Universidade de Aveiro Departamento de Química

Ano 2014

Ana Lúcia Barbosa Azevedo

Impacto do Exercício Físico Moderado no Acetiloma Mitocondrial Cardíaco

Impact of Moderate Exercise Training on Heart Mitochondrial Acetylome

Universidade de Aveiro Departamento de Química

Ano 2014

Ana Lúcia Barbosa Azevedo

Impacto do Exercício Físico Moderado no Acetiloma Mitocondrial Cardíaco

Impact of Moderate Exercise Training on Heart Mitochondrial Acetylome

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, ramo Métodos Biomoleculares, realizada sob a orientação científica do Doutor Rui Miguel Pinheiro Vitorino, Investigador Auxiliar do Departamento de Química da Universidade de Aveiro e da Doutora Rita Maria Pinho Ferreira, Professora auxiliar convidada do Departamento de Química da Universidade de Aveiro.

Apoio financeiro da FCT, do Programa Operacional Temático Fatores de Competitividade (COMPETE) E Comparticipado pelo Fundo Comunitário Europeu (FEDER) aos projetos PEst-C/QUI/UI0062/2013, EXPL/DTP-DES/1010/2013 e FCOMP-01-0124-FEDER-041115.

Dedico este trabalho à minha família.

o júri

presidente

Prof. Doutor Pedro Miguel Dimas Neves Domingues
professor auxiliar do Departamento de Química da Universidade de Aveiro

Prof. Doutora Margarida Sâncio da Cruz Fardilha
professora auxiliar convidada da Secção Autónoma de Ciências da Saúde da Universidade de Aveiro

Prof. Doutor Rui Miguel Pinheiro
investigador auxiliar do Departamento de Química da Universidade de Aveiro

agradecimentos

Aos meus orientadores, Professora Doutora Rita Ferreira e Professor Doutor Rui Vitorino, agradeço a orientação científica que me proporcionaram e principalmente o incentivo, disponibilidade e oportunidades que me deram ao longo deste percurso. Agradeço também a disponibilidade incondicional e o exemplo de dedicação, trabalho e busca de conhecimento.

À Cristina pela ajuda, simpatia e boa disposição.

À Rita pela simpatia com que me recebeu, pela ajuda na parte experimental, amizade, e por todos os bons momentos que passamos.

À Liliana e ao Fábio pela ajuda e disponibilidade.

Aos meus amigos pelo apoio, incentivo, pela partilha de experiências e pelos momentos de diversão.

Aos meus pais, irmã e irmão por terem sido o meu apoio ao longo destes anos todos. Sem o vosso exemplo, a vossa força afeto nunca teria conseguido.

Ao André pela paciência, apoio e incentivo. Agradeço também a força transmitida e o carinho demonstrado.

palavras-chave

exercício físico, remodelação cardíaca, mitocôndria, modificações pós-traducionais, acetilação, sirtuinas.

resumo

O efeito cardioprotetor do exercício físico moderado tem sido associado a uma adaptação benéfica do proteoma mitocondrial do coração. Com o intuito de melhor compreender o impacto do exercício físico praticado ao longo da vida na funcionalidade cardíaca e, especificamente, na regulação da atividade mitocondrial, no presente trabalho utilizou-se uma abordagem proteómica baseada em espectrometria de massa para avaliar as alterações promovidas por 54 semanas de exercício em tapete rolante no perfil proteico mitocondrial do coração de ratos, mais especificamente no acetiloma mitocondrial. Para o efeito utilizaram-se estratégias de enriquecimento baseadas em imunoprecipitação com anticorpos anti-acetil-lisina e anti-SIRT3.
A avaliação da funcionalidade cardíaca suportou o efeito cardioprotetor da atividade física praticada ao longo de 54 semanas. A análise de mitocôndrias isoladas do coração evidenciou um aumento da capacidade de produção de ATP com a prática de exercício físico. Concomitantemente verificou-se um aumento da expressão de SIRT3 e alteração do perfil de proteínas mitocondriais acetiladas. Estas proteínas acetiladas estão envolvidas em vários processos biológicos, sendo de salientar o metabolismo cardíaco que se torna mais dependente da oxidação de hidratos de carbono com a prática de exercício físico. Curiosamente, de acordo com a ferramenta bioinformática STRING, a maioria das proteínas mitocondriais identificadas como acetiladas não parecem ser substrato da SIRT3. A análise por imunoprecipitação com anti-SIRT3 seguida de LC-MS/MS não permitiu identificar os potenciais substratos da SIRT3. No futuro será importante identificar as enzimas envolvidas na regulação do acetiloma mitocondrial para além da SIRT3, as suas proteínas substrato e, consequentemente, o seu impacto nos vários processos biológicos ativados pelo estilo de vida.

keywords physical activity, cardiac remodeling, mitochondria, post-translational modifications, acetylation, sirtuins.

abstract

Physical activity has been suggested to have a cardioprotective role, which seems to be related with mitochondrial adaptation. Aiming a better understanding of the impact of exercise training on heart functionality and, specifically, on the regulation of mitochondrial activity, in the present work we applied a mass spectrometry-based proteomic approach for the characterization of mitochondrial proteome adaptation to 54 weeks of moderate treadmill exercise. Focus was given to mitochondrial acetylome and so, enrichment strategies based on immunoprecipitation with anti-acetyl-lysine and anti-SIRT3 antibodies were used.

Data highlighted the beneficial impact of 54-weeks of treadmill exercise on cardiac functionality. The analysis of mitochondria isolated from rat heart evidenced an exercise-related greater ability to produce ATP. Concomitantly, the mitochondrial expression of SIRT3 increased in exercised animals and was paralleled by alterations in the profile of acetylated proteins in heart mitochondria. These acetylated proteins are involved in a wide range of biological process being of notice the metabolism, which becomes more dependent on carbohydrates metabolism with lifelong exercise training. Curiously, none of the identified acetylated proteins are targets of SIRT3, according to STRING bioinformatics tool. Immunoprecipitation with anti-SIRT3 followed by LC-MS/MS did not allowed the identification of SIRT3 substrates. In the future, it will be important the identification of the enzymes involved in the regulation of mitochondrial acetylome besides SIRT3 to better characterize the impact of this posttranslational modification on the biological processes activated by lifestyle.

Index

Figure Index

Table Index

Abbreviations

ABC	Ammonium bicarbonate
AceCS2	Acetyl-CoA Synthase 2
ANT	Adenine Nucleotide Translocator
ATP	Adenosine Triphosphate
BSA	Bovine serum albumin
CID	Collision-Induced Dissociation
CPS1	Carbamoyl Phosphate Synthetase 1
CVDs	Cardiovascular diseases
Dox	Doxorubicin
DTT	Dithiothreitol
ECD	Electron-Capture dissociation
EDP	End-diastolic pressure
ESI	Electrospray Ionization
ETC	Electron Transport Chain
ETD	Electron Transfer Dissociation
FASP	Filter-Aided Sample Preparation
FMN	Flavin Mononucleotide
GCN5L1	General control of amino acid synthesis 5-like 1
GDH	Glutamate Dehydrogenase
HCD	High Energy Dissociation
HKII	Hexokinase II
HMGCS2	3-Hydroxy-3-Methylglutaryl-CoA Synthase 2
IAA	Iodoacetamide
IDH2	Isocitrate Dehydrogenase 2
InsP3R2	Type 2 inositol 1,4,5-trisphosphate receptor
IT	Ion Trap
iTRAQ	isobaric Tags for Relative and Absolute Quantification
LCAD	Long-chain acyl-CoA Dehydrogenase
MALDI	Matrix-Assisted Laser Desorption/ Ionization
MPTP	Mitochondrial Permeability Transition Pore

MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
NAD^+	Nicotinamide adenine dinucleotide
nanoLC-MS/MS	nanoflow liquid chromatography tandem mass spectrometry
OOADPr	O-acetyl-ADP-ribose
OPA1	Dynamic-like 120 kDa protein
OTC	Ornithine Transcarbamoylase
OXPHOS	Oxidative Phosphorylation
Pmax	Peak systolic pressure
PTMs	Post-Translational Modifications
Q	Quadrupole
RONS	Reactive Oxygen/ Nitrogen Species
SCX	Strong Cation Exchange
SdhA	succinate dehydrogenase flavoprotein
SILAC	Stable Isotopic Labeling by Amino Acids in Cell Culture
SILAM	Stable Isotopic Labeling in Mammals
SIRTs	Sirtuins
SOD2	Superoxide dismutase 2
STZ	Streptozotocin
Tau	Time constant of ventricular pressure decay
TBS-T	Tris buffered saline with Tween-
TBT	Tris buffered saline
TMT	Tandem Mass Tag
TOF	Time-Of-Flight
WHO	World Health Organization

I. Introduction

Introduction

Cardiovascular diseases (CVDs) are the main cause of death worldwide (1). World Health Organization (WHO) estimates that 17.3 million of people died from CVDs in 2008, representing about 30% of all deaths worldwide (2). Of these, 7.3 million were attributed to coronary heart disease and 6.3 million to stroke. Global cardiovascular deaths are projected to increase from 7.1 million in 2002 to 23.3 million by 2030 (3). In Europe approximately half of all deaths (47%) are from CVDs. Besides being the largest contributor to mortality and morbidity in world, particularly in Europe, CVDs have a great social and economic impact (4).

CVDs are mostly associated and causally linked to four particular behaviors: tobacco use, unhealthy diet, physical inactivity and the harmful use of alcohol (2). Recent estimative suggests that physical inactivity may be responsible for up to 6% of all premature mortality worldwide. Regular physical activity and/or aerobic exercise training has been suggested as a preventive and/or treatment strategy for CVDs. Indeed, WHO recommends the practice of 60 minutes of moderate to vigorous intensity physical activity daily for children and adolescents and at least 2.5 hours of moderate physical activity or 75 minutes of vigorous physical activity *per* week for adults aiming to protect the functionality of cardiovascular system (5).

Physical exercise promotes the increase of oxygen consumption (6). Despite the increased generation of reactive oxygen/nitrogen species (RONS), several studies demonstrate that physical exercise can be cardioprotective by defending the myocardium against acute and chronic deleterious insults. The beneficial effects of physical activity seem to occur at different levels of cellular organization, being mitochondria a preferential target of this physiological stimulus (6, 7). Mitochondria play an important role in energy production and cell signaling in many tissues and their dysfunction has been implicated in a wide range of human diseases (8). Changes in energy status have been related to alterations in mitochondrial protein acetylation. Protein acetylation is an important post-translational modification that regulates mitochondrial proteins' activity and overall mitochondria function. More than one-third of all proteins in mitochondria are acetylated, of which the majority are involved in some aspects of energy metabolism (9). Nevertheless, the role of protein acetylation in the regulation of mitochondria functionality is poorly characterized and even less the impact of exercise training on mitochondria acetylome.

3.1. Mitochondria: Molecular Structure and Function

Mitochondria are double-membrane organelles present in almost all cell types. They are responsible for the production of ATP, fatty acid oxidation, and participate in apoptosis, calcium homeostasis and signaling through reactive oxygen species (10). Mitochondria are composed by an outer membrane, the intermembranar space, an inner membrane, contact sites, the cristae space and the matrix (11) (Figure 1).

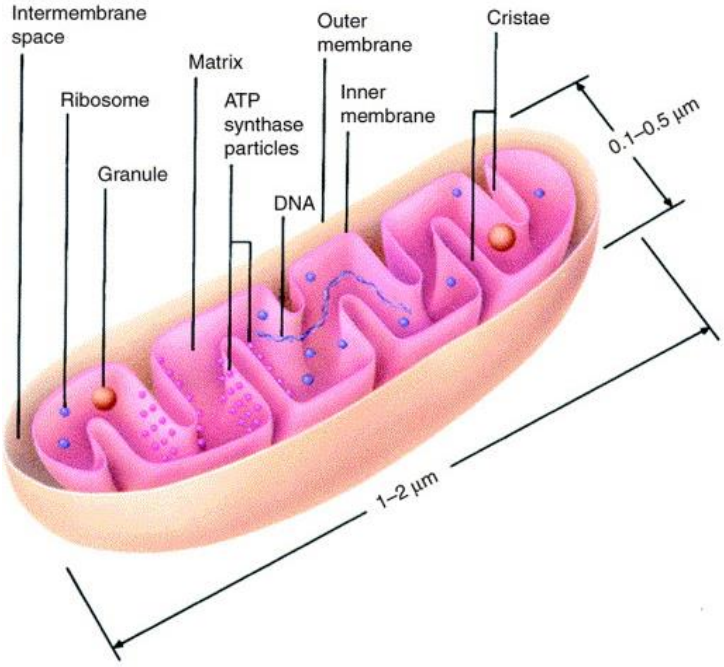

Figure 1. Schematic representation of mitochondria and its compartments (12).

The outer membrane is smooth and contains porin that facilitates the transport of small molecules (< 5 kDa) in and out of mitochondria (11, 13). The intermembranar space is located between the inner and outer membrane. The protein most prominent in the intermembranar space is cytochrome *c* and enzymes such as adenylate kinase and creatine kinase. About 21 % of the total mitochondrial proteome is found in the inner membrane that is largely impermeable to all molecules. Thus, solutes require specific transport proteins to move across it. The inner membrane shows many invaginations called cristae that greatly increase its surface area, enhancing its ability to generate ATP. The cristae contain the electron transport chain (ETC) complexes (complexes I-IV) and ATP synthase (complex V)

(14). The matrix is located within the inner membrane and contain approximately two-thirds of the total protein content in mitochondria. The matrix contain mitochondrial ribosomes, tRNA, mtDNA and various enzymes used for pyruvate oxidation, for the oxidation of fatty acids, for citric acid cycle and for protein synthesis (10, 15).

3.1.1. Oxidative Phosphorylation System

The electron transport chain consists in multi-subunit complexes designed as NADH:ubiquinone oxidoreductase (Complex I), succinate dehydrogenase (Complex II), ubiquinone: cytochrome *c* oxidoreductase (Complex III), cytochrome *c* oxidase (Complex IV) and two connecting redox-active molecules, a lipophilic quinone, designated Coenzyme Q or ubiquinone, and a hydrophilic heme protein, designated cytochrome *c* (16). The mitochondrial oxidative phosphorylation (OXPHOS) system is composed of these complexes along with ATP synthase (complex V) (15) (Figure 2).

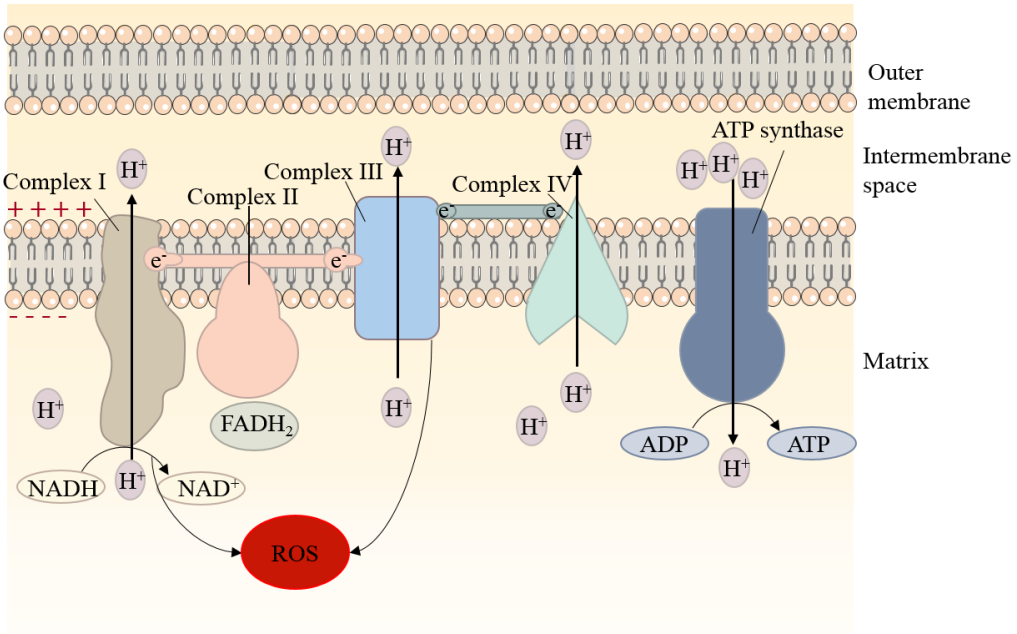

Figure 2. Representation of the oxidative phosphorylation system within mitochondria. The tricarboxylic acid cycle produces reduced co-factors that donate electrons to the electron transport chain. Complexes use energy released from electrons to pump protons into the intermembranar space, which creates a membrane potential that is used to convert ADP to ATP. Figure was made based on Servier Medical Art.

Complex I is composed by 42-43 different polypeptides and it has an L-shaped form with an intermembranar arm and a peripheral one. The redox active centers are located in the peripheral arm of the enzyme, which contains the NADH-biding site, one molecule of flavin mononucleotide (FMN) and 6 iron sulfur centers (10, 15). This complex catalyzes the transference of two electrons from NADH to ubiquinone coupled with the injection of four protons from the mitochondrial matrix to the intermembranar space (10).

Complex II is the membrane-bound component of the citric acid cycle that also functions as a component of the mitochondrial respiratory chain (15). It contains five prosthetic groups of two types and four different proteins subunits, two of which extend into the matrix and contain three Fe-S centers, bound flavin adenine dinucleotide (FAD), and a site for the substrate, succinate. The other two subunits are integral membrane proteins that contain a heme group, heme *b* and a binding site for ubiquinone (17). Succinate dehydrogenase catalyzes succinate oxidation to fumarate, thus reducing the covalently attached FAD. This reaction results in the transfer of 2 electrons from $FADH_2$ to the inner membrane forming ubiquinone. In this complex there is no pumping of protons (10, 15).

Complex III is composed by 9-10 polypeptides (15). The functional unit of complex III is a dimer, with two monomeric units of cytochrome *b* that contains two heme B groups. The enzymatic system has also two quinone-binding domains and an iron-sulfur protein center that reacts with cytochrome c_1 (10, 15). This complex catalyzes the oxidation of ubiquinol and the transfer of electrons to the mobile carrier cytochrome *c* coupled to the transfer of 4 protons to the inner membrane (10).

Complex IV is a large enzyme composed by 13 subunits with four redox metal centers, CuA, heme a, heme a3, and CuB, that are part of a pathway from the substrate cytochrome *c* to oxygen (10, 17). This complex accepts four electrons from cytochrome *c* and transfers them to the molecular oxygen, which is reduced by ferricytochrome *c* to water. At the same time, two protons cross the inner membrane (10).

Complex V is the final component of the oxidative phosphorylation system (15). Mitochondrial ATP synthase is an F-type ATPase and contains two distinct components: F_1, a peripheral membrane protein where catalysis takes place, and F_o, which is integral to membrane and contains a proton channel. Intact F_1F_o-ATP synthase can synthesize and hydrolyze ATP (18, 19). Complex V utilizes the potential created by the increased

concentration of protons in the intermembranar space to produce ATP, through the passage of these protons back into the mitochondrial matrix (10, 15).

OXPHOS has a central role in cell bioenergetics and, consequently, in the homeostasis of several tissues and organs including the heart. Due to its functions in cell, OXPHOS is highly modulated by several pathophysiological conditions as physical activity (20).

3.2. Impact of Exercise Training on Cardiac Mitochondrial Function

Exercise has been considered one of the most effective strategies to promote health. Physical activity in various forms has been shown to be an effective intervention that can provide defense against acute and chronic deleterious insults for the myocardium. These include *in vitro* anoxia reoxygenation (A-R) (21), diabetes mellitus (22, 23), aging (24) and doxorubicin (Dox) treatment (25). It has been suggested that the beneficial effects of increased physical activity occur at different levels of cellular organization, being mitochondria preferential target organelles (reviewed in 26).

Endurance training was shown to limit the functional alterations of heart rat mitochondria submitted to *in vitro* anoxia-reoxygenation (21). Ascensão *et al*. (21) evaluated mitochondria functionality in A-R conditions, which significantly impaired state 3 and state 4 respiration, as well as the respiratory control ratio (RCR) and ADP/O. However, mitochondrial state 3 respiration was significantly higher in trained than in the sedentary group both before and after A-R. The sedentary-related impairments in respiratory control ratio, ADP/O ratio and state 4 induced by A-R were significantly attenuated by endurance-trained. These differences were related with differential oxidative modifications of mitochondrial proteins and phospholipids, as measured through the formation of carbonyl groups and malondialdehyde, respectively, which levels were higher in sedentary animals.

Recent data demonstrates that even an acute bout of exercise protects against calcium-induced cardiac mitochondrial permeability transition pore opening in doxorubicin-treated rats (25). This effect was related to the attenuation of drug-related mitochondrial respiration decrease manifested by impaired state 3, longer phosphorylative lag-phase, reduced maximal transmembrane potential and calcium-induced mitochondrial permeability transition pore (MPTP) opening. Moreover, exercise prevented doxorubicin-induced decrease of cardiac mitochondrial respiratory chain complexes I and V activities (25).

Streptozotocin-induced hyperglycemia is another deleterious stimulus for cardiac mitochondrial functionality against which endurance treadmill training demonstrated a cross-tolerance effect (23). Indeed, 14 week of endurance training was shown to revert in rats the hyperglycemia- related increased susceptibility to MPTP gating of heart mitochondria. The beneficial effect of treadmill training was mediated by alterations in the expression of pore proteins like adenine nucleotide translocator (ANT) and cyclophilin D as

well as in pro- and anti-apoptotic proteins of the Bcl-2 family (23). Endurance training attenuated the increased activities of caspase-3 and -9 as well as the amplified Bax/Bcl-2 ratio observed in the sedentary hyperglycemic group. Furthermore, endurance training reverted the hyperglycemia-induced cyclophilin D elevation, attenuating the decrease of ANT, voltage-dependent anion channel (VDAC) and transcription factor A (Tfam) (23).

In aging, a lifelong voluntary wheel running program significantly reduced heart subsarcolemmal (SSM) and interfibrillar (IFM) mitochondrial hydrogen peroxide production (24). Additionally, MnSOD activity was significantly lowered in SSM and IFM following chronic voluntary exercise, which was interpreted as a sign of a reduction in mitochondrial superoxide production. Nevertheless, wheel running did not altered respiratory parameters (state 3, state 4 and RCR), antioxidant activities or content (catalase, glutathione peroxidase and glutathione reductase) or lipid peroxidation (24). This may suggests a balance between ROS-induced damage to lipids and antioxidant-repair systems (27).

Taken together, these findings demonstrate that exercise training provides protection to heart mitochondria against deleterious insults like A-R, STZ-induced hyperglycemia, doxorubicin and aging. However, some contradictory findings were reported. A vigorous swimming training program (6 h/day, 5 days/week, 8-9 weeks) was related to mitochondrial dysfunction in a post-ischaemic rat heart (28). In this study, a decrease in state 4 respiration was noticed using malate-pyruvate and 2-oxoglutarate as substrates. The dramatic reduction of state 3 respiration rates with both malate-pyruvate and 2-oxoglutarate and the loss of respiratory control with 2-oxoglutarate clearly demonstrate that mitochondria from hypertrophied hearts induced by excessive hours of swimming reduced their potential for oxidative phosphorylation.

In order to better understand the impact of exercise training on mitochondria functionality, an interactive perspective of the signaling pathways harbored in heart mitochondria is crucial. Proteomics offers great advantages, such it can provide global perception into protein expression, localization and interaction of mitochondrial proteins and related them with pathological conditions (29). Mitochondrial proteomics research has been focused on mitochondrial proteome profiling and of their post-translational modifications (PTMs) to create a complete mitochondrial database (30).

3.3. The Role of Post-Translational Modifications in the Mitochondrial Proteome

Several proteins are post-translationally modified and most of these modifications are essential for their proper function in the cell. PTMs are covalent processing events that change the properties of a protein through proteolytic cleavage or by the addition of a modifying group to one or more amino acids residues (31). They can affect catalytic activity, subcellular localization, hydrophobicity and stability as well as association with other metabolically important pathway enzymes (11). There are about 87,308 experimentally identified post-translational modifications of proteins and 234,938 putative modifications on 530,264 proteins (32), which include glycosylation, phosphorylation, acetylation, ubiquitination and oxidation. Together, these PTMs orchestrate the function of mitochondrial proteome.

Phosphorylation is one of the most frequent PTMs with about one-third of eukaryotic proteins estimated to be phosphorylated as a consequence of the regulatory activity of protein kinases and phosphatases. Proteins of eukaryotic cells are mainly phosphorylated on threonine (Thr), tyrosine (Tyr) or serine (Ser) amino acid residues by protein kinases that catalyze the transfer of γ-phosphate group from ATP to the side chains of these specific amino acids (33). The transfer of γ-phosphate generates negatively charged side chains, which significantly changes the structural properties of proteins. The importance of phosphorylation/dephosphorylation in the regulation of mitochondrial processes is supported by the recent findings that mitochondria contain protein kinases, phosphatases, and phosphorylated proteins (34). Kinases and phosphatases comprise approximately 3% of human genome. The number of phosphoproteins identified in mitochondria are more than 3817 considering all tissues/cells from all species (rat, mouse, cow, bovine, rabbit and human) and pathophysiological conditions studied (33). In mitochondria, phosphorylation has been shown to regulate the assembly of the ETC complexes and metabolic flux into the TCA cycle (35).

The ubiquitination is a PTM in which ubiquitin is joined to an amino group on a substrate, either at the free amino terminus or on an internal lysine residue (36). In general ubiquitination occurs through a three sequential steps involving the action of three enzymes (36). In the step one, the C-terminal Gly residue of ubiquitin is activated by a specific enzyme, E1. This step requires ATP and consists in the formation of an ubiquitin adenylate

intermediate with release of PPi, followed by a thioester linkage of ubiquitin to a Cys residue of E1, with release of AMP. In the step two, activated ubiquitin is transferred to an active Cys residue of an ubiquitin-carrier protein, E2. Finally, in the step three, ubiquitin is linked by its C-terminus in an amide isopeptide linkage to a ε-amino group of substrate protein's Lys residues. This step is catalyzed by an ubiquitin-protein ligase (E3 enzyme). Multiple rounds of ubiquitination results in a polyubiquitin (polyUb) tag that often leads to proteolytic degradation by the 26S proteasome (36). The ubiquitin–proteasome system (UPS) is one of the principal system by which cells regulate their proteome and increasing evidences show that the UPS is part of the mitochondrial protein quality control system (36). In several proteomic profilings of ubiquitinated mitochondrial proteins were identified. The proteomic screening of mouse heart allowed to identify 121 proteins that were ubiquitinated, and to detect 33 ubiquitination sites in 21 proteins (37). In this study, mitochondrial proteins represented 38% of all identified proteins, suggesting that the ubiquitination system plays an active role in the regulation of mitochondrial functions. Moreover, the identified mitochondrial proteins were found in all locations, such as the outer membrane, intermembranar space, inner membrane and matrix. The five components of OXPHOS system were identified as ubiquitin substrates (37).

The mitochondrial oxidative phosphorylation is a major source of intracellular reactive oxygen species (ROS) and mitochondrial proteins are main targets for oxidative modifications and consequent loss of function. Protein oxidation results from the reaction of reactive oxygen species (superoxide anion, hydrogen peroxide, and hydroxyl radical) and reactive nitrogen species (nitric oxide and peroxynitrite) with both amino acid side chains and peptidic backbone (38). Reversible modifications occur essentially in the sulfur-containing amino acids, namely cysteine and methionine. The types of oxidation that occur can be divided into reversible modifications, which are likely to be involved in regulatory processes, and irreversible modifications that are unlikely to be directly involved in signaling. Protein carbonylation and nitration are considered major forms of protein oxidation. OXPHOS subunits of heart mitochondria are particularly susceptible to these oxidative modifications (39).

The post-translational acetylation of proteins was reported on a wide array of proteins and occurs by the covalent addition of an acetyl group to a ε-lysine residue. The addition of an acetyl group to lysines neutralizes its positive charge impacting the electrostatic

properties of the protein (40). It was initially reported on histones, but since then several non-histone proteins have been identified to be acetylated (reviewed in 40). In an extensive proteomic survey of cellular proteins, 277 lysine acetylation sites were identified in 133 proteins from two fractions of mouse liver mitochondria, one from fed mice and the other from fasted mice (41). Among the acetylated proteins, 62% were identified in both fractions, 14% were specific to fed mice, and 24% were specific to fasted mice. Most lysine-acetylated proteins from mitochondrial fractions were metabolic enzymes (91 proteins). Interestingly, lysine acetylation was also identified in ATP synthase F_o subunit 8, a protein originated in mtDNA, suggesting that the acetylation reaction can occur in mitochondria (9).

3.3.1. Characterization of Mitochondrial Acetylome by Proteomics

Protein acetylation has recently emerged as an important post-translational modification that regulates mitochondrial proteins and overall organelle function. Despite its recognized importance, few studies have performed acetylome profiling of isolated mitochondria. In one of these studies, 133 mitochondrial proteins were found to be acetylated on lysine. From these, more than half are involved in some aspect of energy metabolism (41). Notably 44% of mitochondrial dehydrogenases (21 proteins complexes) were acetylated suggesting that lysine acetylation serves as a feedback mechanism for the regulation of these enzymes activities in response to cellular energy demands. Moreover, the identified acetyl-lysines in 16 proteins were believed to influence longevity (41).

Recent improvements in proteomic technologies have extended the identification and quantitative analysis of protein expression in different pathophysiological conditions to include the study of their PTMs. Figure 3 deciphers the general workflow of proteomic analysis, including mitochondrial isolation, purification, mass spectrometry (MS) identification and data analysis.

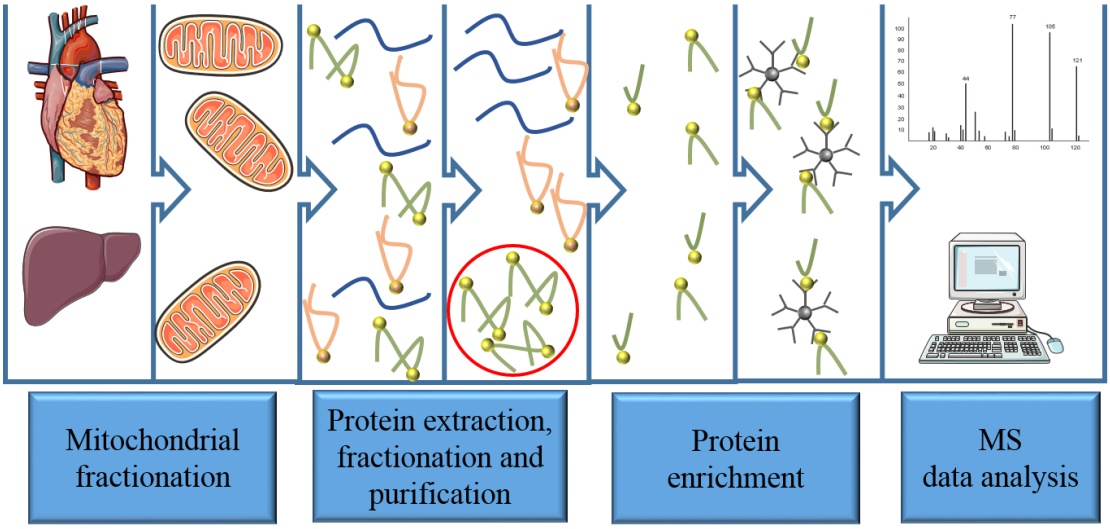

Figure 3. Proteomics workflow for lysine acetylated proteins analysis from mitochondria isolated from tissues. MS: Mass spectrometry. Figure was made based on Servier Medical Art.

An essential first step of mitochondrial PTMs identification is the preparation of pure and functional viable mitochondria. Mitochondrial fractions might be prepared by the disruption of the cellular organization (tissue homogenization) and differential centrifugation of the homogenate (42-45) followed by purification using free-flow electrophoresis or density gradients (e.g. Ficoll, Percoll, or Nycodenz) (41, 46). After these purification steps, some methods are employed for the enrichment of modified proteins/peptides, reducing proteomic complexity of biological samples and increasing the number of identified proteins. The enrichment approaches for protein acetylation have been based on immunoaffinity selection or immunoprecipitation, peptide isoelectric focusing and microchip (47-50). For example, Verdin and coworkers (51) isolated liver mitochondria by differential centrifugation, purified the mitochondrial fraction by density gradient centrifugation, digested mitochondrial matrix lysates with trypsin, immunoprecipitated the acetylated peptides with an anti-acetyllysine antibody and analyzed the resultant peptides by mass spectrometry. This approach allowed the identification of more than 1000 acetylated peptides in liver mitochondria from SIRT3 knockout mice. Immunoprecipitation using a combination of two polyclonal anti-acetyllysine antibodies was used by another research group in order to increase the diversity and efficiency of acetylated lysines peptides detection (45). This experimental strategy allowed the identification of 2187 unique lysine acetylation sites across 483 proteins in the liver mitochondria.

In addition to the enrichment of modified proteins/peptides, further separation methods are usual employed to reduce sample complexity before MS analyses (46). These methods include gel-based approaches such as one dimensional SDS-PAGE (42), two dimensional PAGE (2-DE) (52) for proteins as well as gel free-based approaches such as liquid chromatography (53) using e.g. strong cation exchange (SCX) (44) for trypsin generated peptides. After a separation step, sample is subjected to MS analysis. In recent years, MS has evolved dramatically and is now considered a central technology for the identification and quantification of proteins and PTMs (48). The development of two soft ionization techniques capable of ionizing non-volatile and thermounstable biological compounds, namely electrospray ionization (ESI) and matrix-assisted laser desorption/ ionization (MALDI) has facilitated protein analysis by MS and have been extensively used to study mitochondrial protein PTMs (46, 48). For tandem MS (MS/MS) experiments, fragmentation methods used are collision-induced dissociation (CID), electron-capture dissociation (ECD), electron transfer dissociation (ETD) and high energy dissociation (HCD). CID is still the most common fragmentation method, while the use of ECD and EDT for the characterization of protein PTMs in biological samples is still rare (48). Among mass analyzers, three basic types are often used for PTMs studies: ion trap (IT), time-of-flight (TOF) and quadrupole (Q). Once high resolving power and accuracy are essential for PTMs analysis, the most common tandem mass analyzers used are quadrupole time-of-flight (QqTOF), time-of-flight/time-of-flight (TOF/TOF) and ion trap/orbitrap (IT/orbitrap) mass spectrometers (48). Recently, the orbitrap has acquired a prominent role in the study of acetylated proteins. For example, the study of mitochondrial acetylome in wild-type or SIRT3 knockout mice submitted to caloric restriction was performed using nano reverse phase liquid chromatography coupled to an ESI-Orbitrap (44). In this study, mitochondria were isolated by differential centrifugation and digested with LysC followed by trypsin. Resulting peptides were labeled with tandem mass tag isobaric levels and fractionated by SCX. Acetylated peptides were enriched with pan-acetyllysine antibody-agarose conjugate. Acetyl enriched and protein fractions were analyzed by nano-reverse phase liquid chromatography coupled to an ESI-Orbitrap. Fragmentation method used is HCD (44).

In parallel to protein identification, two main strategies have been applied to the quantitative analysis of mitochondrial protein PTMs: a differential labeling quantitative techniques and MS-based label free approach. Isotope-labeling experiments include isotope-

coded affinity tag, isobaric tags for relative and absolute quantification (iTRAQ) or tandem mass tag (TMT), stable isotopic labeling by amino acid in cell culture (SILAC) and stable isotopic labeling in mammals (SILAM). Post metabolic labeling strategies, such as iTRAQ, might be impractical in some cases. For example, peptide centric workflows that target PTM-containing peptides or immunoaffinity enrichment procedures are incompatible with the chemical tag (48, 54). However, iTRAQ and TMT were used for quantitative mitochondrial acetylome analysis of fasting and refeeding mice (55). Label-free quantitative methods are a suitable alternative for protein PTMs experiments. These quantification methods might be used for mitochondrial acetylome analysis. For example, label-free quantification was used to analyze changes in lysine acetylation from mouse liver mitochondria in the absence of SIRT3 (45). This method revealed that lysine acetylation at 283 sites present in 136 proteins was significantly increased in the absence of SIRT3.

Finally, modified peptides are identified through databases by searching their tandem mass spectrum using pattern matching with a variety of different algorithms, such as Peptide Sequence Tags, Sequest, or Mascot (48). The integration of acquired data in biological and molecular processes allow disclosing the physiological impact of lysine acetylation in mitochondrial proteins. So, it is increasingly recognized the value of bioinformatic tools for this kind of data analysis. DAVID, PANTHER, and REACTOME are examples of tools that allows the integration of proteins datasets, based on Gene Ontology terms, in metabolic pathways (56), giving an integrated picture of the most probable processes modulated by this type of PTM.

3.3.2. Regulation of Mitochondrial Acetylome by Sirtuins

Reversible lysine acetylation is highly regulated by protein deacetylases (9) and acetyltransferases (57). The majority of mitochondrial acetylation studies have been focused on mitochondrial deacetylases. However, recently it was identified a mitochondrial acetyltransferase, the general control of amino acid synthesis 5-like 1 (GCN5L1) protein (57). This protein functions as an essential component of the mitochondrial lysine acetyltransferase machinery and modulates mitochondrial respiration via acetylation of ETC proteins. The GCN5L1 depletion diminishes mitochondrial protein acetylation and augments mitochondrial enrichment of autophagy mediators and consequently increase mitochondrial

turnover and reduced mitochondrial protein content and/or mass (58). Interesting, the GCN5L1 also counters the mitochondrial deacetylase function of SIRT3 (57), a member of a large family of NAD^+-dependent protein deacetylases called sirtuins. Knockdown of SIRT3 increased mitochondrial protein acetylation which was significantly reversed by the knockdown of GCN5L1. As a result, the knockdown of SIRT3 diminished mitochondrial oxygen consumption and cellular ATP levels and the knockdown of GCN5L1 reversed these metabolic phenotypes (57).

Sirtuins are known to deacetylate lysine residues on histones and non-histone proteins (40) and are NAD^+-dependent enzymes. The deacetylation of target residues, coupled to the cleavage of NAD^+, results in the generation of nicotinamide and metabolite O-acetyl-ADP-ribose (OOADPr). Sirtuins in mammals are a family of seven protein deacetylases/ADP ribosyltransferases (SIRT1–SIRT7). These have distinct subcellular localizations, such as nucleus (SIRT1, -2, -3, -6 and -7), cytoplasm (SIRT1 and -2) and mitochondria (SIRT3, -4 and -5) (59). The mitochondrial sirtuins are referred to as mitochondrial stress sensors that can modulate activity of several mitochondrial proteins involved in metabolism (60).

The first report on the functional role of mitochondrial proteins acetylation described the activation of acetyl-CoA synthase (AceCS2), a mitochondrial matrix enzyme, by mitochondrial SIRT3-catalyzed deacetylation of a single lysine residue (LyS^{635}) (61). AceCS2 is abundant in murine heart and skeletal muscle, being inactivated by acetylation of the conserved lysine residues and rapidly reactivated by SIRT-3 mediated deacetylation (62). This target of SIRT3 is activated under caloric-restriction and functions during ketogenic states (Figure 4).

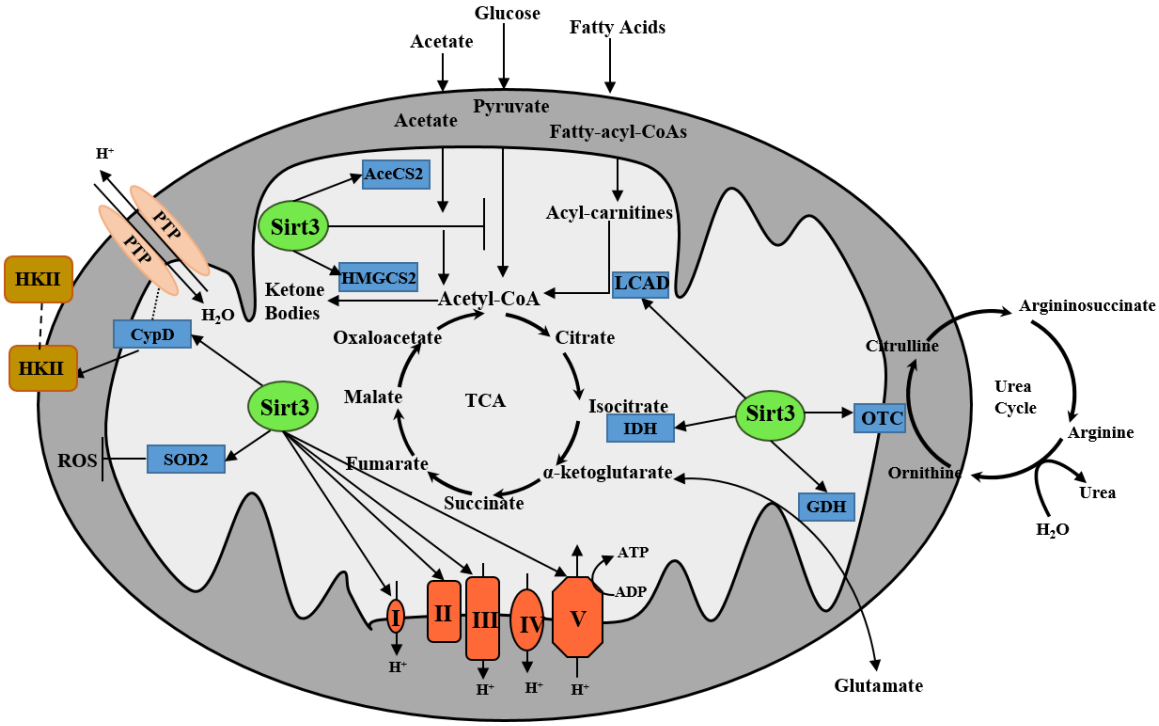

Figure 4. Representation of mitochondrial SIRT3 role in mitochondrial pathways. Mitochondrial SIRT3 influences metabolism by affecting several aspects of mitochondrial physiology. Adapted from (63). Figure was made based on Servier Medical Art.

Three additional mitochondrial matrix proteins have been identified as substrates of SIRT3 and in all, lysine deacetylation results in the increase of their activities. These proteins include glutamate dehydrogenase (GDH), which catalyzes the oxidative deamination of glutamate to α-ketoglutarate; the TCA enzyme isocitrate dehydrogenase 2 (IDH2), which promotes regeneration of antioxidants (64); and the urea cycle enzyme ornithine transcarbamoylase (65). GDH is also a target of SIRT4. But in contrast to SIRT3, SIRT4 inhibits the activity of this enzyme via ADP-ribosylation preventing the usage of amino acids as an energy source under basal dietary conditions (Figure 5). On the other hand, SIRT4 activity is suppressed during caloric restriction resulting in the activation of GDH, which fuels the TCA cycle and possibly also gluconeogenesis (66). Another mitochondrial matrix enzyme involved in the urea cycle and regulated by acetylation/ deacetylation is the enzyme carbamoyl phosphate synthetase 1 (CPS1) (67). This enzyme detoxifies and disposes

ammonia in the organism, and it is deacetylated and activated by SIRT5. Indeed, fasted SIRT5 knockout mice revealed elevated serum ammonia levels (Figure 5).

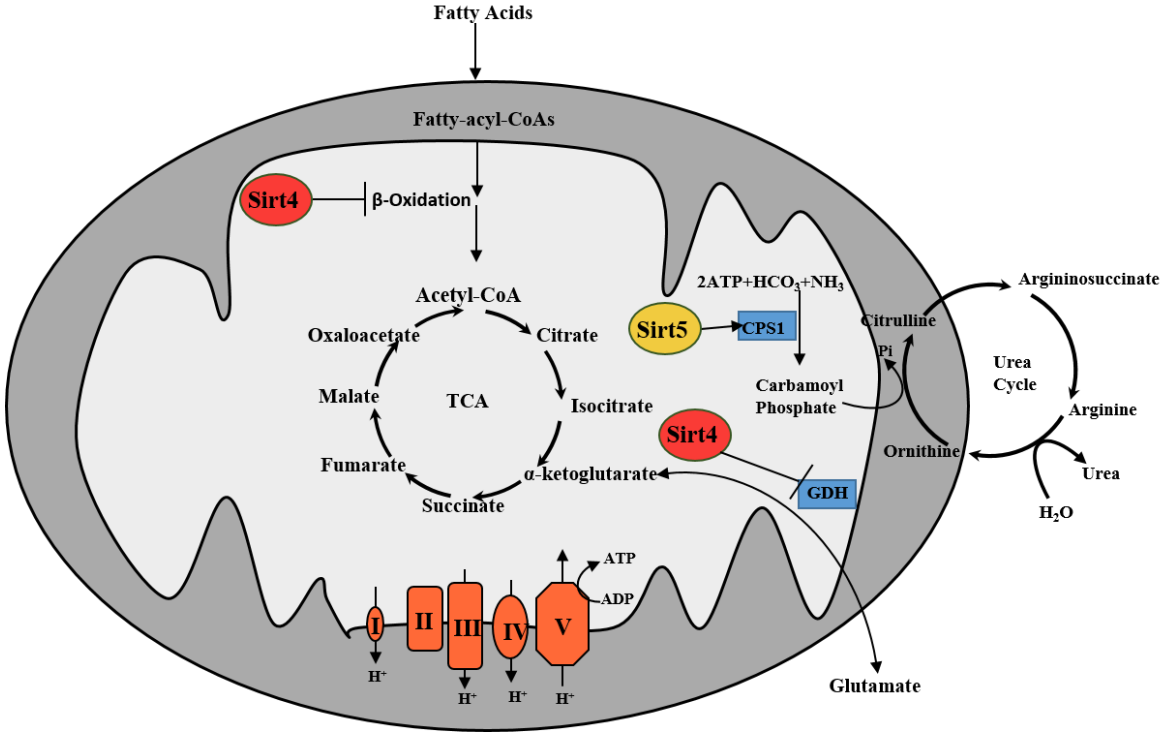

Figure 5. Representation of SIRT4 and SIRT5 role in mitochondrial pathways. Mitochondrial SIRT4 and -5 influence metabolism by affecting several aspects of mitochondrial physiology. Adapted from (63). Figure was made based on Servier Medical Art.

Moreover, the mitochondrial sirtuins SIRT3 and SIRT4 have been suggested to modulate fatty acid oxidation in skeletal muscle. SIRT3 was shown to stimulate fatty acid deacetylation on one acetylation site of long-chain acyl-CoA dehydrogenase (LCAD) out of eight total acetylation sites, thereby activating the fatty acid oxidation pathway (Figure 4) (51). LCAD was found hyperacetylated at lysine 42 in the absence of SIRT3, and deacetylated by this sirtuin in wild-type mice under fasted conditions. Furthermore, fasted SIRT3-knockout mice exhibited hallmarks of fatty acid oxidation disorders including reduced ATP levels and cold intolerance (51).

Another study showed that SIRT3 deacetylates and stimulates the catalytic activity of 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2), a mitochondrial enzyme that catalyzes the conversion of acetoacetyl-CoA in 3-hydroxy-3-methylglutaryl-CoA, the rate-

limiting step in β-hydroxybutyrate production (Figure 4) (68). In fasted SIRT3 knockout mice HMGCS2 is hyperacetylated and its enzymatic activity is reduced, resulting in the decrease of β-hydroxybutyrate synthesis. The sites of acetylation in HMGCS2 were shown to be regulated by SIRT3. Of eleven acetylation sites identified only three are targets of SIRT3 (Lys^{310}, Lys^{447} and Lys^{473}). Acetylation of Lys^{310}, Lys^{447} and Lys^{473} leads to substantial changes in conformation and dynamics of the enzyme, primarily in two regions close to the active site, leaving the remainder of the protein largely unchanged. Such as HMGCS2, the AceCS2 is deacetylated and activated by SIRT3 and involved in ketogenesis, suggesting that SIRT3 have an important role as regulator of ketone bodies (62, 68).

Interestingly, SIRT3 also deacetylates and inactivates cyclophilin D, a mitochondrial matrix protein, diminishing its peptidyl-propyl cis-trans isomerase activity and inducing its dissociation from the ANT, which then promotes the detachment of hexokinase II (HKII) from mitochondria, stimulating oxidative phosphorylation (69) (Figure 4). Moreover, SIRT3 can regulated MPTP through the deacetylation of cyclophilin D at lysine 166, suppressing age-related cardiac hypertrophy (70). Cardiac myocytes from SIRT3 knockout mice were reported to exhibit increased mitochondrial swelling due to increased MPTP opening and accelerated signs of aging in the heart including cardiac hypertrophy and fibrosis (70). These data show that a loss of SIRT3 activity leads to increased activation of MPTP, which, in turn promotes mitochondrial alterations resulting in enhanced ROS production and cell dysfunction. It is also evident that SIRT3 activity is necessary to prevent mitochondrial and cardiac hypertrophy during aging (70). SIRT3 also regulates the activity of superoxide dismutase 2 (SOD2). Overexpression of SIRT3 in wild type mouse was shown to reduce cellular ROS levels by 40%.This diminishing of cellular ROS by SIRT3 was blunted in SOD2 knockout mouse, indicating that SOD2 is the major downstream mediator of SIRT3 in reducing cellular ROS. SIRT3 activates SOD2 via deacetylation of Lys^{53} and Lys^{89} that are highly conserved across species (Figure 4) (71). Sirtuins have also been shown to deacetylate and activate multiple protein subunits of OXPHOS. SIRT3 can physically interact with complex I NDUF9 subunit, succinate dehydrogenase flavoprotein (SdhA) subunit, core I of complex III and with the ATPase subunit α of complex V (42, 43, 72, 73). SIRT3 activates these complexes and so, the genetic depletion of SIRT3 commits OXPHOS activity, mitochondrial oxygen consumption, and ATP production (Figure 4) (42, 43, 72, 73).

3.4. Aims

Considering the role of protein acetylation on the regulation of metabolism and the effect of physical activity in heart metabolic remodeling, the aim of the present thesis was to evaluate the impact of moderate exercise training on heart mitochondrial acetylome. To achieve this goal, we characterized the heart mitochondrial proteome and acetylome of sedentary (control) and lifelong exercised animals using liquid chromatography coupled to tandem mass spectrometry – nanoLC-MS/MS and related with SIRT3 and ATP synthase expression and ATP synthase activity.

II. Materials and Methods

2.1. Chemicals and Reagents

Chemicals reagents such sodium orthovanadate, Triton X-100, SDS, glycerol 99%, bromophenol blue, ethylenediaminetetraacetic acid disodium salt (EDTA), ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), iodoacetamide (IAA), dithiothreitol (DTT), HEPES, ammonium molybdate, oligomycin, protease subtilopeptidase A, protease inhibitor cocktail (#P8340), phosphatase inhibitor cocktail 2 (#P5726), phosphatase inhibitor cocktail 3 (#P0044), and bovine serum albumin (BSA) fat free were purchased from Sigma (Karlsruhe, Germany). Sequencing grade modified trypsin (porcine) was from Promega (Madsion, U.S.). Rabbit monoclonal anti-Sirt3 antibody (#2627), rabbit acetylated-lysine antibody (#9441) and protein A magnetic beads (#8687) were purchased from Cell Signaling Technology (Massachusetts, USA). Rabbit IgG (sc-2027) was acquired from Santa Cruz Biotechnology. Mouse monoclonal anti-ATPB antibody (ab14730) was obtained from Abcam (Cambridge, U.K.). Secondary peroxidase-conjugated antibody (anti-rabbit IgG) was obtained from GE Healthcare (Buckinghamshire, UK).

2.2. Experimental design

In order to understand the impact of lifelong exercise training on heart mitochondrial acetylome, the experimental protocol presented in the Figure 6 was followed.

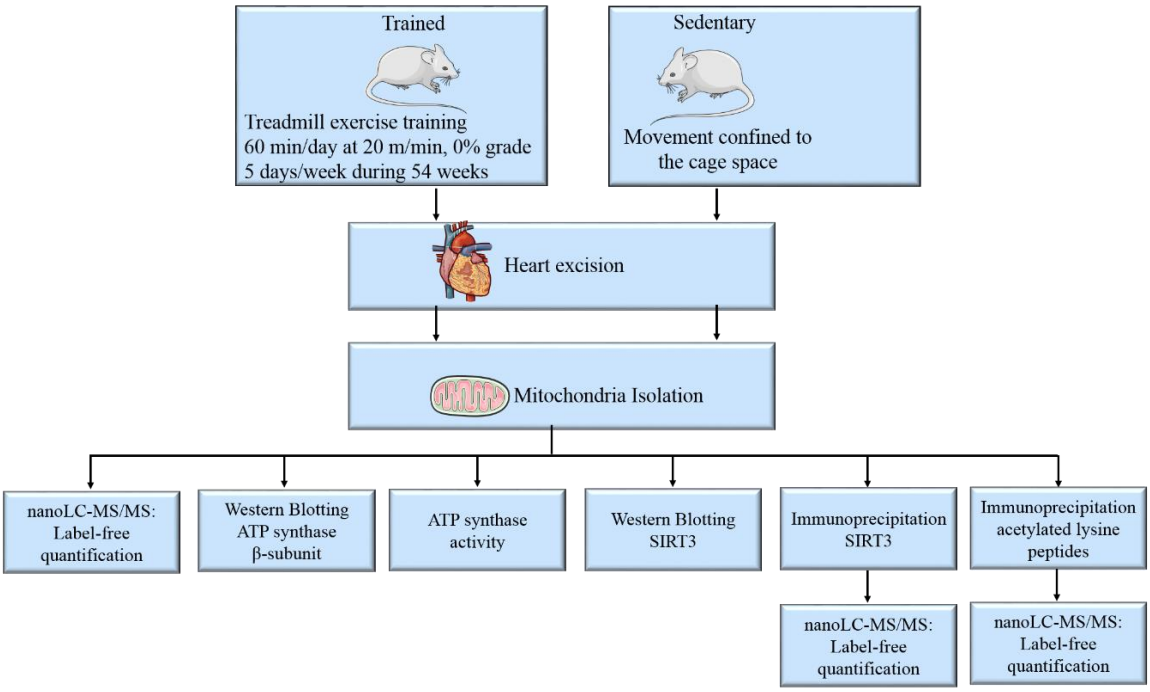

Figure 6. Experimental design followed in the analysis of the effect of exercise training in the mitochondrial acetylome. Eight animals were used *per* group. Figure was made based on Servier Medical Art.

2.3. Animals and Exercise Protocol

Animal experiments were performed according to the Portuguese law on animal welfare and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, Revised 1996). Female Sprague-Dawley rats (n= 20; with the age of 5 weeks and an average body weight of 132.46 g at the beginning of the experiment; provided by Harlan Laboratories Models, Barcelona, Spain), were randomly separated into two groups: i) trained (*Ex* group; n=10; submitted to treadmill exercise training) and ii) sedentary (Cont group; n=10; with movement confined to the cage). Animals were maintained on a 12:12-h light-dark cycle and received food at *ad libitum*. Two animals from each group died during the time-course of the experimental protocol. At the end, 8 animals *per* group were considered for the study.

Animals from the *Ex* group were adapted to treadmill exercise for two consecutive weeks, involving a gradual increase in the running time till 60 min/day at 20 m/min, 0% grade, 5 days/week. This exercise program remained constant during 54 weeks.

Twenty-four hours after the end of the last exercise training for *Ex* group rats were anaesthetized by inhalation with a mixture of 4% sevoflurane with oxygen, intubated for mechanical ventilation (respiratory frequency 100 min^{-1} and weight adjusted tidal volume; Harvad Small Animal Ventilator-Model 683) and placed over a heating pad (37ºC) for hemodynamic evaluation. Once completed this analysis, the heart was excised for mitochondria isolation.

2.4. Mitochondria Isolation

Mitochondria isolation was performed as previously described (74). All procedures were performed at 4 ºC. Briefly, after excised the hearts were immediately washed with 0.5 mM EGTA, 10 mM HEPES-KOH (pH 7.4) and minced in an ice-cold isolation medium containing 250 mM sucrose, 0.5mM EGTA, 10 mM HEPES-KOH (pH 7.4) and 0.1% defatted BSA. The minced blood-free tissue was resuspended in isolation medium containing protease subtilopeptidase A type VIII (1 mg/g tissue) and homogenized with tightly fitted Potter-Elvehjen homogenizer and Teflon pestle. The suspension was incubated for 1 min (4ºC) and re-homogenized. Homogenate was centrifuged at 14500 *g* during 10 min. the supernatant fluid was decanted, and the pellet, essentially devoid of protease, was gently resuspended in solution medium. The suspension was centrifuged (750 *g*, 10 min) and resulting supernatant was centrifuged again (12000 *g*, 10 min). The pellet containing the mitochondrial fraction, was gently resuspended in a washing medium containing 250 mM sucrose and 10 mM HEPES-KOH (pH 7.4). Phosphatase and protease inhibitors were added and all the procedures were performed at 4 ºC.

Mitochondrial protein concentration was estimated with a colorimetric method (RC DC protein assay, Bio-Rad) using BSA as standard. This assay is based on a modification of the Lowry *et al.*(75) protocol, allowing the quantification of the protein in the presence of reducing agents and detergents.

2.5. Determination of ATP synthase activity

For spectrophotometric determination of respiratory chain complex V activity, mitochondrial fractions were disrupted by a combination of freeze–thawing cycles in

hypotonic media (25 mM potassium phosphate, pH 7.2) and the activity was measured as previously described. The phosphate produced by hydrolysis of ATP reacts with ammonium molybdate in the presence of reducing agents to form a blue-color complex, the intensity of which is proportional to the concentration of phosphate in solution. Oligomycin was used as an inhibitor of mitochondrial ATPase activity. Each sample was analyzed in duplicate.

2.6. Immunoprecipitation

For immunoprecipitation, mitochondrial fractions (with 500 µg of protein) were incubated with the antibodies anti-SIRT3 or anti-acetyl lysine. The antibody was added to the sample (at dilution recommended by the supplier) and the mixture was incubated overnight with rotation at 4ºC. Next, immunocomplexes were captured with protein A magnetic beads: 15 µL Protein A magnetic beads was added to the sample. After a 30 min incubation with rotation at 4ºC, the supernatant was discharged. The beads were washed five times with 500 µL of cell lysis buffer (20 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 2.5 mM Na_2HPO_4, 1 mM Na_3VO_4, 1 µg/mL leupeptin, 1% Triton X-100 and 1 mM PMSF). Between washes, the sample was kept on ice. Then, captured proteins were eluted from the beads. To elute the bound proteins for western blot analysis, the beads were resuspended with 20 µL of SDS sample buffer (187.5 mM Tris·HCl (pH 6.8), 6% (w/v) SDS, 30% glycerol and 0.03% (w/v) bromophenol blue) with 1/10 volume of 1.25 M DTT. Then, samples were centrifuged for 30 sec at 14000 *g*, boiled at 100ºC for 5 min and centrifuged at 14000 *g* for 1 min. For negative control of immunoprecipitation experiments was used normal rabbit IgG (sc-2027).

2.7. Western blotting

Fifteen µL of sample were electrophoresed on a 12.5 % SDS-PAGE, as described by Laemmli (76), and then transferred to a nitrocellulose membrane (Whatman®, Proton) in transfer buffer during 2 hours (200 mA). Then, nonspecific binding was blocked with 5% (w/v) dry nonfat milk in Tris buffered saline with Tween-20 (TBS-T (20 mM Tris-HCl pH 8, 150 mM NaCl and 0.05% Tween-20) and the membrane was incubated with primary antibody (anti-SIRT3 or anti-ATP synthase subunit β, 1:1000 in blocking solution) for 2

hours at 4ºC, washed and incubated with secondary horseradish peroxidase-conjugated anti-rabbit (GE Healthcare) for 2 hours. Immunoreactive bands were detected by enhanced chemiluminescence ECL (GE Healthcare) according to the manufacturer's procedure and images were recorded using X-ray film (Kodak Biomax Light Film, Sigma). Films were scanned in Molecular Images Gel Doc XR+ System (Bio-Rad) and analyzed with Quantity One Software (v 4.6.3 Bio-Rad).

2.8. In-solution digestion

Mitochondrial protein extracts were reduced with dithiothreitol, alkylated with iodoacetamide and digested with trypsin using filter-aided sample preparation (FASP) procedure (77). Briefly, 100 µg of each sample was solubilized in 4 % SDS. Protein were reduced with 0.1 M of DTT and 0.1M HEPES, and incubated for 30 min at 60ºC. After reduction with DTT, samples were mixed with 0.2 mL of a solution of 8 M Urea, 0.1 M HEPES, pH 8.5 (UH solution), loaded into filtration devices (3K Micron, Milipore) centrifuged at 14000 *g* for 20 min, and washed twice with UH solution. Then, the concentrates were alkylated with 50 mM iodoacetamide (IAA) in UH solution during 20 min at 25ºC in dark, and washed twice with UH solution. The concentrate was diluted with 0.1 mL of 50 mM ammonium bicarbonate (ABC) and digested 18 h at 37ºC with 2 µg of trypsin diluted in 30 µL of 50 mM ABC. Finally, eluted peptides were acidified with 10 µL of formic acid and cleaned up on a homemade column filled with C18 disk (3M, Empore, USA) for subsequent analysis by LC-MS/MS.

2.9. Peptide separation by nano-HPLC

The peptides were analyzed by nanoflow liquid chromatography tandem mass spectrometry (nanoLC-MS/MS) on an EAY-nLC system (Proxeon Biosystems, Odense, Denmark) connected to the LTQ Orbitrap Velos instrument (Thermo Fisher Scientific, Bremen, Germany) through a nanoelectrospray ion source, as previously described (78). Briefly, one microgram of the peptide mixture for each sample was autosampled directly onto the analytical HPLC column (120 mm X75 µm i.d., 3 µm particle size (Nikkyo Technos Co., Ltd.). Chromatographic gradients started at 97% buffer A (0.1% formic acid in water)

and 3% buffer B (0.1% formic acid in acetonitrile) with a flow rate of 300 nL/min, and gradually increased to 93% buffer A and 7% buffer B in 1 min, and to 65% buffer A/35% buffer B in 120 min. After each analysis, the column was washed for 10 min with 10% buffer A/90% buffer B. The effluent obtained from the HPLC column was directly electrosprayed into the mass spectrometer. The mass spectrometer was operated in positive ionization mode with nanospray voltage set at 2.2 kV and source temperature at 325 °C. Ultramark 1621 for the FT mass analyzer was used for external calibration prior the analyses. Moreover, an internal calibration was also performed using the background polysiloxane ion signal at m/z 445.1200. The instrument was operated in DDA mode and full MS scans with 1 microscans at resolution of 60,000 were used over a mass range of m/z 250-2000 with detection in the Orbitrap. Auto gain control (AGC) was set to 1E6, dynamic exclusion (60 seconds) and charge state filtering disqualifying singly charged peptides was activated. In each cycle of DDA analysis, following each survey scan the top ten most intense ions with multiple charged ions above a threshold ion count of 5,000 were selected for fragmentation at normalized collision energy of 35%. Fragment ion spectra produced via collision-induced dissociation (CID) were acquired in the Ion Trap, AGC was set to 5e4, isolation window of 2.0 m/z, activation time of 0.1ms and maximum injection time of 100 ms was used. All data were acquired with Xcalibur software v2.2.

2.10. Protein identification and annotation

Raw files were processed using Proteome Discoverer version 1.3 (Thermo Fisher Scientific, Bremen). Peak lists were searched using Mascot software version 2.3 (Matrix Science, UK) against a SwissProt database containing entries corresponding to *Rattus norvegicus* (version of July 2012) containing 7,755 protein entries, a list of 598 common contaminants, and all the corresponding decoy entries. Trypsin was chosen as enzyme and a maximum of three miscleavages were allowed. Carbamidomethylation (C) was set as a fixed modification, whereas oxidation (M), acetylation (N-terminal) and phosphorylation (STY) were used as variable modifications. Searches were performed using a peptide tolerance of 7 ppm, a product ion tolerance of 0.5 Da. Resulting data files were filtered for Mascot IonScore > 20 for the acetylated dataset.

2.11. Label-free quantitation of acetylated peptides

For each identified acetylated protein, the peak area was extracted using the 'Precursor Ions Area Detector' module of Proteome Discoverer (v1.4) with a mass tolerance of 2 ppm. Areas were normalized to ensure that the medians of all peptide areas were the same in all samples: (log(area)normalized =log(area)raw - (median(log(area))sample – median(log(area))dataset)). Protein abundance was estimated using the median of the three most intense peptides per protein. Observed changes in the abundance of acetylated peptides were corrected with observed protein fold-change: acetylated peptide_logFC =|log(acetylated peptideFC)raw - log(proteinFC)|.

2.12. Statistical Analysis

Regarding western blotting data, the optical densities of each protein band were exported, after normalization, from QuantityOne® imaging software (V4.6.3, BioRad) to GraphPad Prism5 and the unpaired Student’s t-test was used at 95% confidence degree, in order to evaluate differences of protein expression between the two groups (Control and Exercised group). Unpaired Student t-test was also applied to ATP synthase activity data.

III. Results

3.1. Characterization of animal's response to exercise training

In order to evaluate the effect of lifelong exercise training on cardiac function it was performed hemodynamic analysis, which confirmed that 54 week-treadmill running improves cardiac function. Indeed, the exercised animals exhibited a significant increase in peak systolic pressure (Pmax), and a decrease in end-diastolic pressure (EDP) and time constant of ventricular pressure decay (Tau) (data not shown). Moreover, to analyze the oxidative activity in heart, the ATP synthase subunit β expression and ATP synthase activity was evaluated in mitochondria isolated from heart of rats from both groups. There was a significant increase of complex V activity in exercised animals (Figure 7), suggesting that exercised animals exhibited better ability to produce ATP.

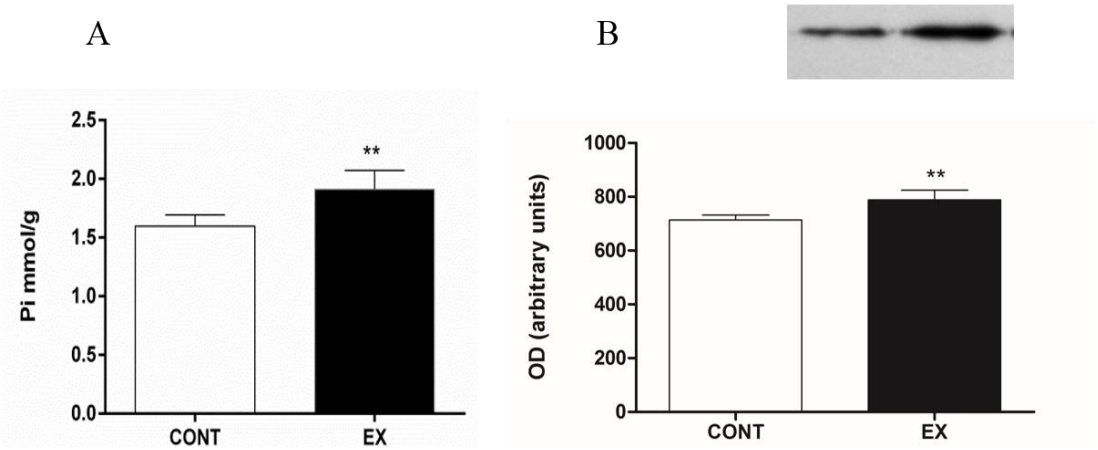

Figure 7. Evaluation of ATP synthase activity (A) and of ATP synthase subunit β expression evaluated by western blotting (B). A representative immunoblot is presented above the graph. Values are expressed as mean ± standard deviation (**p<0.01 vs. *Cont*).

To understand the effect of physical training on the regulation of mitochondria proteome, nanoLC-MS/MS analysis of mitochondria isolated from heart of exercised animals and sedentary ones was performed. This analysis resulted in the identification of 638 proteins, most of which with catalytic activity, binding, structural molecule activity and transporter activity (Figure 8, A). The most representative biological process clusters were localization, metabolic process and cellular metabolic process (Figure 8, B). From the identified proteins, 287 were common to all animals analyzed, 32 were only identified in the

control group and 34 were exclusively observed in exercised animals. The majority of these unique proteins were involved in metabolism.

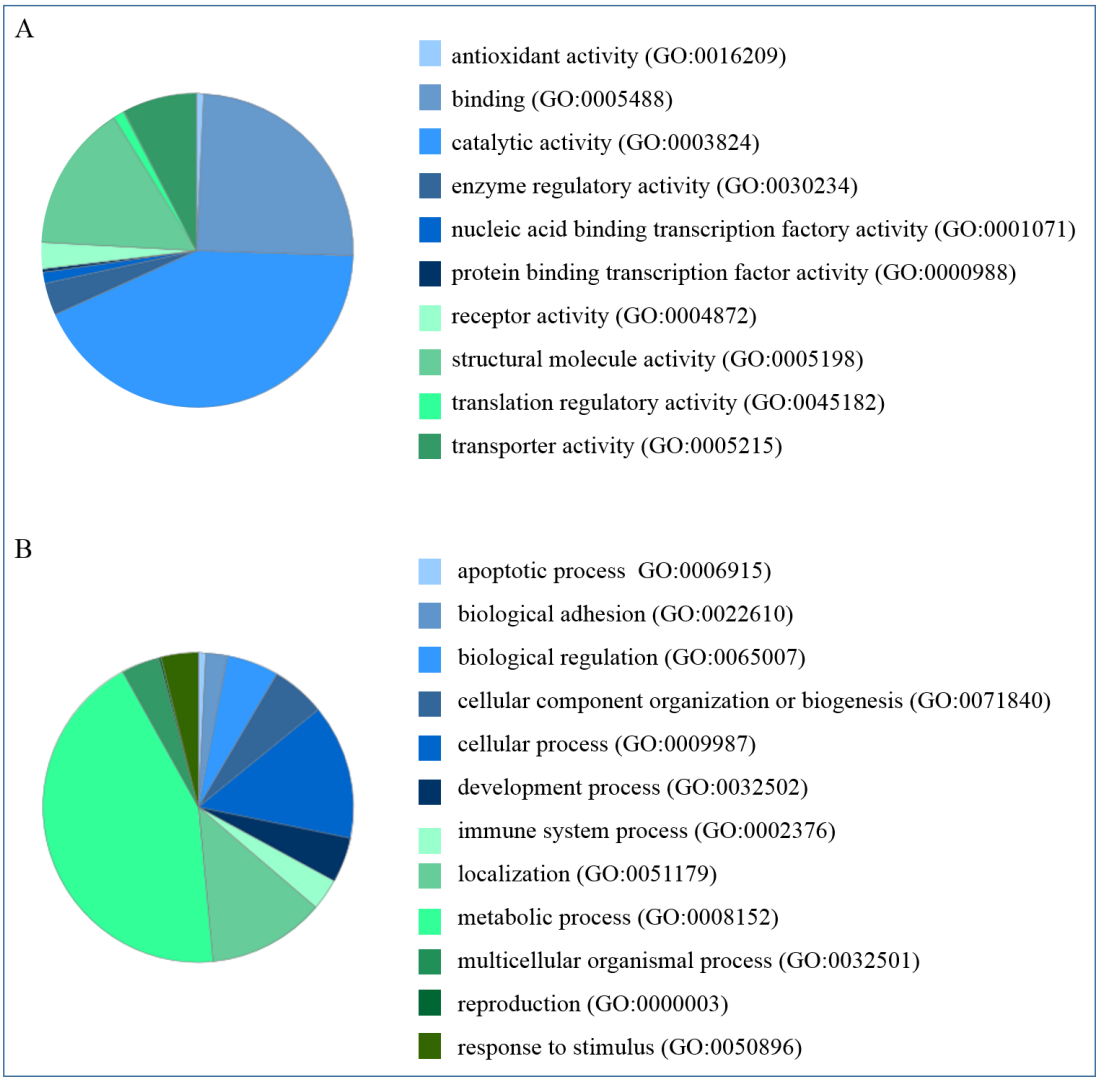

Figure 8. Categorical analysis of identified proteins based on molecular function (A) and biological process (B) assigned by PANTHER.

3.2. Impact of exercise in the regulation of mitochondrial acetylome

In order to evaluate the effect of exercise in the regulation of mitochondrial acetylome, nanoLC-MS/MS analysis of isolated mitochondria was performed targeting acetylated proteins. This approach resulted in the identification of 9 lysine acetylated peptides from 9 proteins (Supplemental Table A- 1). The two most significantly molecular

function clusters to which these proteins belong were binding and catalytic activity (Figure 9, A). Similarly, the most representative biological process was metabolism (Figure 9, B).

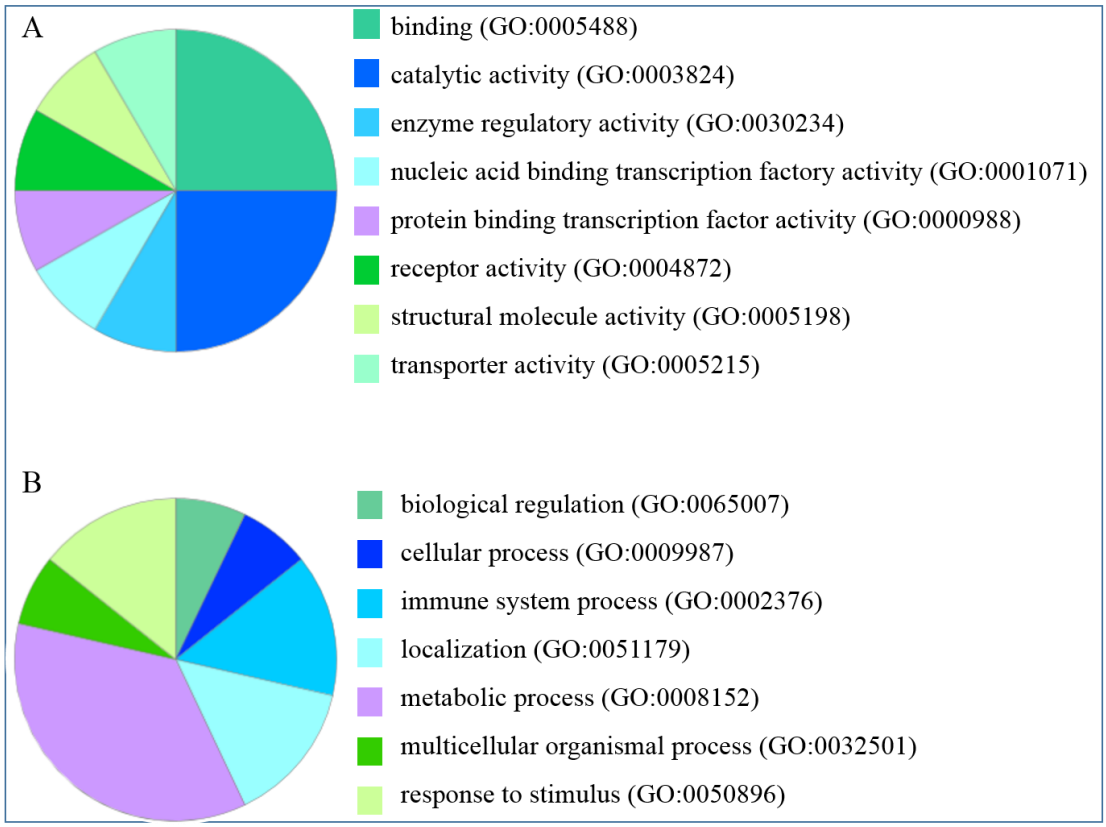

Figure 9. Categorical analysis of lysine acetylated proteins based on molecular function (A) and biological process (B) assigned by PANTHER.

From the identified proteins, 5 were common to all animals analyzed, 3 were exclusively identified in exercised animals and 1 was only observed in sedentary rats (Table 1). Interestingly, serine/threonine-protein kinase 10, inositol 1,4,5-trisphosphate receptor type 2 and protein phosphatase 1 regulatory subunit 14B were only found acetylated in exercised animals. These proteins are not traditionally located in mitochondria but they seem to interact with this organelle in the heart of exercised animals. Indeed, it is nowadays recognized that a significant portion of the identified proteins has more than one subcellular location, which biological significance remains to be disclosed. Multi-residential proteins seem to support mitochondrial protein trafficking and communication with other organelles

(78). Additional analysis of protein domains using Pfam database (http://pfam.sanger.ac.uk) revealed that some of identified acetylation sites are located in known protein domains such as polo kinase kinase domain identified on serine/threonine-protein kinase 10 and PKC-activated protein phosphatase-1 inhibitor domain identified on protein phosphatase 1 regulatory subunit 14B. This domain family of polo kinase kinase is essential during mitosis for the activation of Cdc25C, for spindle assembly, and for cyclin B degradation.

Table 1. List of identified acetylated proteins unique in control (Cont) and unique in exercised group (Ex), including acetylated lysine site, Domain Pfam and functional role.

Accession number	Protein name	Acetylated lysine	Domain (Pfam)	Functional Role	Express in Ex/ Cont
E9PTG8	Serine/threonine-protein kinase 10	K703; K710	Polo Kinase Kinase	Polo-like kinase 1 (Plx1) is essential during mitosis for the activation of Cdc25C, for spindle assembly, and for cyclin B degradation. This family is Polo kinase kinase (PKK) which phosphorylates Polo kinase and Polo-like kinase to activate them.	Ex
P29995	Inositol 1,4,5-trisphosphate receptor type 2	K1113	-	-	Ex
Q8K3F3	Protein phosphatase 1 regulatory subunit 14B	K140	PKC-activated protein phosphatase-1 inhibitor	Signaling pathways activate kinases such as PKC or Rho-dependent kinases that phosphorylate the myosin phosphatase inhibitor protein called CPI-17. Phosphorylation of CPI-17 at Thr-38 enhances its inhibitory potency 1000-fold, creating a molecular switch for regulating contraction	Ex
Q4FZU3	Nuclear speckle splicing regulatory protein 1	K123;K124	DUF2040	The proteins do contain a coiled-coil domain, but the function is unknown.	Cont

In order to evaluate the involvement of SIRT3, the most well characterized mitochondrial deacetylase, in the regulation of these identified acetylated proteins, a protein-protein interaction analysis was performed with STRING 9.1 (http://string-db.org/)

bioinformatic tool (Figure 10). Curiously, according to this analysis none of the identified acetylated proteins interact with SIRT3.

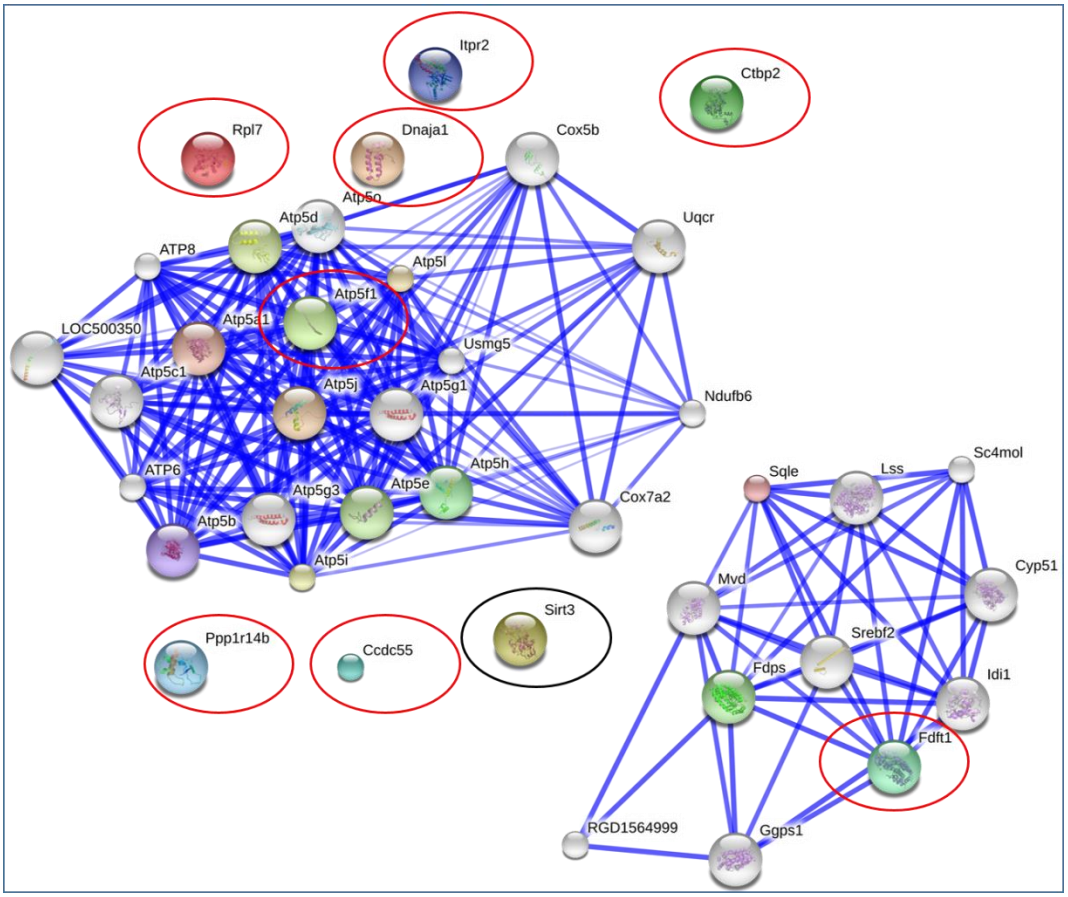

Figure 10. Protein-protein interaction of identified acetylated proteins with SIRT3 (String 9.1). Deacetylase SIRT3 and the identified acetylated proteins are shown with black and red circles, respectively.

Moreover, label-free quantitative analysis evidenced differences among groups for common peptides (Table A- 2). Acetylated peptides distribution among groups was depicted in a radar-plot based on label-free levels (Figure 11). The acetylated peptides DLHHTLILVNNK (N-terminal) from decorin, LLQLGHKac from PH domain leucine-rich repeated protein phosphatase 1, QMKacIIHKacNGYSK from guanine nucleotide-binding protein G(t) subunit alpha-3 and VKLLTGKac from dynamic-like 120 kDa protein were found to have an increase in magnitude change of 0.03, 0.004, 0.004 and 0.0015, respectively, being more abundant in mitochondria of exercised rats. In mitochondria of

sedentary animals, the acetylated peptides LTLASKLKacR from NEDD8-conjugating enzyme UBE2F and SSGALLPKPQMR (N-terminal) from cytochrome c oxidase subunit 6C-2 were found in higher amounts (0.002 and 0.002 magnitude change). According to ClueGo + CluePedia comparative analysis, the most representative biological processes on exercise animals are G-protein signaling, coupled to cyclic nucleotide second messenger and embryonic heart tube formation. In sedentary rats the most representative biological process is transition between slow and fast fibers (Figure 12).

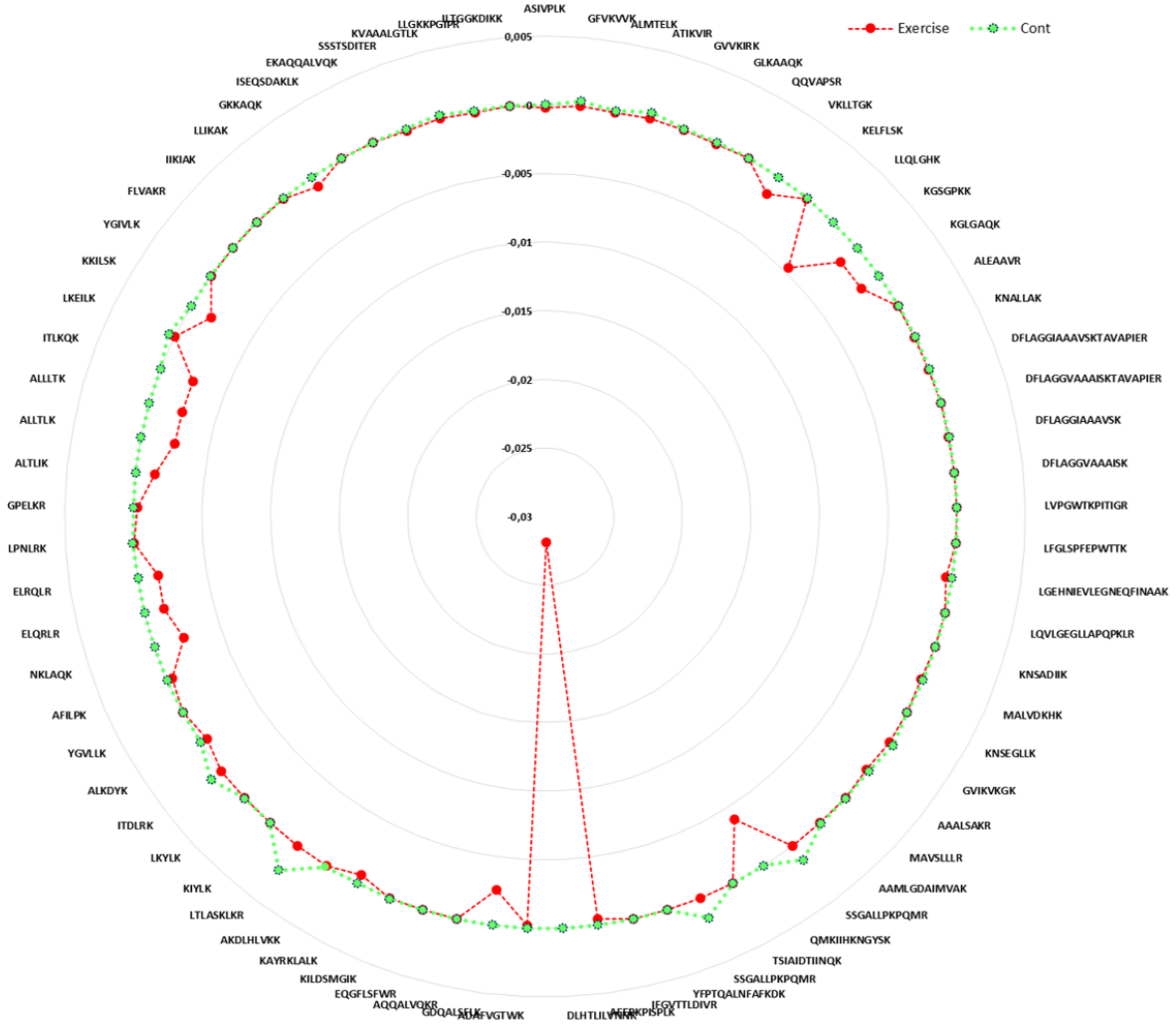

Figure 11. Radar plot of frequency analysis of acetylated peptides modulated by exercise training. Red nodes refer to exercised group whereas green nodes refer to control group.

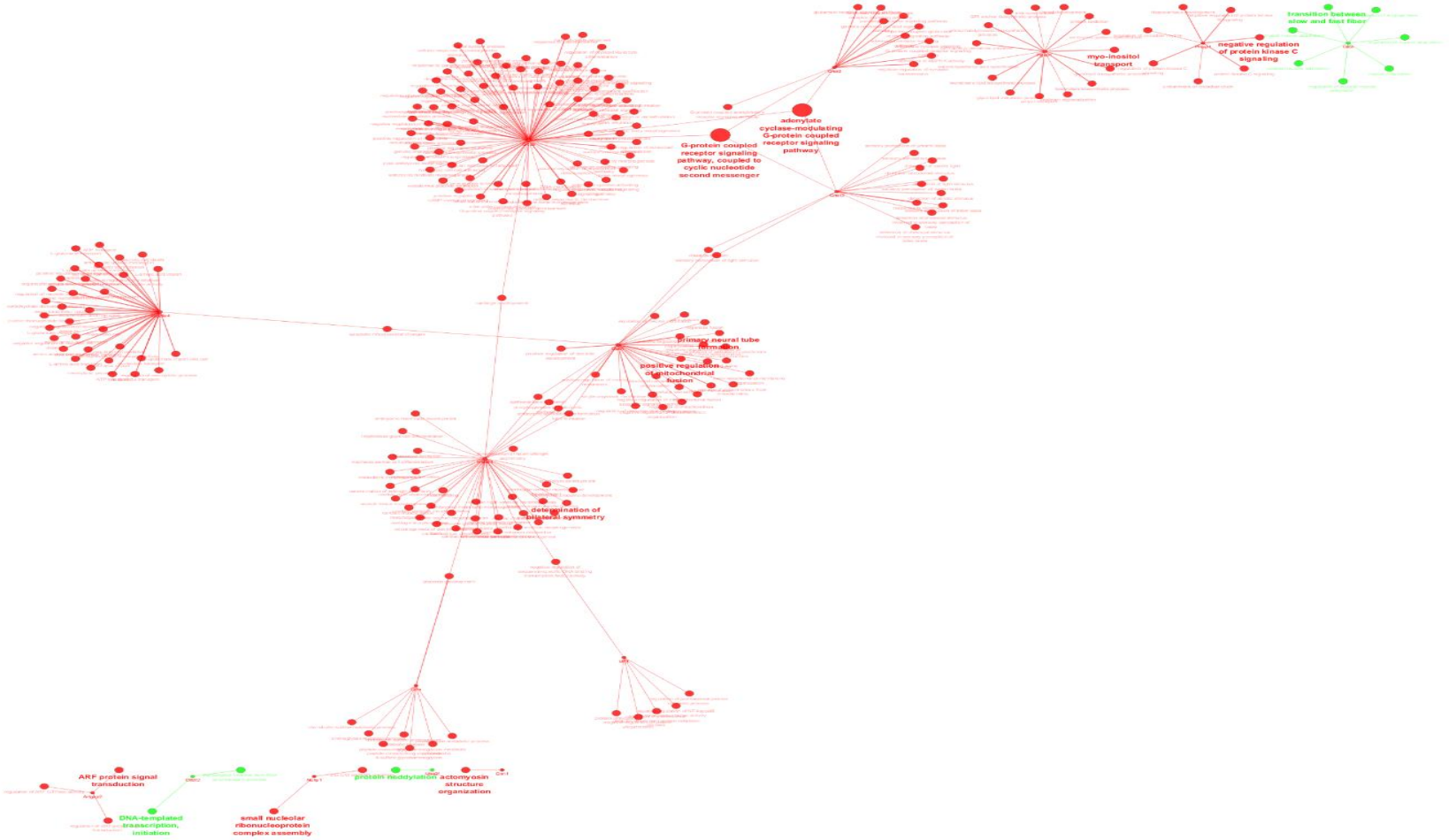

Figure 12. Comparative ClueGo + CluePedia analysis of increased acetylated proteins in each group according biological processes. Red nodes refer to exercised group whereas green nodes refer to control group.

Using an experimental approach for the enrichment of acetylated proteins based on acetyl-lysine immunoprecipitation followed by LC-MS/MS analysis with label-free quantification it was possible to identify 111 proteins, from which 4 *N*-terminal acetylated and 7 lysine acetylated proteins were considered for comparative analysis between groups once the acetylated peptides were detected (Supplemental Table A- 3). The most significant molecular function cluster to which these proteins belong was catalytic activity (Figure 13, A). Similarly, the most representative biological process was metabolism (Figure 13, B).

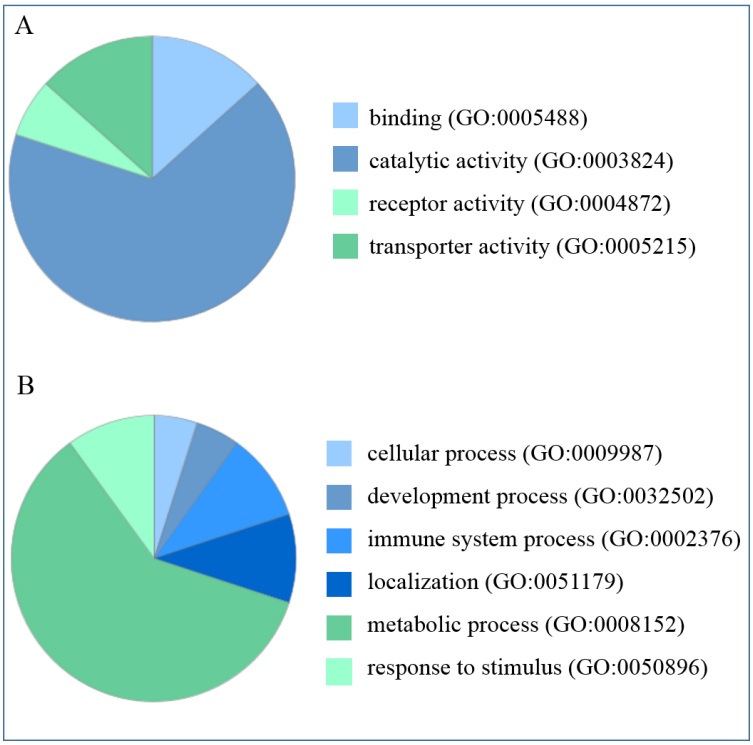

Figure 13. Categorical analysis of enriched lysine acetylated proteins based on molecular function (A) and biological process (B) assigned by PANTHER.

From these, 5 acetylated peptides from 5 proteins were found lysine acetylated in heart mitochondria from exercised rats (Supplemental Table A- 3). The two most significant molecular function clusters to which these proteins belong were catalytic activity and binding (Figure 14, A). The majority of these acetylated proteins are involved in metabolism (Figure 14, B). Indeed, the largest changes in lysine acetylation were observed in key metabolic pathways including ketogenesis (hydroxymethylglutaryl-CoA lyase (HMGCL)), in tricarboxylic acid cycle (TCA) (citrate synthase (CS)), and fatty acid oxidation (enoyl-CoA isomerase 1 (ECI1) and short-chain specific acyl-CoA dehydrogenase) (Supplemental

Table A- 3). The acetylation of K48 residue of HMGCL, K327 of citrate synthase and K410 of dihydrolipoyl dehydrogenase was previously reported (45); however, its effect on activity is not known.

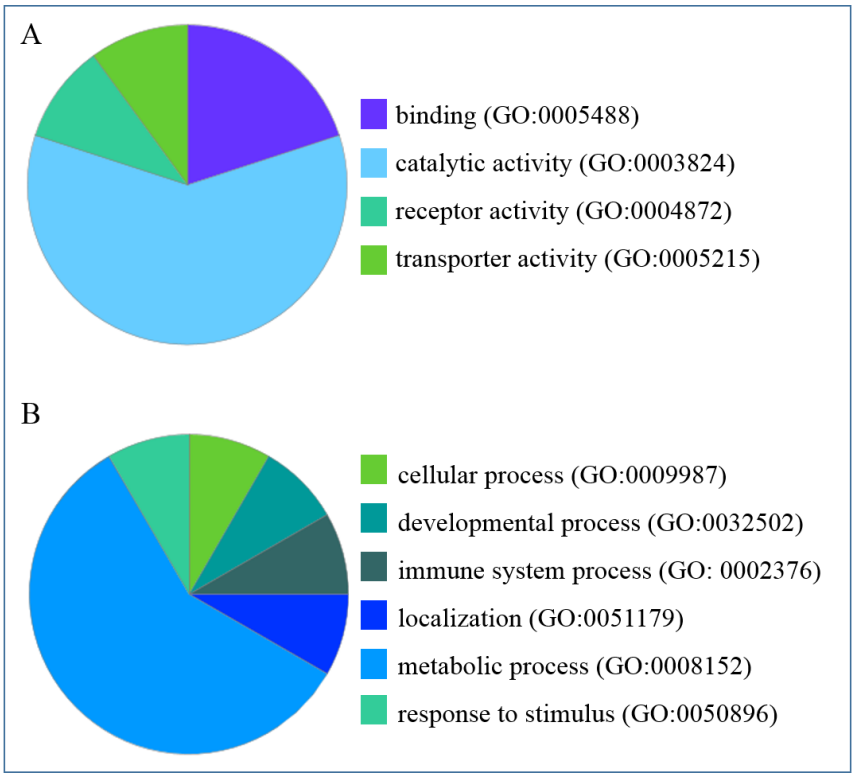

Figure 14. Categorical analysis of lysine acetylated proteins of exercised animals based on molecular function (A) and biological process (B) assigned by PANTHER.

Additional protein domain analysis performed with Pfam database (http://pfam.sanger.ac.uk) revealed that some of the identified acetylation sites are located in known protein domains such as acyl-CoA dehydrogenase, c-terminal domain identified on short-chain specific acyl-CoA dehydrogenase (Supplemental Table A- 3). Moreover, protein-protein interaction network analysis performed with String (9.1) showed complex interaction among acetylated proteins. However, according to this analysis none of the identified acetylated proteins interact with SIRT3 (Figure 15).

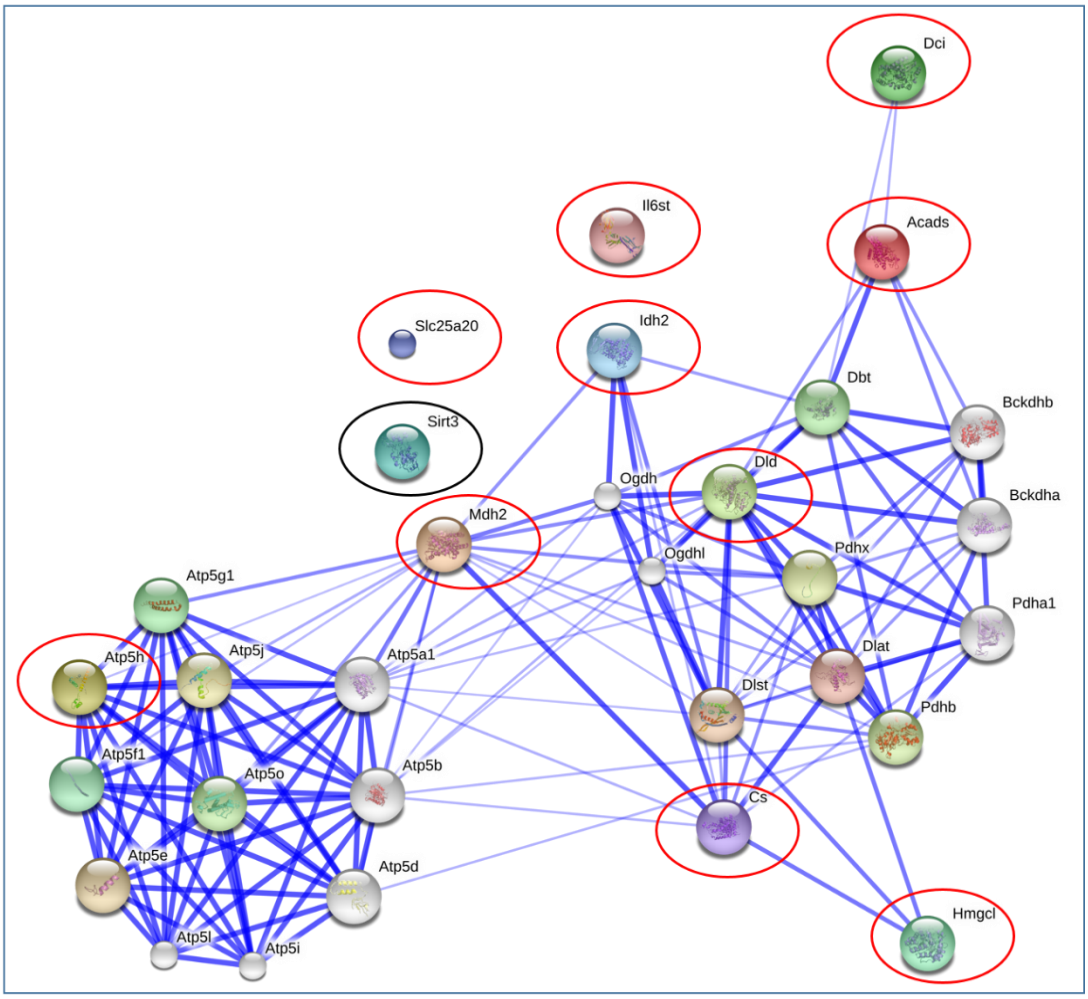

Figure 15. Protein-protein interaction of lysine acetylated proteins with SIRT3 (String 9.1). Deacetylase SIRT3 and identified acetylated proteins using enrichment with an anti-acetyllysine antibody followed by LC-MS/MS are shown with black and red circles, respectively.

Considering the role of SIRT3 on the regulation of mitochondrial acetylome, the expression of this mitochondrial deacetylase was evaluated in mitochondria isolated from heart of rats from both groups. Data showed that 54 weeks of treadmill exercise promoted a significant increase of mitochondrial SIRT3 in the heart (Figure 16).

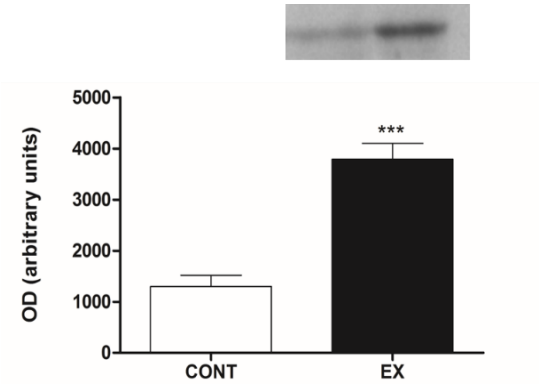

Figure 16. Western blotting analysis of SIRT3 expression in mitochondria isolated from heart. A representative immunoblot is presented above the graph. Values are expressed as mean ± standard deviation (***p<0,001 vs. Cont).

To better explore the role of SIRT3 in the regulation of cardiac mitochondrial acetylome, immunoprecipitation was performed followed by LC-MS/MS; however, no positive identifications were obtained, possibly justified by the low amount of protein obtained. Future work should focus on the optimization of immunoprecipitation's conditions.

IV. Discussion

Exercise has been considered one of the most effective strategies to promote health (78). Indeed, lifelong exercise improves cardiac function and, consequently, promotes health, quality and extension of the life span (78). Increasing evidences suggest that the beneficial effects of increased physical activity occur in mitochondria (7), namely through a significant remodeling of the mitochondrial cardiac proteome towards carbohydrate metabolism, protein translation, redox protection and apoptotic resistance (78-80). Several studies show that endurance training results in a reduction of mitochondrial oxidant capacity (24, 81) and enhanced mitochondrial antioxidant activity. Eight weeks of endurance training was reported to increase the levels of glutathione peroxidase 1 suggesting that heart tissue has a strong enzymatic antioxidant defense against exercise-induced oxidative stress (82). Since energy metabolism is at the heart of mitochondria, the link between cardioprotection and mitochondria is likely. Indeed, the shifting of mitochondrial oxidation preference from fatty acids to glucose results in a relatively greater production of ATP *per* unit of oxygen consumed and has been related to improve cardiac function and clinical outcomes of ischemic heart disease (83). During endurance training heart energy turnover increases several fold when compared with resting conditions, with a consequent rise in mitochondrial ATP production. The increased activity of ATP synthase (Figure 7) in heart mitochondria of lifelong exercised rats supports the increased mitochondrial ability to produce ATP promoted by exercise (25).

Mitochondria are highly dynamic organelles that continuously adjust ATP generation to match changing bioenergetics demands of cells. So, the regulation of proteins involved in energy production may help to satisfy the increased energy demands promoted by exercise and maintain and/or enhance cardiac function in resting conditions (79). Among the benefits in cardiac function, the deacetylation of OXPHOS complexes was reported with the consequent enhance of its oxidative phosphorylative state. In heart mitochondria, the major regulator of ATP synthesis is mitochondrial deacetylase SIRT3 (42). SIRT3 can physically interact with complex I NDUF9 subunit, SdhA subunit, core I of complex III and with the ATPase subunit α of complex V, activating them (42, 43, 72, 73). In the present study, 54 week of treadmill exercise increased the levels of SIRT3 (Figure 16), which might explain, at least in part, the increase of ATP synthase activity and levels observed in the heart of exercised animals (Figure 7). Previous studies have also reported increased activity of the respiratory chain complex in the heart of mice subjected to swimming (84) and endurance

treadmill exercise (85). In heart, SIRT3 also protects against oxidative stress in a Foxo3a – dependent manner, and has also been shown to regulate opening of the MPTP via deacetylation of cyclophilin D (70, 86). So, it is evident that exercise induces a cardiac mitochondrial phenotype that resists to apoptotic stimuli (87). The mechanism underlying the increased expression of SIRT3 is not completely understood; however, sirtuin enzymatic activity requires NAD^+ as a cofactor which levels increase during exercise (60).

Despite increased levels of the deacetylase SIRT3, more mitochondrial proteins were found acetylated in the heart of exercised rats, either using or not enrichment strategies. Though allowing the identification of more post-translational modified proteins, stoichiometry is loss with enrichment strategies, as the one used in the present study, which makes difficult to understand its biological meaning (88). Knowledge of acetylation stoichiometry is often needed to understand the mechanism and impact of this modification in the control of protein functions, such as enzymatic activity (45, 89). However, proteomic experiments carried out without enrichment resulted in the identification of very few acetylation sites, consistent with low to moderate levels of lysine acetylation (45). More recently, Nakayasu et al. (89) described a sophisticated method using a mass spectrometry method with a combination of isotope labeling and detection of a diagnostic fragment ion to determine the stoichiometry of acetylated lysine residues. Indeed, in our study, more proteins were identifies using immunoprecipitation with an anti-acetyl lysine antibody, most of which are involved in metabolic pathways. However, exercise training only impacted 8 proteins, 3 of which involved in fatty acid oxidation, short-chain specific acyl-CoA dehydrogenase, enoyl-CoA delta isomerase 1 and carnitine/acylcarnitine carrier protein (Supplemental Table A- 3). The acetylation of these enzymes, and eventually the inhibition of their activity, might support an exercise-induced metabolic adaptation towards carbohydrate oxidation (78). The acetylation of dynamic-like 120 kDa protein (OPA1) at K^{931} was reported to be a target of SIRT3 (90). Higher levels of acetylated OPA1 were observed in exercised heart (Supplemental Table A- 2). In mammals, this protein is responsible for mitochondrial inner membrane fusion and is also involved in the maintenance of cristae structure and protection of cells from death stimuli. In heart under stress conditions, this protein was found hyperacetylated, reducing its GTPase activity (90). OPA1 appeared to be necessary for the adaptive response to exercise and mitochondrial biogenesis (91). Moreover, it can be speculated whether increased SIRT3 activity during exercise enhanced energy metabolism

is related to organelle fission. Indeed, in the context of mitochondrial dynamics, exercise promotes dynamin related protein 1 (DRP1) phosphorylation at serine 637 by protein kinase A (PKA), a process thought to inhibit DRP1-induced mitochondrial fission and apoptotic cell death (92). Taken together, the acetylation of proteins involved in metabolism and mitochondrial biogenesis seems to underlie heart adaptive remodeling in response to lifelong exercise training.

V. Conclusion

In order to evaluate the effects of exercise training in heart mitochondrial acetylome an animal model was used and involved a training program of 54 weeks of moderate treadmill exercise. Data obtained allowed to conclude that exercise improves cardiac function through adaptation of mitochondrial proteome that resulted in an increased ability to produce ATP. This cardiac adaptive remodeling involved the regulation of mitochondrial proteome acetylome in a process that seems to involve SIRT3 deacetylase. Data didn't allowed to clearly infer on the protein substrates of this deacetylase. Among the main targets of protein acetylation modulated by lifelong physical activity are metabolic proteins, as for example enzymes from in fatty acid oxidation.

Future work will be necessary to clearly identify the mitochondrial proteins regulated by acetylation in exercised heart and the enzymes involved in such regulation, envisioning a better molecular comprehension of the cardiac adaptive remodeling promoted by exercise training.

VI. References

References

1. Global Atlas on Cardiovascular Disease Prevention and Control. Geneva: World Health Organization in collaboration with the World Heart Federation and the World Stroke Organization; 2011.

2. Reel B., Sala-Newby G. B., Huang W.-C., Newby A. C. Diverse patterns of cyclooxygenase-independent metalloproteinase gene regulation in human monocytes. British Journal of Pharmacology. 2011; 163(8): 1679-90.

3. Mathers C. D., Loncar D. Projections of Global Mortality and Burden of Disease from 2002 to 2030. PLoS Medicine. 2006; 3(11): 442.

4. Nichols M., Townsend N., Scarborough P., Rayner M. Cardiovascular disease in Europe: epidemiological update. European Heart Journal. 2013; 34(39): 3028-34.

5. World Health Organization. Global Recommendations on Physical Activity for Health. Geneva: World Health Organization; 2010.

6. Ascensão A., Ferreira R., Magalhães J. Exercise-induced cardioprotection - biochemical, morphological and functional evidence in whole tissue and isolated mitochondria. International Journal of Cardiology. 2007; 117(1): 16-30.

7. Ascensão A., Lumini-Oliveira J., Oliveira P. J., Magalhães J. Mitochondria as a Target for Exercise-Induced Cardioprotection. Curr Drug Targets. 2011; 12(6): 860-71.

8. Hausenloy D. J., Ruiz-Meana M. Not just the powerhouse of the cell: emerging roles for mitochondria in the heart. Cardiovascular Research. 2010; 88(1): 5-6.

9. Anderson K. A., Hirschey M. D. Mitochondrial protein acetylation regulates metabolism. In: Scott I, editor. Lysine-Based Post-Translational Modification of Proteins; 2012. p. 23-35.

10. Schaffer S. W., Suleiman S. Mitochondria: The Dynamic Organelle. Springer; 2007.

11. Distler A. M., Kerner J., Hoppel C. L. Proteomics of mitochondrial inner and outer membranes. Proteomics. 2008; 8(19): 4066-82.

12. Lodish H., Berk A., Zipursky S. L., Matsudaira P., Baltimore D., Darnell J. Molecular Cell Biology. New York: W. H. Freeman; 2000.

13. Henrikson R. C., Kaye G. I., Mazurkiewicz J. E. Histology. Lippincott Williams & Wilkins; 1997.

14. Gilkerson R. W., Selker J. M. L., Capaldi R. A. The cristal membrane of mitochondria is the principal site of oxidative phosphorylation. FEBS Letters. 2003; 546(2–3): 355-8.

15. Navarro A., Boveris A. The mitochondrial energy transduction system and the aging process. American Journal of Physiology - Cell Physiology. 2007; 292(2): C670-C86.

16. Lenaz G., Genova M. L. Structural and functional organization of the mitochondrial respiratory chain: A dynamic super-assembly. The International Journal of Biochemistry & Cell Biology. 2009; 41(10): 1750-72.

17. Cecchini G. Function and structure of complex II of the respiratory chain. Annual Review of Biochemistry. 2003; 72: 77-109.

18. Boyer P. D. The ATP synthase - A splendid molecular machine. Annual Review of Biochemistry. 1997; 66: 717-49.

19. Lutter R., Saraste M., Vanwalraven H. S., Runswick M. J., Finel M., Deatherage J. F., Walker J. E. F_1F_O-ATP Synthase from bovine heart-mitochondria - Development of the purification of a monodisperse oligomycin-sensitive ATPase. Biochemical Journal. 1993; 295: 799-806.

20. Korzeniewski B., Zoladz J. A. Training-induced adaptation of oxidative phosphorylation in skeletal muscles. Biochemical Journal. 2003; 374(Pt 1): 37-40.

21. Ascensão A., Magalhães J., Soares J. M. C., Ferreira R., Neuparth M. J., Marques F., Oliveira P. J., Duarte J. A. Endurance training limits the functional alterations of heart rat mitochondria submitted to in vitro anoxia-reoxygenation. International Journal of Cardiology. 2006; 109(2): 169-78.

22. Mokhtar N., Lavoie J. P., Rousseaumigneron S., Nadeau A. Physical-training reverses defect in mitochondrial energy-production in heart of chronically diabetic rats. Diabetes. 1993; 42(5): 682-7.

23. Lumini-Oliveira J., Magalhães J., Pereira C. V., Moreira A. C., Oliveira P. J., Ascensão A. Endurance training reverts heart mitochondrial dysfunction, permeability transition and apoptotic signaling in long-term severe hyperglycemia. Mitochondrion. 2011; 11(1): 54-63.

24. Judge S., Jang Y. M., Smith A., Selman C., Phillips T., Speakman J. R., Hagen T., Leeuwenburgh C. Exercise by lifelong voluntary wheel running reduces subsarcolemmal and interfibrillar mitochondrial hydrogen peroxide production in the heart. American Journal of Physiology-Regulatory Integrative and Comparative Physiology. 2005; 289(6): R1564-R72.

25. Ascensão A., Lumini-Oliviera J., Machado N. G., Ferreira R. M., Gonçalves I. O., Moreira A. C., Marques F., Sardão V. A., Oliveira P. J., Magalhães J. Acute exercise protects against calcium-induced cardiac mitochondrial permeability transition pore opening in doxorubicin-treated rats. Clinical Science. 2011; 120(1-2): 37-49.

26. Ascensao A., Lumini-Oliveira J., Oliveira P. J., Magalhaes J. Mitochondria as a Target for Exercise-Induced Cardioprotection. Curr Drug Targets. 2011; 12(6): 860-71.

27. Radák Z., Kaneko T., Tahara S., Nakamoto H., Ohno H., Sasvári M., Nyakas C., Goto S. The effect of exercise training on oxidative damage of lipids, proteins, and DNA in rat skeletal muscle: evidence for beneficial outcomes. Free Radical Biology and Medicine. 1999; 27(1–2): 69-74.

28. Leichtweis S. B., Leeuwenburgh C., Parmelee D. J., Fiebig R., Ji L. L. Rigorous swim training impairs mitochondrial function in post-ischaemic rat heart. Acta Physiologica Scandinavica. 1997; 160(2): 139-48.

29. Jiang Y., Wang X. Comparative mitochondrial proteomics: perspective in human diseases. Journal of Hematology & Oncology. 2012; 5(1): 11.

30. Chen X., Li J., Hou J., Xie Z., Yang F. Mammalian mitochondrial proteomics: insights into mitochondrial functions and mitochondria-related diseases. Expert Review of Proteomics. 2010; 7(3): 333-45.

31. Mann M., Jensen O. N. Proteomic analysis of post-translational modifications. Nature Biotechnology. 2003; 21(3): 255.

32. Khoury G. A., Baliban R. C., Floudas C. A. Proteome-wide post-translational modification statistics: frequency analysis and curation of the swiss-prot database. Scientific Reports. 2011; 1.

33. Padrão A. I., Vitorino R., Duarte J. A., Ferreira R., Amado F. Unraveling the Phosphoproteome Dynamics in Mammal Mitochondria from a Network Perspective. Journal of Proteome Research. 2013; 12(10): 4257-67.

34. Salvi M., Brunati A. M., Toninello A. Tyrosine phosphorylation in mitochondria: A new frontier in mitochondrial signaling. Free Radical Biology and Medicine. 2005; 38(10): 1267-77.

35. Boja E. S., Phillips D., French S. A., Harris R. A., Balaban R. S. Quantitative Mitochondrial Phosphoproteomics Using iTRAQ on an LTQ-Orbitrap with High Energy Collision Dissociation. Journal of Proteome Research. 2009; 8(10): 4665-75.

36. Livnat-Levanon N., Glickman M. H. Ubiquitin–Proteasome System and mitochondria — Reciprocity. Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms. 2011; 1809(2): 80-7.

37. Jeon H. B., Choi E. S., Yoon J. H., Hwang J. H., Chang J. W., Lee E. K., Choi H. W., Park Z. Y., Yoo Y. J. A proteomics approach to identify the ubiquitinated proteins in mouse heart. Biochemical and Biophysical Research Communications. 2007; 357(3): 731-6.

38. Friguet B. Oxidized protein degradation and repair in ageing and oxidative stress. FEBS Letters. 2006; 580(12): 2910-6.

39. Padrão A. I., Ferreira R. M. P., Vitorino R., Alves R. M. P., Neuparth M. J., Duarte J. A., Amado F. OXPHOS susceptibility to oxidative modifications: The role of heart

mitochondrial subcellular location. Biochimica et Biophysica Acta (BBA) - Bioenergetics. 2011; 1807(9): 1106-13.

40. Glozak M. A., Sengupta N., Zhang X., Seto E. Acetylation and deacetylation of non-histone proteins. Gene. 2005; 363(0): 15-23.

41. Kim S. C., Sprung R., Chen Y., Xu Y., Ball H., Pei J., Cheng T., Kho Y., Xiao H., Xiao L., Grishin N. V., White M., Yang X.-J., Zhao Y. Substrate and Functional Diversity of Lysine Acetylation Revealed by a Proteomics Survey. Molecular Cell. 2006; 23(4): 607-18.

42. Ahn B.-H., Kim H.-S., Song S., Lee I. H., Liu J., Vassilopoulos A., Deng C.-X., Finkel T. A role for the mitochondrial deacetylase Sirt3 in regulating energy homeostasis. Proceedings of the National Academy of Sciences of the United States of America. 2008; 105(38): 14447-52.

43. Cimen H., Han M.-J., Yang Y., Tong Q., Koc H., Koc E. C. Regulation of Succinate Dehydrogenase Activity by SIRT3 in Mammalian Mitochondria. Biochemistry. 2010; 49(2): 304-11.

44. Hebert Alexander S., Dittenhafer-Reed Kristin E., Yu W., Bailey Derek J., Selen Ebru S., Boersma Melissa D., Carson Joshua J., Tonelli M., Balloon Allison J., Higbee Alan J., Westphall Michael S., Pagliarini David J., Prolla Tomas A., Assadi-Porter F., Roy S., Denu John M., Coon Joshua J. Calorie Restriction and SIRT3 Trigger Global Reprogramming of the Mitochondrial Protein Acetylome. Molecular Cell. 2013; 49(1): 186-99.

45. Rardin M. J., Newman J. C., Held J. M., Cusack M. P., Sorensen D. J., Li B., Schilling B., Mooney S. D., Kahn C. R., Verdin E., Gibson B. W. Label-free quantitative proteomics of the lysine acetylome in mitochondria identifies substrates of SIRT3 in metabolic pathways. Proceedings of the National Academy of Sciences of the United States of America. 2013; 110(16): 6601-6.

46. Zhang J., Lin A., Powers J., Lam M. P., Lotz C., Liem D., Lau E., Wang D., Deng N., Korge P., Zong N. C., Cai H., Weiss J., Ping P. Mitochondrial proteome design: From molecular identity to pathophysiological regulation. Journal of General Physiology. 2012; 139(6): 395-406.

47. Hirschey M. D., Shimazu T., Huang J. Y., Verdin E. Acetylation of mitochondrial proteins. In: Allison WS, Murphy AN, editors. Mitochondrial Function, Part B: Mitochondrial Protein Kinases, Protein Phosphatases and Mitochondrial Diseases; 2009. p. 137-47.

48. Silva A. M. N., Vitorino R., Domingues M. R. M., Spickett C. M., Domingues P. Post-translational Modifications and Mass Spectrometry Detection. Free Radical Biology and Medicine. 2013; 65(0): 925-41.

49. Xie H., Bandhakavi S., Roe M. R., Griffin T. J. Preparative peptide isoelectric focusing as a tool for improving the identification of lysine-acetylated peptides from complex mixtures. Journal of Proteome Research. 2007; 6(5): 2019-26.

50. Xu L., Wang W., Zhang Z., Yang P., Fan H., Kong J. Microchip-based strategy for enrichment of acetylated proteins. Microchimica Acta. 2013; 180(7-8): 613-8.

51. Hirschey M. D., Shimazu T., Goetzman E., Jing E., Schwer B., Lombard D. B., Grueter C. A., Harris C., Biddinger S., Ilkayeva O. R., Stevens R. D., Li Y., Saha A. K., Ruderman N. B., Bain J. R., Newgard C. B., Farese R. V., Jr., Alt F., Kahn C. R., Verdin E. SIRT3 regulates mitochondrial fatty-acid oxidation by reversible enzyme deacetylation. Nature. 2010; 464(7285): 121-U37.

52. Grillon J. M., Johnson K. R., Kotlo K., Danziger R. S. Non-histone lysine acetylated proteins in heart failure. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease. 2012; 1822(4): 607-14.

53. Lundby A., Lage K., Weinert Brian T., Bekker-Jensen Dorte B., Secher A., Skovgaard T., Kelstrup Christian D., Dmytriyev A., Choudhary C., Lundby C., Olsen Jesper V. Proteomic Analysis of Lysine Acetylation Sites in Rat Tissues Reveals Organ Specificity and Subcellular Patterns. Cell Reports. 2012; 2(2): 419-31.

54. Schilling B., Rardin M. J., MacLean B. X., Zawadzka A. M., Frewen B. E., Cusack M. P., Sorensen D. J., Bereman M. S., Jing E., Wu C. C., Verdin E., Kahn C. R., MacCoss M. J., Gibson B. W. Platform-independent and Label-free Quantitation of Proteomic Data Using MS1 Extracted Ion Chromatograms in Skyline: Application to protein acetylation and phosphorylation. Molecular & Cellular Proteomics. 2012; 11(5): 202-14.

55. Still A. J., Floyd B. J., Hebert A. S., Bingman C. A., Carson J. J., Gunderson D. R., Dolan B. K., Grimsrud P. A., Dittenhafer-Reed K. E., Stapleton D. S., Keller M. P., Westphall M. S., Denu J. M., Attie A. D., Coon J. J., Pagliarini D. J. Quantification of mitochondrial acetylation dynamics highlights prominent sites of metabolic regulation. Journal of Biological Chemistry. 2013.

56. Maudsley S., Chadwick W., Wang L., Zhou Y., Martin B., Park S.-S. Bioinformatic Approaches to Metabolic Pathways Analysis. In: Luttrell LM, Ferguson SSG, editors. Signal Transduction Protocols: Humana Press; 2011. p. 99-130.

57. Scott I., Webster B. R., Li J. H., Sack M. N. Identification of a molecular component of the mitochondrial acetyltransferase programme: a novel role for GCN5L1. Biochemical Journal. 2012; 443: 655-61.

58. Webster B. R., Scott I., Han K., Li J. H., Lu Z. P., Stevens M. V., Malide D., Chen Y., Samsel L., Connelly P. S., Daniels M. P., McCoy J. P., Combs C. A., Gucek M., Sack M. N. Restricted mitochondrial protein acetylation initiates mitochondrial autophagy. J Cell Sci. 2013; 126(21): 4843-9.

59. Michishita E., Park J. Y., Burneskis J. M., Barrett J. C., Horikawa I. Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins. Molecular Biology of the Cell. 2005; 16(10): 4623-35.

60. Huang J.-Y., Hirschey M. D., Shimazu T., Ho L., Verdin E. Mitochondrial sirtuins. Biochimica Et Biophysica Acta-Proteins and Proteomics. 2010; 1804(8): 1645-51.

61. Hallows W. C., Lee S., Denu J. M. Sirtuins deacetylate and activate mammalian acetyl-CoA synthetases. Proceedings of the National Academy of Sciences of the United States of America. 2006; 103(27): 10230-5.

62. Fujino T., Kondo J., Ishikawa M., Morikawa K., Yamamoto T. T. Acetyl-CoA synthetase 2, a mitochondrial matrix enzyme involved in the oxidation of acetate. Journal of Biological Chemistry. 2001; 276(14): 11420-6.

63. Pirinen E., Lo Sasso G., Auwerx J. Mitochondrial sirtuins and metabolic homeostasis. Best Practice & Research Clinical Endocrinology & Metabolism. 2012; 26(6): 759-70.

64. Schlicker C., Gertz M., Papatheodorou P., Kachholz B., Becker C. F. W., Steegborn C. Substrates and Regulation Mechanisms for the Human Mitochondrial Sirtuins Sirt3 and Sirt5. Journal of Molecular Biology. 2008; 382(3): 790-801.

65. Hallows W. C., Yu W., Smith B. C., Devires M. K., Ellinger J. J., Someya S., Shortreed M. R., Prolla T., Markley J. L., Smith L. M., Zhao S. M., Guan K. L., Denu J. M. Sirt3 Promotes the Urea Cycle and Fatty Acid Oxidation during Dietary Restriction. Molecular Cell. 2011; 41(2): 139-49.

66. Haigis M. C., Mostoslavsky R., Haigis K. M., Fahie K., Christodoulou D. C., Murphy Andrew J., Valenzuela D. M., Yancopoulos G. D., Karow M., Blander G., Wolberger C., Prolla T. A., Weindruch R., Alt F. W., Guarente L. SIRT4 Inhibits Glutamate Dehydrogenase and Opposes the Effects of Calorie Restriction in Pancreatic β Cells. Cell. 2006; 126(5): 941-54.

67. Nakagawa T., Lomb D. J., Haigis M. C., Guarente L. SIRT5 Deacetylates Carbamoyl Phosphate Synthetase 1 and Regulates the Urea Cycle. Cell. 2009; 137(3): 560-70.

68. Shimazu T., Hirschey M. D., Hua L., Dittenhafer-Reed K. E., Schwer B., Lombard D. B., Li Y., Bunkenborg J., Alt F. W., Denu J. M., Jacobson M. P., Verdin E. SIRT3 Deacetylates Mitochondrial 3-Hydroxy-3-Methylglutaryl CoA Synthase 2 and Regulates Ketone Body Production. Cell Metabolism. 2010; 12(6): 654-61.

69. Shulga N., Wilson-Smith R., Pastorino J. G. Sirtuin-3 deacetylation of cyclophilin D induces dissociation of hexokinase II from the mitochondria. J Cell Sci. 2010; 123(6): 894-902.

70. Hafner A. V., Dai J., Gomes A. P., Xiao C.-Y., Palmeira K. C. M., Rosenzweig A., Sinclair D. A. Regulation of the mPTP by SIRT3-mediated deacetylation of

CypD at lysine 166 suppresses age-related cardiac hypertrophy. Aging-Us. 2010; 2(12): 914-23.

71. Qiu X., Brown K., Hirschey M. D., Verdin E., Chen D. Calorie Restriction Reduces Oxidative Stress by SIRT3-Mediated SOD2 Activation. Cell Metabolism. 2010; 12(6): 662-7.

72. Jing E. X., Emanuelli B., Hirschey M. D., Boucher J., Lee K. Y., Lombard D., Verdin E. M., Kahn C. R. Sirtuin-3 (Sirt3) regulates skeletal muscle metabolism and insulin signaling via altered mitochondrial oxidation and reactive oxygen species production. Proceedings of the National Academy of Sciences of the United States of America. 2011; 108(35): 14608-13.

73. Bao J., Scott I., Lu Z., Pang L., Dimond C. C., Gius D., Sack M. N. SIRT3 is regulated by nutrient excess and modulates hepatic susceptibility to lipotoxicity. Free Radical Biology and Medicine. 2010; 49(7): 1230-7.

74. Ascensao A., Magalhaes J., Soares J. M. C., Ferreira R., Neuparth M. J., Marques F., Oliveira P. J., Duarte J. A. Moderate endurance training prevents doxorubicin-induced in vivo mitochondriopathy and reduces the development of cardiac apoptosis. American Journal of Physiology-Heart and Circulatory Physiology. 2005; 289(2): H722-H31.

75. Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J. Protein measurement with Folin phenol reagent. Journal of Biological Chemistry. 1951; 193(1): 265-75.

76. Laemmli U. K. Cleavage of structural proteins during assembley of head of bacteriophage T4. Nature. 1970; 227(5259): 680-&.

77. Wisniewski J. R., Zougman A., Nagaraj N., Mann M. Universal sample preparation method for proteome analysis. Nat Meth. 2009; 6(5): 359-62.

78. Ferreira R., Vitorino R., Padrao A. I., Espadas G., Mancuso F. M., Moreira-Gonçalves D., Castro-Sousa G., Henriques-Coelho T., Oliveira P. A., Barros A. S., Duarte J. A., Sabidó E., Amado F. Lifelong exercise training modulates cardiac mitochondrial phosphoproteome in rats. Journal of Proteome Research. 2014.

79. Kavazis A. N., Alvarez S., Talbert E., Lee Y., Powers S. K. Exercise training induces a cardioprotective phenotype and alterations in cardiac subsarcolemmal and intermyofibrillar mitochondrial proteins. American Journal of Physiology-Heart and Circulatory Physiology. 2009; 297(1): H144-H52.

80. Rocha L. A. O., Petriz B. A., Borges D. H., Oliveira R. J., de Andrade R. V., Domont G. B., Pereira R. W., Franco O. L. High molecular mass proteomics analyses of left ventricle from rats subjected to differential swimming training. BMC physiology. 2012; 12: 11-.

81. Starnes J. W. B. B. D. O. M. E. Exercise training decreases rat heart mitochondria free radical generation but does not prevent Ca2+-induced dysfunction. Journal of Applied Physiology. 2007; 102(5): 1793-8.

82. Sun B., Wang J. h., Lv Y. y., Zhu S. s., Yang J., Ma J. z. Proteomic adaptation to chronic high intensity swimming training in the rat heart. Comparative Biochemistry and Physiology Part D: Genomics and Proteomics. 2008; 3(1): 108-17.

83. Stanley W. C., Lopaschuk G. D., Hall J. L., McCormack J. G. Regulation of myocardial carbohydrate metabolism under normal and ischaemic conditions - Potential for pharmacological interventions. Cardiovascular Research. 1997; 33(2): 243-57.

84. Sundaresan N. R., Gupta M., Kim G., Rajamohan S. B., Isbatan A., Gupta M. P. Sirt3 blocks the cardiac hypertrophic response by augmenting Foxo3a-dependent antioxidant defense mechanisms in mice. The Journal of Clinical Investigation. 2009; 119(9): 2758-71.

85. Li L., Muehlfeld C., Niemann B., Pan R., Li R., Hilfiker-Kleiner D., Chen Y., Rohrbach S. Mitochondrial biogenesis and PGC-1 alpha deacetylation by chronic treadmill exercise: differential response in cardiac and skeletal muscle. Basic Research in Cardiology. 2011; 106(6): 1221-34.

86. Sundaresan N. R., Gupta M., Kim G., Rajamohan S. B., Isbatan A., Gupta M. P. Sirt3 blocks the cardiac hypertrophic response by augmenting Foxo3a-dependent antioxidant defense mechanisms in mice. Journal of Clinical Investigation. 2009; 119(9): 2758-71.

87. Kavazis A. N., McClung J. M., Hood D. A., Powers S. K. Exercise induces a cardiac mitochondrial phenotype that resists apoptotic stimuli. American Journal of Physiology-Heart and Circulatory Physiology. 2008; 294(2): H928-H35.

88. Amado F. M., Barros A., Azevedo A. L., Vitorino R., Ferreira R. An integrated perspective and functional impact of the mitochondrial acetylome. Expert Review of Proteomics. 2014; 11(3): 383-94.

89. Nakayasu E. S., Wu S., Sydor M. A., Shukla A. K., Weitz K. K., Moore R. J., Hixson K. K., Kim J.-S., Petyuk V. A., Monroe M. E., Pasa-Tolic L., Qian W.-J., Smith R. D., Adkins J. N., Ansong C. A method to determine lysine acetylation stoichiometries. International journal of proteomics. 2014; 2014(0): 1-8.

90. Samant S. A., Zhang H. J., Hong Z., Pillai V. B., Sundaresan N. R., Wolfgeher D., Archer S. L., Chan D. C., Gupta M. P. SIRT3 Deacetylates and Activates OPA1 To Regulate Mitochondrial Dynamics during Stress. Molecular and Cellular Biology. 2014; 34(5): 807-19.

91. Caffin F., Prola A., Piquereau J., Novotova M., David D. J., Garnier A., Fortin D., Alavi M. V., Veksler V., Ventura-Clapier R., Joubert F. Altered skeletal muscle mitochondrial biogenesis but improved endurance capacity in trained OPA1-deficient mice. Journal of Physiology-London. 2013; 591(23): 6017-37.

92. Cribbs J. T., Strack S. Reversible phosphorylation of Drp1 by cyclic AMP-dependent protein kinase and calcineurin regulates mitochondrial fission and cell death. Embo Reports. 2007; 8(10): 939-44.

VII. Appendix

Table A- 1. List of identified acetylated proteins including acetylated lysine sites, Domain Pfam and functional role.

Acession number	Protein Name	Peptide		Acetylated lysine	Domain (Pfam)	Functional Role	Express in Ex/ Cont
E9PTG8	Serine/threonine-protein kinase 10	QKaEDLELAMKK		K703; K711	PKK (Polo Kinase Kinase)	Polo-like kinase 1 (Plx1) is essential during mitosis for the activation of Cdc25C, for spindle assembly, and for cyclin B degradation. This family is Polo kinase kinase (PKK) which phosphorylates Polo kinase and Polo-like kinase to activate them. PKK is a serine/threonine kinase.	Ex
P29995	Inositol 1,4,5-trisphosphate receptor type 2	QIKADLDQLR		K1113	-	-	Ex
Q8K3F3	Protein phosphatase 1 regulatory subunit 14B	IRGMQK		K140	PP1 inhibitor (PKC-activated protein phosphatase-1 inhibitor)	Contractility of vascular smooth muscle depends on phosphorylation of myosin light chains, and is modulated by hormonal control of myosin phosphatase activity. Signaling pathways activate kinases such as PKC or Rho-dependent kinases that phosphorylate the myosin phosphatase inhibitor protein called CPI-17. Phosphorylation of CPI-17 at Thr-38 enhances its inhibitory potency 1000-fold, creating a molecular switch for regulating contraction	Ex
Q4FZU3	Nuclear speckle splicing regulatory protein 1	MEKKIQR		K123 ;K124	DUF2040	The proteins do contain a coiled-coil domain, but the function is unknown.	Cont
P05426	60S ribosomal protein L7	KVAAALGTLK		K19	-	-	Ex and Cont
P19511	ATP synthase F(0) complex subunit B1, mitochondrial	EKAQQALVQK		K154	Mt_ATP-synt_B	The Fo sector of the ATP synthase is a membrane bound complex which mediates proton transport. It is composed of nine different polypeptide subunits (a, b, c, d, e, f, g F6, A6L)	Ex and Cont

P63036	DnaJ homolog subfamily A member 1	KAYRKLALK		K24;K28;K32	DnaJ	DnaJ domains (J-domains) are associated with hsp70 heat-shock system and it is thought that this domain mediates the interaction. DnaJ-domain is therefore part of a chaperone (protein folding) system.	Ex and Cont
Q02769	Squalene synthase	GVVKIRK		K318	SQS_PSY	-	Ex and Cont
Q9EQH5	C-terminal-binding protein 2	MALVDKHK		K6	-	-	Ex and Cont

Table A- 2. Label-free quantitative analysis of common acetylated proteins.

Acession number	**Protein name**	**Effect exercise**	**Peptide**	**Lysine acetylation**	**Domain (Pfam)**	**Functional Role**
Q01129	Decorin	-0,03	DLHTLILVNNK			
Q9WTR8	PH domain leucine-rich repeat protein phosphatase 1	-0,005	LLQLGHK	K214	-	-
P04897 P63095 P29348	Guanine nucleotide-binding protein G(i) subunit alpha-2; Guanine nucleotide-binding protein G(s) subunit alpha isoforms short; Guanine nucleotide-binding protein G(t) subunit alpha-3	-0,004	QMKIIHKNGYSK	K54;k58	G-protein alpha subunit	G proteins couple receptors of extracellular signals to intracellular signaling pathways. The G protein alpha subunit binds guanyl nucleotide and is a weak GTPase.
Q05962	ADP/ATP translocase 1	-0,003	GDQALSFLK	-	-	-
Q641W3	Nuclear fragile X mental retardation-interacting protein 1	-0,003	ITLKQK	k428	-	-
Q765A7	GPI inositol-deacylase	-0,003	ALLTLK	-	-	-
Q08290	Calponin-1	-0,002	NKLAQK	K21	-	-

B2GV24	E3 UFM1-protein ligase 1	-0,002	KKILSK	K608;609	-	-
P97832	Heart- and neural crest derivatives-expressed protein 1	-0,002	KGSGPKK	K96	Helix-loop-helix DNA-binding domain	The helix-loop-helix (HLH) DNA-binding domain consists of a closed bundle of four helices in a left-handed twist with two crossover connections. The HLH domain directs dimerisation, and is juxtaposed to basic regions to create a DNA interaction interface surface that recognises specific DNA sequences. Basic region/HLH (bHLH) proteins regulate diverse biological pathways
Q3MID3	ADP-ribosylation factor GTPase-activating protein 2	-0,002	KGLGAQK	K228	-	-
Q9WUJ3	Myomegalin	-0,001	ELRQLR		-	-
Q2TA68	Dynamin-like 120 kDa protein, mitochondrial	-0,002	VKLLTGK	K931	-	-
Q66HC3	Protein C9orf72 homolog	-0,001	ALTLIK		-	-
Q5U203	NEDD8-conjugating enzyme UBE2F	0,002	LTLASKLKR	K9	-	-
Q5U2Y1	General transcription factor II-I	0,001	ITDLRK	K715	GTF2I	This region of sequence similarity is found up to six times in a variety of proteins including GTF2I. It has been suggested that this may be a DNA binding domain
Q63489	general transcription factor IIF subunit 2	0,0004	LKEILK	K218	TFIIF_beta	Accurate transcription in vivo requires at least six general transcription initiation factors, in addition to RNA polymerase II. Transcription initiation factor IIF (TFIIF) is a tetramer of two beta subunits associate with two alpha subunits which interacts directly with RNA polymerase II. The beta subunit of TFIIF is required for recruitment of RNA polymerase II onto the promoter.

Appendix

Table A- 3. List of identified acetylated proteins using acetyl-lysine immunoprecipitation

Acession number	Name	Post-Translational Modifications	Domain (Pfam)	Functional Role	Effect Exercise
P18163	Long-chain-fatty-acid--CoA ligase 1	N-Term(Ac)	-	-	↔
P56574	Isocitrate dehydrogenase [NADP]	K282 (Ac)	Iso_dh	Isocitrate dehydrogenase (IDH), is an important enzyme of carbohydrate metabolism which catalyses the oxidative decarboxylation of isocitrate into alpha-ketoglutarate.	↓
P04636	Malate dehydrogenase	N-Term(Ac)	-	-	↑
P15651	Short-chain specific acyl-CoA dehydrogenase	K262 (Ac)	Acyl-CoA_dh_1	C-terminal domain of Acyl-CoA dehydrogenase is an all-alpha, four helical up-and-down bundle.	↑
P23965	Enoyl-CoA delta isomerase 1	K76(Acetyl)	ECH	Enoyl-CoA hydratase catalyses the hydratation of 2-trans-enoyl-CoA into 3-hydroxyacyl-CoA.	↑
P40190	Interleukin-6 receptor subunit beta	N-Term(Acetyl)	-	-	↑
P97519	Hydroxymethylglutaryl-CoA lyase	K48(Acetyl)	HMGL-like	Key enzyme in ketogenesis. Terminal step in leucine catabolism. Ketone bodies (beta-hydroxybutyrate, acetoacetate and acetone) are essential as an alternative source of energy to glucose, as lipid precursors and as regulators of metabolism	↑
P97521	Mitochondrial carnitine/acylcarnitine carrier protein	N-Term(Acetyl)	-	-	↑
Q6P6R2	Dihydrolipoyl dehydrogenase	K410(Acetyl)	Pyr_redox_dim	This family includes both class I and class II oxidoreductases and also NADH oxidases and peroxidases.	↑
Q8VHF5	Citrate synthase	K327(Acetyl)	Citrate_synt	-	↑
P31399	ATP synthase subunit d	K63(Acetyl); K73(Acetyl)	Mt_ATP-synt_D	This family consists of several ATP synthase D chain, mitochondrial (ATP5H) proteins.	↔