteins. Yellow represents upregulated proteins, and blue represents downregulated proteins. D, COG classification of differential modified Kbh proteins. (blue: cellular processes and signaling, green: information storage and processing, yellow: metabolism, purple: poorly characterized). GO, gene ontology; COG/KOG, clusters of orthologous groups/eukaryotic ortholog groups.

involved in energy metabolism and cardiomyocyte function contribute to cardiac aging.

To further investigate the features of Kbh sites, we used the MoMo software and hierarchical clustering to analyze the flanking sequences from -10 to +10 of the Kbh site. Our results revealed that the hydrophobic amino acids F (phenylalanine), L (leucine), W (tryptophan), Y (tryptophan) at +1 positions and A (alanine), E (glutamic acid), G (glycine) at -1 positions had the highest frequency around the Lys Kbh sites (Fig. 8C). The amino acid sequences of Kbh modified peptides were further analyzed using the Motif-X program

(Supplementary Data S2). Consistent with the results of the amino acid heat map (Fig. 8C), a total of 13 conserved motifs were obtained (Supplementary Data S2, sheet S8). These conserved sequences matched 1563 identified modified peptides with varying abundance (Supplementary Data S2, sheet S9).

PPI Network Between Global Proteome and Kbh-Modified Proteome in Aged Heart

Since altered total protein levels may interfere with the alteration on Kbh-modified sites, we drew a nine-quadrant

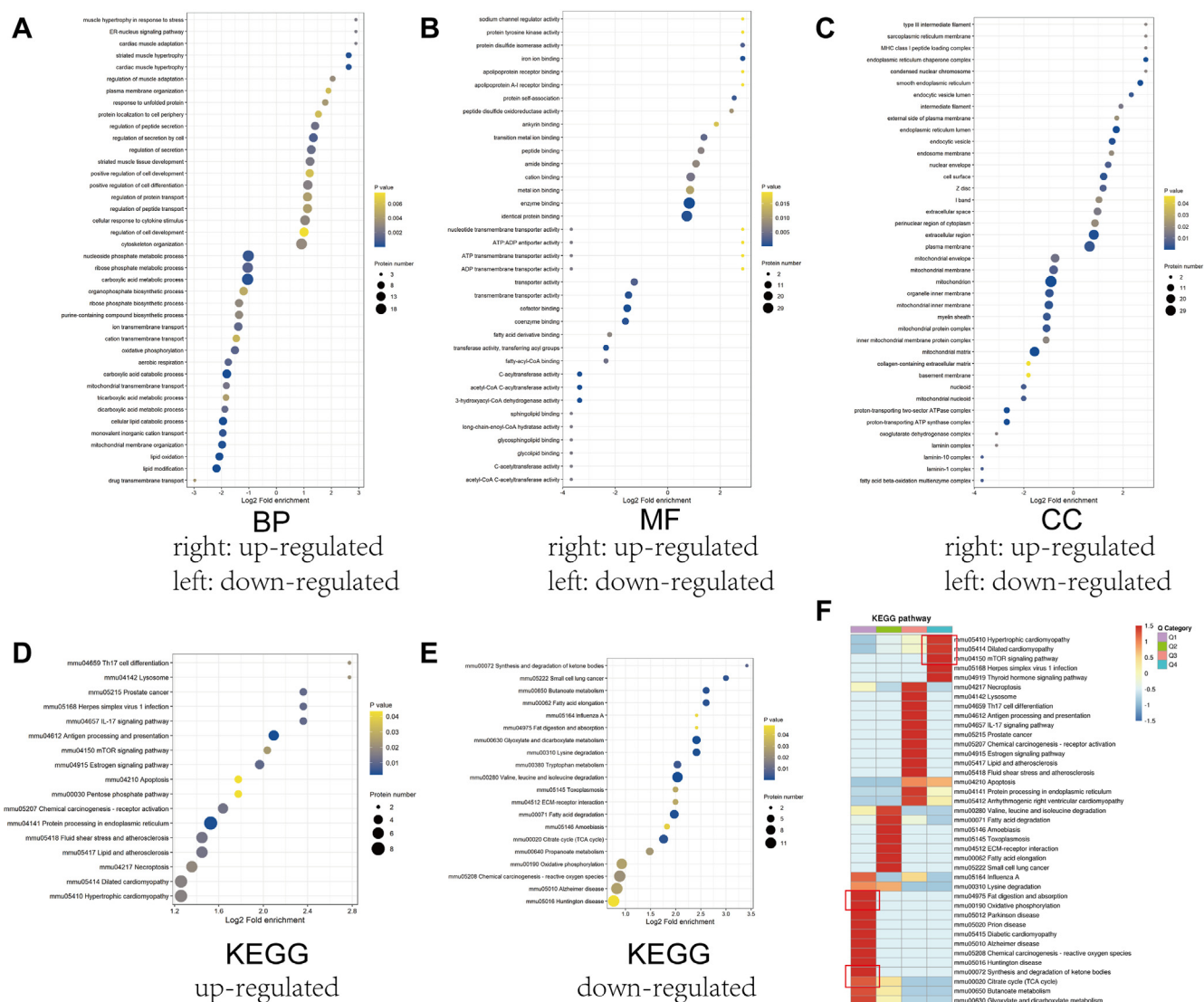


FIG. 7. Functional enrichment analysis of differential Kbbh proteins in aged hearts. A, functional enrichment of biological process of differential upregulated (*right*) and downregulated (*left*) Kbbh proteins, respectively. B, functional enrichment of molecular function of differential upregulated (*right*) and downregulated (*left*) Kbbh proteins, respectively. C, functional enrichment of cellular component of differential upregulated (*right*) and downregulated (*left*) Kbbh proteins, respectively. D, functional enrichment of KEGG pathway of differential upregulated Kbbh proteins. E, functional enrichment of KEGG pathway of differential downregulated Kbbh proteins. F, KEGG pathways of differential modified Kbbh proteins depended on four categories. (Q1: $FC < 0.5$; Q2: $0.5 < FC < 1/1.5$; Q3: $1.5 < FC < 2$; Q4: $FC > 2$). KEGG, Kyoto Encyclopedia of Genes and Genomes.

diagram using the log2 (FC) of identified proteins and log2 (FC) of identified Kbbh sites (Fig. 9A). The sites with prominent FC neither in Kbbh modification nor at the protein level are concentrated in the middle region E. The sites in the D quadrant represent the downregulated Kbbh-modified sites ($FC < 1/1.5$) without any significant change in the protein level ($1/1.5 < FC < 1.5$). In contrast, the upregulated Kbbh-modified sites ($FC > 1.5$) without significant change in protein level ($1/1.5 < FC < 1.5$) were located in F. According to this data, the sites with large changes at the Kbbh sites, at the meantime with large changes at the protein level only account for 4.3%

of all identified Kbbh sites, as shown in (Fig. 9A, quadrants C and G).

To uncover the comprehensive connection between the proteome and Kbbh-modified proteins, we conducted PPI network analysis to identify the critical nodes and significant biological processes associated with the DEPs and Kbbh-modified proteins (Fig. 9B). Based on the KEGG enrichment analysis and KEGG category, we observed that down-regulated Kbbh-modified proteins were highly enriched in mitochondrial oxidative phosphorylation, fatty acid degradation, and the TCA cycle (Tables 1–3). Therefore, we analyzed

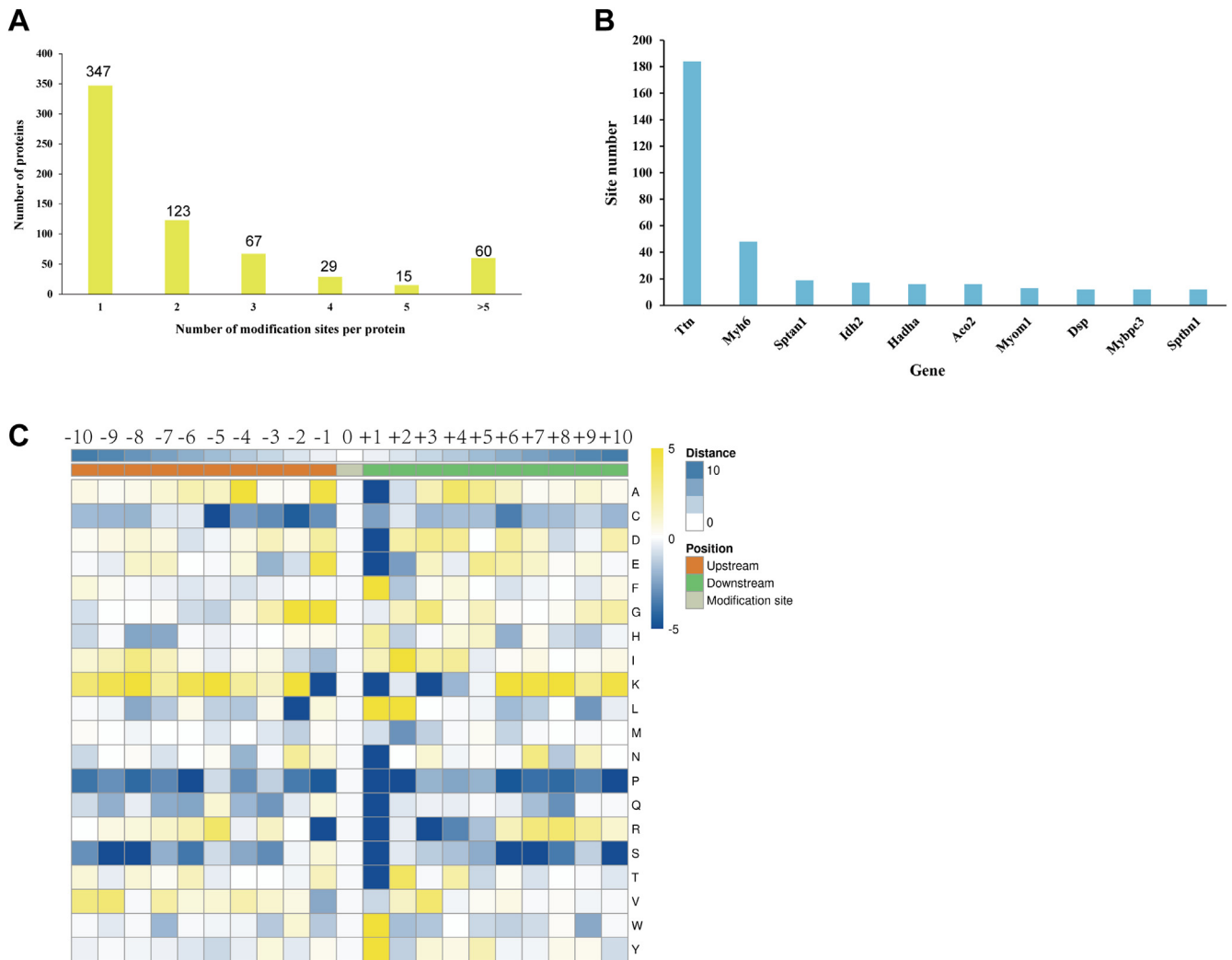


FIG. 8. **Characteristics of Kbh sites in aged hearts.** A, distribution of the Kbh sites number per protein. B, statistical map of proteins with >10 Kbh sites. C, heat map of the ±10 amino acid compositional frequencies surrounding the Kbh residues.

the interaction network of differential proteins and Kbh-modified proteins related to these three pathways using the interaction gene/protein retrieval tool (STRING v.11.0) database.

In the PPI network, we identified 29 proteins related to oxidative phosphorylation, 12 proteins related to fatty acid degradation, and 8 proteins related to the TCA cycle (Fig. 9C). In the oxidative phosphorylation pathway, Kbh modification significantly altered the sites of 10 proteins without any change in their protein levels, such as protein ATP synthase subunit d (Atp5pd) at sites lysine 117 and 72 and ATP synthase-coupling factor 6 (Atp5pf) at site lysine 94 (down-regulated), as indicated by blue arrow marks (Fig. 9C (a)). Regarding the proteins involved in the fatty acid degradation, most were significantly altered at their Kbh mediated sites, while the protein remained unaltered. For example, lysine 385 and 157 at aldehyde dehydrogenase (Aldh2) were upregulated without alteration in protein level. Medium-chain-specific

acyl-CoA dehydrogenase (Acadm) was upregulated at site lysine 301 and downregulated at lysine 212 without changes in protein level. Only one protein, ATP-citrate synthase (Acy), in TCA cycle, was altered in protein expression instead of Kbh sites (Fig. 9C (b, c)). These findings suggested that Kbh modification of these critical enzymes involved in energy metabolism, rather than the enzymes themselves, maybe the key regulatory mechanism in the process of cardiac aging.

DISCUSSION

Aging plays a pivotal role in the development of CVD, the leading cause of death and disability in the elderly population worldwide (15, 16). PTMs are major regulatory forms of protein functions and have been implicated in cardiac aging and various CVDs (4, 5). Kbh is one of the PTM processes, in which β-hydroxybutyrate is covalently attached to lysine ε-amino groups (6). However, the alterations and effect of

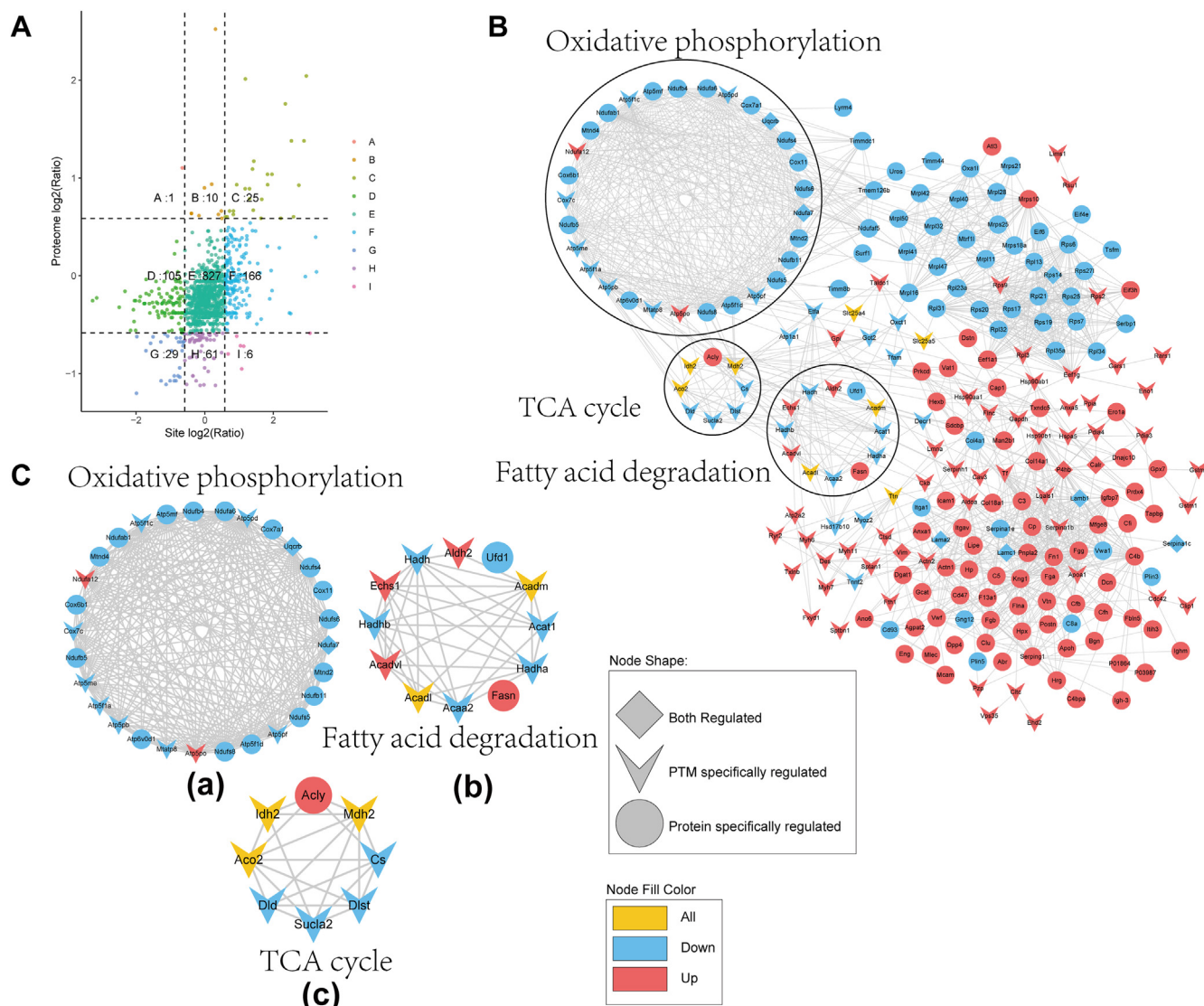


FIG. 9. Protein-protein interactions network between global proteome and Kbbh modified proteome in aged heart. A, Nine-quadrant diagram of the correlation of log₂ (FC) of DEPs in WTA mice and log₂ (FC) of differentially Kbbh in WTA mice (log FC > 1.5; *p*-value < 0.05). Quadrant-A (QA) were those proteins that were upregulated in the proteome, whereas declined in Kbbh; Quadrant-B (QB) were proteins upregulated in the proteome, whereas they were unaltered in Kbbh; Quadrant-C (QC) were proteins that were upregulated in proteome and Kbbh; Quadrant-D (QD) were proteins that were unaltered in the proteome, whereas downregulated in Kbbh; Quadrant-E (QE) were proteins unaltered in proteome and Kbbh; Quadrant-F (QF) were proteins that were unaltered in proteome, but increased in Kbbh; Quadrant-G (QG) were proteins downregulated in proteome and Kbbh, Quadrant-H (QH) were proteins downregulated in proteome, but were unaltered in Kbbh; Quadrant-I (QI) were proteins downregulated in proteome, but were upregulated in Kbbh. B, the PPI network of proteome and Kbbh-modified proteome. C, the PPI network of proteome and Kbbh modified proteome of oxidative phosphorylation (a), free fatty degradation (b), and TCA cycle (c) pathway. The circle represents specifically differentially expressed protein without differentially modified Kbbh sites, the arrow represents specifically differentially modified Kbbh site without change in proteome, and the diamond represents both differentially expression in proteome and Kbbh modification. Blue represents downregulation in proteome or Kbbh modification; Red represents upregulation in proteome or Kbbh modification; Yellow represents both downregulation and upregulation in proteome or Kbbh modification.

Kbbh modification during cardiac aging are yet unknown. In the present study, based on high-throughput and quantitative proteomics analysis, we identified 590 significant DEPs and 183 differentially modified sites in 134 proteins in the aged hearts and demonstrated that most of the Kbbh-modified sites at key enzymes were involved in energy metabolism; for

instance, mitochondrial oxidative phosphorylation, fatty acid degradation, and the TCA cycle were significantly down-regulated in aged hearts.

Phenotypically, we observed prominent changes associated with aging in 22-month-old mice in the present study. These changes included reduced grip strength (Fig. 1),

TABLE 1
Downregulated Kbbh sites of oxidative phosphorylation pathway

Protein description	Gene name	Amino acid	Sites	WTA/WTY ratio	WTA/WTY <i>p</i> value
ATP synthase protein 8	Mtstp8	K	48	0.467	0.00136
Cytochrome c oxidase subunit 7C	Cox7c	K	34	0.466	0.00169
ATP synthase-coupling factor 6	Atp5pf	K	94	0.531	0.01419
ATP synthase subunit alpha	Atp5f1a	K	167	0.24	0.01977
ATP synthase subunit e	Atp5me	K	12	0.601	0.00557
ATP synthase subunit gamma	Atp5f1c	K	55	0.641	0.00593
ATP synthase subunit gamma	Atp5f1c	K	49	0.495	0.04448
ATP synthase F(0) complex subunit B1	Atp5pb	K	225	0.44	0.000002
Cytochrome b-c1 complex subunit 7	Uqcrb	K	12	0.583	0.00099
ATP synthase subunit d	Atp5pd	K	117	0.268	0.00015
ATP synthase subunit d	Atp5pd	K	72	0.487	0.00154
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 7	Ndufa7	K	23	0.254	0.02144

Kbbh, Lysine β-hydroxybutyrylation; WTA, wild type aged; WTY, wild type young.

deterioration of ventricular diastolic function (Fig. 2), and cardiomyocyte hypertrophy (Fig. 3). Grip strength, which can be measured by a grip strength meter, is a strong indication of upper limb strength and muscle function (17). It is regarded as a predictive biomarker for all-cause and disease-specific mortality associated with hospitalization and helps to identify older adults at risk of poor health status (17, 18). Typically, grip strength declines after middle age, with the loss accelerating in an age-dependent manner (19). In our study, a significant decline in grip strength was observed in 22-month-old mice (Fig. 1), consistent with previous findings (17, 20). Therefore, we speculated that declined grip strength might be one of the prominent phenotypical characteristics of aging mice, which is primarily attributed to age-related loss of muscle mass and strength (17, 20).

Cardiac diastolic function is assessed using echocardiographic indicators, such as the ratio of E/A and E'/A'. On the other hand, cardiac systolic function is represented by parameters, such as LVEF, fractional shortening (FS), and

cardiac output (CO). As individuals age, both cardiac diastolic and systolic functions tend to decline gradually; however, the deterioration of diastolic function emerges earlier than systolic function in cardiac aging (21). Reportedly, compared to young rats (4 months old), 18-month-old rats (equivalent to early-elder humans, around 60 years old) exhibited only impaired diastolic function while maintaining a normal systolic function (22). However, in the 24-month-old rats, equivalent to middle-elder humans, both diastolic and systolic functions were significantly compromised (22). We also noticed a decreased E/A ratio and E'/A' in the 22-month-old mice compared to the 3-month-old mice (Fig. 2B). Strikingly, the LVEF just showed only a slight decrease that was not statistically significant (Fig. 2B). These findings align with the observation that the deterioration of diastolic function emerges earlier than systolic function in cardiac aging (21). This pattern is similar to age-associated diastolic dysfunction with preserved EF in older humans (23). The deterioration of ventricular diastolic function with age can be attributed to decreased ventricular

TABLE 2
Downregulated Kbbh sites of fatty acid degradation

Protein description	Gene name	Amino acid	Sites	WTA/WTY ratio	WTA/WTY <i>p</i> value
Medium-chain specific acyl-CoA dehydrogenase	Acadm	K	212	0.543	0.00293
Long-chain specific acyl-CoA dehydrogenase	Acadl	K	322	0.552	0.00066
Hydroxyacyl-coenzyme A dehydrogenase	Hadh	K	81	0.616	0.00012
Trifunctional enzyme subunit alpha	Hadha	K	728	0.348	0.00006
Trifunctional enzyme subunit alpha	Hadha	K	644	0.561	0.00101
Trifunctional enzyme subunit alpha	Hadha	K	60	0.429	0.00006
Trifunctional enzyme subunit alpha	Hadha	K	415	0.475	0.00111
Trifunctional enzyme subunit alpha	Hadha	K	214	0.57	0.00006
Trifunctional enzyme subunit alpha	Hadha	K	519	0.601	0.00725
3-ketoacyl-CoA thiolase	Acaa2	K	214	0.514	0.00041
Acetyl-CoA acetyltransferase	Acat1	K	265	0.396	0.00023
Trifunctional enzyme subunit beta	Hadhb	K	202	0.624	0.00425
Trifunctional enzyme subunit beta	Hadhb	K	189	0.515	0.00280

Kbbh, Lysine β-hydroxybutyrylation; WTA, wild type aged; WTY, wild type young.

TABLE 3
Downregulated Kbbh sites of TCA cycle

Protein description	Gene name	Amino acid	Sites	WTA/WTY ratio	WTA/WTY <i>p</i> value
Dihydrolipoyl dehydrogenase	Dld	K	430	0.587	0.00599
Dihydrolipoyl dehydrogenase	Dld	K	277	0.606	0.02961
Malate dehydrogenase	Mdh2	K	239	0.382	0.00033
Malate dehydrogenase	Mdh2	K	157	0.531	0.00026
Isocitrate dehydrogenase	Idh2	K	199	0.619	0.00017
Isocitrate dehydrogenase	Idh2	K	180	0.519	0.00001
Aconitate hydratase	Aco2	K	517	0.56	0.00033
Aconitate hydratase	Aco2	K	730	0.374	0.00876
Citrate synthase	Cs	K	321	0.422	0.00013
Citrate synthase	Cs	K	52	0.293	0.01559
Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex	Dl1st	K	268	0.39	0.00016
Succinate-CoA ligase [ADP-forming] subunit beta	Suc1a2	K	88	0.248	0.00082

Kbbh, Lysine β -hydroxybutyrylation; TCA, tricarboxylic acid; WTA, wild type aged; WTY, wild type young.

compliance, primarily influenced by a decrease in the number of cardiomyocytes, compensatory hypertrophy of the remaining cardiomyocytes, and the presence of senescent fibroblasts in the left ventricle (24, 25). Our histological analysis revealed a decreased number of cardiomyocytes and reduced cardiomyocyte hypertrophy, which serves as the structural basis for the deterioration of ventricular diastolic function in aged mice.

In our present study, five types of PTMs, lysine succinylation, crotonylation, malonylation, β -hydroxybutyrylation, and lactylation, were analyzed in the hearts of both young and aged mice based on WB analysis. Lysine succinylation, crotonylation, malonylation, and β -hydroxybutyrylation are the newly discovered short-chain Lys acetylations, involved in gene expression, tissue injury, and metabolic-induced disease (6). Quantitative proteomics analysis revealed that the succinylation of proteins in the cardiac tissue from patients with atrial fibrillation is related to energy metabolism, oxidative stress, and myocardial contraction (26). Proteomics analysis of the crotonylation profile of mice after acute cardiac I/R injury revealed that extensive crotonylation of non-histone proteins was associated with disruption of cardiomyocyte mitochondrial, cardiomyocyte autophagy, and apoptosis (27). Lysine lactylation refers to the process by which the lactyl groups of lactyl-CoA are covalently transferred to lysine residues under the action of acyltransferase (6). Lactylation of α -myosin is involved in cytoskeleton interaction and leads to impaired cardiac structure and function (28). However, the changes in these PTMs in cardiac aging are unknown. In the present study, the abundance of lysine β -hydroxybutyrylation proteins was significantly reduced in the cardiac tissue of aged mice, with no difference in lysine succinylation, crotonylation, or malonylation. Our findings indicated that Kbbh, instead of other forms of acetylation PTM, plays a critical role in cardiac aging.

To comprehensively investigate the alterations in the global Kbbh modification in aged hearts, we employed quantitative proteomics and Kbbh PTM omics in both young and aged mice. The quantitative proteomics analysis identified a total of 5113 proteins, of which 590 showed significant differential expression between aged and young mice, using a cutoff of 1.5-FC and *p*-value <0.05. The analysis of Kbbh modification identified 1710 β -hydroxybutyrylated lysine sites in 641 proteins in the cardiac tissue of young and aged mice, and finally, a total of 183 differentially modified sites were identified in 134 proteins (1.5-fold cutoff, *p* < 0.05). Since the changes in Kbbh-modified sites may be affected by the altered levels of total protein, we evaluated the influence of protein expression on the levels of Kbbh sites. Most of the sites are concentrated in the middle region (D, E, F) in the nine-quadrant diagram, wherein the changes in protein expression were not prominent ($1/1.5 < FC < 1.5$). Conversely, the large changes in the Kbbh sites accompanied by large changes in the same direction at the protein level only account for 4.3%. Moreover, many studies on the modification are not standardized by proteomics, thus not standardizing protein levels is a common practice (29, 30). Together, our data indicated that Kbbh modification, instead of total protein expression, plays a critical role in the process of cardiac aging.

Mitochondria are highly enriched in cardiomyocytes and are key organelles involved in energy supply (31). Mitochondria regulate myocardial function by modulating energy metabolism and play a role in protein PTMs, such as succinylation, crotonylation, malonylation, and β -hydroxybutyrylation, by supplying donors acyl-CoA (32, 33). Proteomics analysis of crotonylation modification, another form of Lys acylation, revealed that most of these modified proteins were localized in cardiomyocyte mitochondria (27). In our study, a majority of Kbbh-modified proteins were localized in the

mitochondria (34.4%). A large number of mitochondrial proteins exhibited extensive Kbh modification in cardiac aging and were associated with oxidative phosphorylation, fatty acid metabolism, and the TCA cycle. For example, lysine 117 and 72 sites of ATP synthase subunit d (Atp5pd) were downregulated, which was involved in oxidative phosphorylation in the mitochondria. Lysine 385 and 157 sites of aldehyde dehydrogenase (Aldh2), a mitochondrial protein related to fatty acid degradation, were upregulated. Succinate-CoA ligase (ADP-forming) subunit beta (Succla2) participated in the mitochondrial TCA cycle and was found to be significantly downregulated. Kbh proteomics analysis in AMPK α 2 knockout mice also revealed that most of the Kbh-modified proteins are located in the mitochondria, which is consistent with this present study (10). Thus, we deduced that the mitochondrial proteins exhibit remarkable changes in Kbh modification, and mitochondria are the primary locations of Kbh modification in aged hearts.

Similar to that observed for Kbh in the hearts of AMPK knockout mice (10), several pathways related to energy metabolism were enriched in Kbh, including fatty acid β -oxidation, glyoxylate, and dicarboxylate metabolism, TCA cycle, and oxidative phosphorylation. Several critical enzymes have at least one Kbh site. Trifunctional enzyme subunit alpha (HADHA) is a mitochondrial β -oxidation enzyme involved in β HB production (13), which exhibited downregulated Kbh modification at multiple sites in aged hearts. This finding suggested that Kbh modification affects the activity and function of HADHA, potentially influencing β HB production. Aspartate aminotransferase (got2) is involved in ATP energy synthesis in cellular energy deficiency cases containing 4 Kbh sites (14). IDH2, a key enzyme of the TCA cycle, contains 2 downregulated (K199 and K180) sites. The K34 site of cytochrome C oxidase, a rate-limiting enzyme in mitochondrial respiration, exhibited downregulation of Kbh modification in aged hearts. This phenomenon implies that Kbh modification may affect cytochrome C oxidase activity, potentially affecting mitochondrial respiration and energy production. Overall, these findings suggested that Kbh modification regulates energy metabolism pathways in cardiac aging, including β HB production, ATP synthesis, the TCA cycle, and mitochondrial respiration (34).

In conclusion, our comprehensive quantitative analysis of Kbh modifications in the context of cardiac aging provides novel insights into the involvement of Kbh in this process, especially through the regulation of proteins related to energy metabolism. To the best of our knowledge, this is the first study of Kbh in the context of cardiac aging. The findings open up new avenues for potential therapeutic strategies by targeting and reversing aberrant Kbh modifications in the treatment of cardiac aging and aging-related CVDs. However, further studies are required to elucidate the specific functions and implications of individual Kbh modification sites in these processes.

DATA AVAILABILITY

Annotated spectra for identified β -hydroxybutyrylation modifications have been deposited on MS-viewer with the search key "jaumousqgp". Mass spectrometry proteomics data have been deposited on the ProteomeXchange Consortium via the iProX partner repository. The project name is "Changes of Lysine β -hydroxybutyrylation in the heart of naturally senescent mice", and the dataset identifier is PXD034269.

Supplemental data—This article contains [supplemental data](#).

Acknowledgments—This research was funded by the Key Technology Research and Development Program of Shandong, grant number 2017GSF218101; the National Natural Science Foundation of China, grant number 81700725; the Natural Science Foundation of Shandong Province, grant number ZR2017BH003; the Natural Science Foundation of Shandong Province, grant number ZR2021MH008; and the Key Technology Research and Development Program of Shandong, grant number 2022CXGC010510.

Author contributions—X. Y., X. L., R. Y., C. T., W. D., Yiping S., and H. G. data curation; X. Y., X. L., N. Y., R. Y., Yan S., S. W., Z. Z., and Y. X. software; X. Y., X. L., and N. Y. validation; X. Y. writing-original draft; X. L. and N. Y. writing-review and editing; N. Y., C. T., W. D., and M. L. investigation; Yan S., M. L., and Yiping S. visualization; W. G. resources; J. F., Z. Z., and Y. X. methodology; Z. Z. and Y. X. supervision; Y. X. conceptualization.

Conflict of interest—The authors declare no competing interests.

Abbreviations—The abbreviations used are: β HB, β -hydroxybutyrate; AMPK α 2, AMP-activated protein kinase alpha 2; BP, Biological process; CC, Cellular component; CHO, Cholesterol; COG, Cluster of orthologous; CVD, Cardiovascular disease; CO, Cardiac output; DDA, Data-dependent acquisition; DEPs, Differentially expressed proteins; ELISA, Enzyme-linked immunosorbent assay; FC, Fold change; FFA, Free fatty acid; FDR, False discovery rate; GO, Gene ontology; GOT2, Aspartate aminotransferase 2; HADHA, Trifunctional enzyme subunit alpha; HPLC, High-performance liquid chromatography; IDH2, Isocitrate dehydrogenase 2; Kbh, Lysine β -hydroxybutyrylation; K-Crot, Lysine crotonylation; KEGG, Kyoto encyclopedia of genes and genomes; K-mal, Lysine malonylation; K-Lact, Lysine lactylation; K-Suss, Lysine succinylation; LC-MS/MS, Liquid chromatography-mass spectrometry; LV, Left ventricle; LVEF, Left ventricle ejection fraction; LVFS, Left ventricle fractional shortening; MF, Molecular function; PASEF, Parallel accumulation serial fragmentation; PPI, Protein-protein interaction; PTM, Post-translational modification; TCA cycle, Tricarboxylic acid cycle;

TG, Triglyceride; Ttn, Titin; WTY, Wild-type young; WTA, Wild-type aged.

Received March 14, 2023, and in revised form, October 1, 2023
Published, MCPRO Papers in Press, October 5, 2023, <https://doi.org/10.1016/j.mcpro.2023.100659>

REFERENCES

- Obas, V., and Vasan, R. S. (2018) The aging heart. *Clin. Sci. (Lond)*. **132**, 1367–1382
- Marian, A. J., and Braunwald, E. (2017) Hypertrophic cardiomyopathy: genetics, pathogenesis, clinical manifestations, diagnosis, and therapy. *Circ. Res.* **121**, 749–770
- Aging Biomarker, C., Bao, H., Cao, J., et al. (2023) Biomarkers of aging. *Sci. China Life Sci.* **66**, 893–1066
- Beltrao, P., Bork, P., Krogan, N. J., and van Noort, V. (2013) Evolution and functional cross-talk of protein post-translational modifications. *Mol. Syst. Biol.* **9**, 714
- Gao, J., Shao, K., Chen, X., Li, Z., Liu, Z., Yu, Z., et al. (2020) The involvement of post-translational modifications in cardiovascular pathologies: focus on SUMOylation, neddylation, succinylation, and prenylation. *J. Mol. Cell Cardiol.* **138**, 49–58
- Xie, Z., Zhang, D., Chung, Z., Tang, Z., Huang, H., Dai, L., et al. (2016) Metabolic regulation of gene expression by histone lysine beta-hydroxybutyrylation. *Mol. Cell* **62**, 194–206
- Chong, D., Gu, Y., Zhang, T., Xu, Y., Bu, D., Chen, Z., et al. (2022) Neonatal ketone body elevation regulates postnatal heart development by promoting cardiomyocyte mitochondrial maturation and metabolic reprogramming. *Cell Discov.* **8**, 106
- [preprint] Tsusaka, T., Osés-Prieto, J. A., Lee, C., DeFelice, B. C., Burlingame, A. L., and Goldberg, E. (2023) Non-specific recognition of histone modifications by H3K9bhb antibody. *bioRxiv*. <https://doi.org/10.1101/2023.04.12.536655>
- Liu, K., Li, F., Sun, Q., Lin, N., Han, H., You, K., et al. (2019) p53 beta-hydroxybutyrylation attenuates p53 activity. *Cell Death Dis.* **10**, 243
- Ding, W. J., Li, X. H., Tang, C. M., Yang, X. C., Sun, Y., Song, Y. P., et al. (2023) Quantification and proteomic characterization of beta-hydroxybutyrylation modification in the hearts of AMPKalpha2 knockout mice. *Mol. Cell Proteomics* **22**, 100494
- Chou, M. F., and Schwartz, D. (2011) Biological sequence motif discovery using motif-x. *Curr. Protoc. Bioinform.* **35**. <https://doi.org/10.1002/0471250953.bi1315s35>
- Loescher, C. M., Hobbach, A. J., and Linke, W. A. (2022) Titin (TTN): from molecule to modifications, mechanics, and medical significance. *Cardiovasc. Res.* **118**, 2903–2918
- Pan, A., Sun, X. M., Huang, F. Q., Liu, J. F., Cai, Y. Y., Wu, X., et al. (2022) The mitochondrial beta-oxidation enzyme HADHA restrains hepatic glucagon response by promoting beta-hydroxybutyrate production. *Nat. Commun.* **13**, 386
- Li, Y., Li, B., Xu, Y., Qian, L., Xu, T., Meng, G., et al. (2022) GOT2 silencing promotes reprogramming of glutamine metabolism and sensitizes hepatocellular carcinoma to glutaminase inhibitors. *Cancer Res.* **82**, 3223–3235
- Liberal, L., Badimon, L., Montecucco, F., Luscher, T. F., Libby, P., and Camici, G. G. (2022) Inflammation, aging, and cardiovascular disease: JACC review topic of the week. *J. Am. Coll. Cardiol.* **79**, 837–847
- North, B. J., and Sinclair, D. A. (2012) The intersection between aging and cardiovascular disease. *Circ. Res.* **110**, 1097–1108
- Graber, T. G., Maroto, R., Fry, C. S., Brightwell, C. R., and Rasmussen, B. B. (2021) Measuring exercise capacity and physical function in adult and older mice. *J. Gerontol. A. Biol. Sci. Med. Sci.* **76**, 819–824
- Porto, J. M., Nakaishi, A. P. M., Cangussu-Oliveira, L. M., Freire Junior, R. C., Spilla, S. B., and Abreu, D. C. C. (2019) Relationship between grip strength and global muscle strength in community-dwelling older people. *Arch. Gerontol. Geriatr.* **82**, 273–278
- Roberts, H. C., Denison, H. J., Martin, H. J., Patel, H. P., Syddall, H., Cooper, C., and Sayer, A. A. (2011) A review of the measurement of grip strength in clinical and epidemiological studies: towards a standardised approach. *Age Ageing* **40**, 423–429
- Seldeen, K. L., Berman, R. N., Pang, M., Lasky, G., Weiss, C., MacDonald, B. A., et al. (2020) Vitamin D insufficiency reduces grip strength, grip endurance and increases frailty in aged C57Bl/6J mice. *Nutrients* **12**, 3005
- Jakovljevic, D. G. (2018) Physical activity and cardiovascular aging: physiological and molecular insights. *Exp. Gerontol.* **109**, 67–74
- Lu, X., Gong, Y., Hu, W., Mao, Y., Wang, T., Sun, Z., et al. (2022) Ultrastructural and proteomic profiling of mitochondria-associated endoplasmic reticulum membranes reveal aging signatures in striated muscle. *Cell Death Dis.* **13**, 296
- Pagel, P. S., Tawil, J. N., Boettcher, B. T., Izquierdo, D. A., Lazicki, T. J., Crystal, G. J., and Freed, J. K. (2021) Heart failure with preserved ejection fraction: a comprehensive review and update of diagnosis, pathophysiology, treatment, and perioperative implications. *J. Cardiothorac. Vasc. Anesth.* **35**, 1839–1859
- Nakayama, H., Nishida, K., and Otsu, K. (2016) Macromolecular degradation systems and cardiovascular aging. *Circ. Res.* **118**, 1577–1592
- Yoneyama, K., Venkatesh, B. A., Bluemke, D. A., McClelland, R. L., and Lima, J. A. C. (2017) Cardiovascular magnetic resonance in an adult human population: serial observations from the multi-ethnic study of atherosclerosis. *J. Cardiovasc. Magn. Reson.* **19**, 52
- Bai, F., Tu, T., Qin, F., Ma, Y., Liu, N., Liu, Y., et al. (2019) Quantitative proteomics of changes in succinylated proteins expression profiling in left appendages tissue from valvular heart disease patients with atrial fibrillation. *Clinica Chim. Acta* **495**, 345–354
- Cai, W., Xu, D., Zeng, C., Liao, F., Li, R., Lin, Y., et al. (2022) Modulating lysine crotonylation in cardiomyocytes improves myocardial outcomes. *Circ. Res.* **131**, 456–472
- Zhang, N., Zhang, Y., Xu, J., Wang, P., Wu, B., Lu, S., et al. (2023) α-myosin heavy chain lactylation maintains sarcomeric structure and function and alleviates the development of heart failure. *Cell Res.* **33**, 679–698
- Trulsson, F., Akimov, V., Robu, M., van Overbeek, N., Berrocal, D. A. P., Shah, R. G., et al. (2022) Deubiquitinating enzymes and the proteasome regulate preferential sets of ubiquitin substrates. *Nat. Commun.* **13**, 2736
- Liu, P., Cong, X., Liao, S., et al. (2021) Global identification of phospho-dependent SCF substrates reveals a FBXO22 phosphodegron and an ERK-FBXO22-BAG3 axis in tumorigenesis. *Cell Death Differ.* **29**, 1–13
- Picca, A., Mankowski, R. T., Burman, J. L., Donisi, L., Kim, J. S., Marzetti, E., and Leeuwenburgh, C. (2018) Mitochondrial quality control mechanisms as molecular targets in cardiac ageing. *Nat. Rev. Cardiol.* **15**, 543–554
- Cai, K., Wang, F., Lu, J. Q., Shen, A. N., Zhao, S. M., Zang, W. D., et al. (2022) Nicotinamide mononucleotide alleviates cardiomyopathy phenotypes caused by short-chain enoyl-coa hydratase 1 deficiency. *JACC Basic Transl. Sci.* **7**, 348–362
- Tang, X., Chen, X. F., Sun, X., Xu, P., Zhao, X., Tong, Y., et al. (2021) Short-chain enoyl-CoA hydratase mediates histone crotonylation and contributes to cardiac homeostasis. *Circulation* **143**, 1066–1069
- Koronowski, K. B., Greco, C. M., Huang, H., Kim, J. K., Fribourgh, J. L., Crosby, P., et al. (2021) Ketogenesis impact on liver metabolism revealed by proteomics of lysine beta-hydroxybutyrylation. *Cell Rep.* **36**, 109487