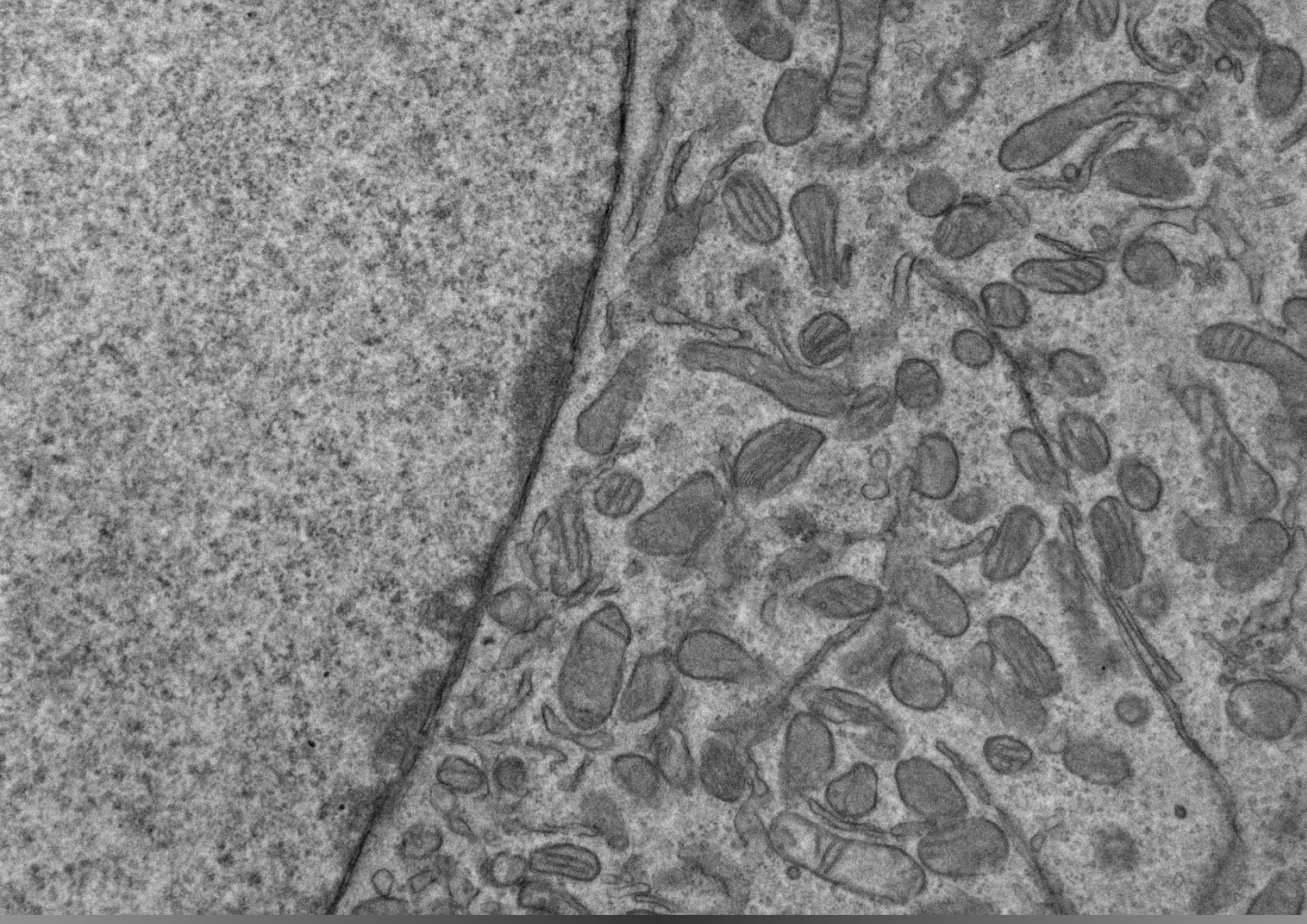

Unraveling New Roles for SIRT1 in Mitochondrial Biology

Ana Patrícia da Silva Gomes

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Universidade de Coimbra

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Dissertação de Doutoramento na Área Científica de Biologia, especialidade de Biologia Celular, orientada pelos Professores Doutores Carlos Manuel Marques Palmeira, Anabela Pinto Rolo e João António Nave Laranjinha e apresentada ao Departamento de Ciências da Vida da Universidade de Coimbra

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“There is no happiness except in the realization that we have accomplished something.”

Henry Ford

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Abstract

Mitochondria are key players in the maintenance of cellular homeostasis, as they provide the energy required for the cellular processes. As such, disruption in mitochondrial homeostasis is closely associated with disease state, caused by subtle alterations in the function of tissues or by major defects, which might lead to death. Therefore, regulation of mitochondrial homeostasis is extremely important for the cell, and is dependent on the regulation of mitochondrial mass through its production (biogenesis). Indeed, physiologic stimuli can activate signaling pathways that coordinate the communication between the nuclear and mitochondrial genomes to produce efficient mitochondria, and adapt the metabolism of the cell. Sirtuin1 (SIRT1) is a metabolic sensor that has been shown to be associated with several metabolic and age-related diseases, which are also correlated with disruption of mitochondrial homeostasis. SIRT1 has been shown to regulate mitochondrial biogenesis by activating the master regulator of mitochondrial biogenesis, peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC-1α), however the role of SIRT1 in mitochondrial biology *in vivo* remains yet to be studied, due to lack of appropriated animal models. As so, this work aimed to investigate the role of SIRT1 in mitochondrial biology, as well as its potential involvement in the mitochondrial-related beneficial effects of two natural compounds with therapeutic potential, resveratrol and berberine.

Using an adult-inducible whole body SIRT1 knockout mouse, the work in this thesis showed that SIRT1 has a dual role in the regulation of mitochondrial metabolism. On one hand, in conditions of low nutrient supply, SIRT1 activates PGC-1α and thereby induces mitochondrial biogenesis. On the other hand, in this thesis is presented for the first time, that in conditions of normal nutrient supply SIRT1 regulates mitochondrial DNA and the expression of mitochondrial-encoded genes through regulation of mitochondrial transcription factor A (TFAM) promoter. This mechanism is PGC-1α independent and mediated by a hypoxia-inducible factor 1 alpha (HIF1α) and myelocytomatosis viral oncogene homolog" (c-Myc) pathway. Indeed, loss of SIRT1 leads to HIF1α stabilization and consequently to an imbalance between the expression of nuclear and mitochondrial-encoded genes that culminates in mitochondrial dysfunction. In addition, its shown that this PGC-1α independent pathway is also relevant for the development of age-related mitochondrial dysfunction. Indeed, here its shown that increasing SIRT1 activity in old mice reverses mitochondrial dysfunction, promotes functional communication between

nuclear and mitochondrial-encoded genes as well as reverses the age-induced HIF1α stabilization.

Lastly, here is also analysed the role of SIRT1 in the effects of both resveratrol and berberine in the maintenance of mitochondrial homeostasis. Resveratrol and berberine are natural compounds that have been demonstrated to protect from metabolic disturbances frequently associated with decline in mitochondrial function. Importantly, and in accordance with the pivotal role of SIRT1 in mitochondrial biology, both resveratrol and berberine were unable to preserve mitochondrial biogenesis and function from either high fat diet or hyperglycemic conditions in the absence of SIRT1. Giving further support to this, overexpression of SIRT1 *in vivo* showed an increase in mitochondrial function and biogenesis in the skeletal muscle. Together, these results show that increasing SIRT1 leads to increase mitochondrial biogenesis and consequently to more efficient mitochondria and represents an advantage in conditions of mitochondrial overload, such as high nutrient supply. Importantly, both resveratrol and berberine were previously shown to activate another metabolic sensor, the AMP-activated kinase (AMPK). AMPK and SIRT1 have many common targets as well as overlapping effects in the cell, therefore the epistasis of SIRT1 and AMPK have been hard to dissect. Here its shown not only that SIRT1 overexpression is sufficient to activate AMPK in the skeletal muscle, but also that SIRT1 is required for the ability of resveratrol and berberine to activate AMPK at moderated doses.

Together the work presented in this thesis, show for the first time the role of SIRT1 in mitochondrial biology *in vivo*, as well as the importance of SIRT1 for the biological effects of resveratrol and berberine. In addition, this work also unravels a new pathway, regulated by SIRT1 and independent of PGC-1α, that regulates mitochondrial homeostasis and plays an important role in the development of age-related mitochondrial dysfunction. As such, the work presented in this thesis supports the idea that activation of SIRT1 is an important therapeutic strategy for the treatment of age and metabolic-related disorders where mitochondrial homeostasis is disturbed.

Resumo

As mitocôndrias são consideradas as centrais energéticas da célula, e desempenham um papel central na manutenção da homeostasia celular, uma vez que são elas que produzem a energia necessária para a execução dos processos celulares. Desta forma, distúrbios na homeostasia mitocondrial estão intimamente associados a estados de doença, causados por alterações subtis na função de cada órgão, sendo que alterações mais dramáticas podem culminar em morte. Assim sendo, a regulação da homeostasia mitocondrial é em última análise extremamente importante para a manutenção do equilíbrio celular. Tal está dependente da regulação da massa mitocondrial (biogénese). Estímulos fisiológicos diversos ativam vias de sinalização que coordenam a comunicação entre os genomas nuclear e mitocondrial, permitindo a formação de mitocôndrias eficientes. A Sirtuina 1 (SIRT1) é um sensor metabólico que tem sido implicado no desenvolvimento de patologias metabólicas e relacionadas com a idade, as quais também se relacionam com perturbações no equilíbrio mitocondrial. A SIRT1 modula a biogénese mitocondrial mediante a ativação do principal regulador deste processo, o co-activador do receptor activado por proliferadores de peroxissoma gamma 1 alfa (PGC-1α). No entanto, o papel da SIRT1 na biologia mitocondrial *in vivo* permanece ainda por estudar, devido à falta de modelos animais adequados. Assim sendo, este trabalho teve como objetivo investigar o papel da SIRT1 na biologia mitocondrial, bem como o seu possível envolvimento nos efeitos benéficos mitocondriais de dois compostos naturais com potencial terapêutico, o resveratrol e a berberina.

Estudos conduzidos em murganhos em que o knockout para a SIRT1 é induzido na idade adulta, demostram um papel duplo da SIRT1 na regulação do metabolismo mitocondrial. Por um lado, nesta tese é demonstrado que, em condições de reduzida disponibilidade de nutrientes, a SIRT1 ativa o PGC-1α e, assim, induz a biogénese mitocondrial. Por outro lado, é também demonstrado pela primeira vez que, em condições normais de disponibilidade de nutrientes, a SIRT1 regula o ADN mitocondrial e a expressão de genes codificados pelo ADN mitocondrial, através da regulação do promotor do factor de transcrição mitocondrial A (TFAM). Este mecanismo é independente da ativação do PGC-1α, sendo regulado por uma via de sinalização mediada pelo factor de transcrição induzível por hipóxia 1 alfa (HIF1α) e pelo proto-oncogene "myelocytomatosis viral oncogene homolog" (c–Myc). De fato, a perda de

SIRT1 leva à estabilização do HIF1α e, consequentemente, a um desequilíbrio entre a expressão de genes codificados pelos genomas nuclear e mitocondrial, culminando em disfunção mitocondrial. Adicionalmente, é também demonstrado nesta tese que esta via independente de PGC-1α parece ser relevante para a disfunção mitocondrial associada ao envelhecimento. De facto, os resultados aqui apresentados, demostram que o aumento da atividade da SIRT1 em murganhos idosos consegue reverter a disfunção mitocondrial, promovendo a comunicação funcional entre os genomas nuclear e mitocondrial, bem como reverte a estabilização do HIF1α causada pelo envelhecimento.

Por último, é também aqui analisado o papel da SIRT1 nos efeitos do resveratrol e da berberina na manutenção da homeostasia mitocondrial. O resveratrol e a berberina são compostos naturais protetores em situações de distúrbio metabólico, frequentemente associados ao declínio da função mitocondrial. De facto, e em conformidade com o papel central da SIRT1 na biologia mitocondrial, tanto o resveratrol como a berberina, na ausência de SIRT1, não têm a capacidade de preservar a biogénese e função mitocondriais do dano causado por uma dieta rica em gordura ou por condições de hiperglicémia. Da mesma forma, a sobreexpressão de SIRT1 *in vivo*, resulta num aumento da biogénese e função mitocondriais no músculo esquelético. Estes resultados demostram que o aumento da SIRT1 leva a um aumento da biogénese mitocondrial e consequentemente a mitocôndrias mais eficientes, o que é benéfico por exemplo em condições de sobrecarga de nutrientes, evitando assim o dano mitocondrial existente nestas situações e consequentemente mantendo a homeostasia celular. Foi previamente demonstrado que o resveratrol e a berberina conseguem ativar outro sensor metabólico, a cinase activada por AMP (AMPK). Atendendo a que AMPK e SIRT1 partilham alvos moleculares, os seus efeitos celulares muitas vezes sobrepõem-se, dificultando a clarificação da epistasia entre SIRT1 e AMPK. Neste trabalho, é não só demonstrado que a sobrexpressão da SIRT1 é suficiente para activar a AMPK no músculo esquelético, como também que a SIRT1 é necessária para a activação da AMPK em condições de tratamento com doses moderadas de resveratrol e de berberina.

Em conclusão, o trabalho apresentado nesta tese, demonstra, pela primeira vez, o papel da SIRT1 na biologia mitocondrial *in vivo*, bem como a importância da SIRT1 para os efeitos biológicos do resveratrol e da berberina. Além disso, este estudo identifica também uma nova via, regulada pela SIRT1 e independente do PGC-1α, que modula a homeostasia mitocondrial e desempenha um papel importante no

desenvolvimento da disfunção mitocondrial associada ao envelhecimento. Desta forma, os resultados apresentados nesta tese sustentam a ativação da SIRT1 como uma importante estratégia para o tratamento de doenças relacionadas com o envelhecimento e distúrbios metabólicos, em que existam desequilíbrios na função mitocondrial.

Abreviation List

ACC, Acetyl-CoA carboxylase

Acetyl CoA, Acetyl coenzyme A

ADP, Adenosine diphosphate

AMP, Adenosine monophosphate

AMPK, AMP-activated protein kinase

ANT, Adenine nucleotide translocator

ARNT, Aryl hydrocarbon receptor nuclear translocator; HIF1β

AROS, Active regulator of SIRT1

ATP, Adenosine triphosphate

ATP5a1, ATP synthase, H^+ transporting, mitochondrial F1 complex, alpha subunit 1

ATP5b1, ATP synthase subunit beta 1

ATP6, ATP synthase F0 subunit 6

ATP8, ATP synthase F0 subunit 8

BBR, Berberine

bFGF, Bovine fibroblast growth factor

c-Myc, Myelocytomatosis viral oncogene homolog

Ca^{2+}, Calcium

cAMP, cyclic adenosine monophosphate

CDK1, Cyclin-dependent kinase 1

CoQ, Coenzyme Q

COX, Cytochrome c oxidase

COX1, Cytochrome c oxidase subunit 1

COX2, Cytochrome c oxidase subunit 2

COX3, Cytochrome c oxidase subunit 3

COX5b, Cytochrome c oxidase subunit 5b

Abreviation List

COX6a1, Cytochrome c oxidase subunit 6a1

CPT1b, Carnitine palmitoyltransferase I

CR, Caloric restriction

CREB, cAMP response element binding protein

Cu/ZnSOD, Copper/zinc superoxide dismutase

Cytb, Cytochrome b

D Loop, displacement loop

DBC1, Deleted in breast cancer 1

DCHC, 3-(2,4-Dichlorophenyl)-7-hydroxy-4H-chromen-4-one

DFO, Desferrioxamine

DMEM, Dubelcco's modified medium

DMOG, Dimethyloxaloylglycine

DMSO, Dimetylsulphoxide

EGF, Epidermal growth factor

ERRα, Estrogen related receptor alpha

ETC, Mitochondrial electron transport chain

$FADH_2$, Flavin adenine dinucleotide, reduced form

FCCP, Carbonylcyanide-p-trifluoromethoxyphenylhydrazon

FIH1, Factor inhibiting HIF1α

FOXO1, Forkhead box subgroup O 1

Glut1, Glucose transporter 1

Glut4, Glucose transporter 4

H_2O, Water

H_2O_2, Hydrogen peroxide

HFD, High fat diet

HIF1α, Hypoxia-inducible factor 1 alpha

HIF2α, Hypoxia-inducible factor 2 alpha

HIF3α, Hypoxia-inducible factor 3 alpha

HK2, Hexokinase 2

HPS, Mitochondrial DNA heavy strand

HRE, Hypoxia response element

JNK, Jun N-terminal kinase

KCN, Potassium cyanide

LCAD, Long chain acyl coA dehydrogenase

LDHA, Lactate dehydrogenase A

LKB1, Liver kinase B1

LPS, Mitochondrial DNA light strand

MCAD, Medium chain acyl coA dehydrogenase

miR, microRNA

MnSOD, Manganese superoxide dismutase

MPTP, Mitochondrial permeability transition pore

mtDNA, mitochondrial DNA

MTERF1, Mitochondrial transcription termination factor 1

mTOR, Mammalian target of rapamycin

MyHCI, Myosisn-heavy chain type 1

MyHCIIa, Myosisn-heavy chain type 2a

MyHCIIb, Myosisn-heavy chain type 2b

NAD^+, Nicotinamide adenine dinucleotide, oxidized form

NADH, Nicotinamide adenine dinucleotide, reduced form

NAM, Nicotinamide

NAMPT, Nicotinamide phosphoribosyltransferase

NAO, N-nonyl acridine orange

ND1, NADH dehydrogenase 1

ND2, NADH-ubiquinone oxidoreductase chain 2

ND3, NADH-ubiquinone oxidoreductase chain 3

ND4, NADH dehydrogenase subunit 4

ND4l, NADH-ubiquinone oxidoreductase chain 4l

ND5, NADH dehydrogenase subunit 5

ND6, NADH-ubiquinone oxidoreductase chain 6

nDNA, nuclear DNA

NDUFAB1, NADH dehydrogenase 1, alpha/beta subcomplex 1

NDUFS8, NADH dehydrogenase Fe-S protein 8

NF-kB, Nuclear factor-kappa B

NMN, Nicotinamide mononucleotide

NMNAT, Nicotinamide/Nicotinic acid mononucleotide adenylyltransferase

NRF-1, Nuclear respiratory factor 1

NRF-2, Nuclear respiratory factor 2

Nrf2, NF-E2-related factor 2

O-AADPR, O-acetyl-ADP ribose

O_2, Molecular oxygen

$O_2^{\cdot-}$, Superoxide anion

ODDD, Oxygen-dependent degradation domain

O_H, origin of replication of the mitochondrial DNA heavy strand

$OH^{\cdot}$, hydroxyl radical

PARP1, Poly(ADP-ribose) polymerase 1

PARP2, Poly(ADP-ribose) polymerase 2

PBS, Phosphate buffer saline

PCR, Polymerase chain reaction

PDE, Phosphodiesterases

PDK1, Pyruvate dehydrogenase kinase 1

PFKM, 6-phosphofrutokinase muscle isozyme

PGC-1α, Peroxisome proliferation-activated receptor gamma co-activator 1 alpha

PGC-1β, Peroxisome proliferation-activated receptor gamma co-activator 1 beta

PGK-1, phosphoglycerate kinase 1

PHD, Prolyl hydroxylase domain enzyme

Pi, Phosphate

PKA, Protein kinase A

PKM, Pyruvate kinase muscle isozyme

PMS, Phenazinemetasulfate

Pol γ, mitochondrial polymerase gamma

POLRMT, Mitochondrial RNA polymerase

PPARα, Peroxisome proliferation-activated receptor alpha

PPARγ, Peroxisome proliferation-activated receptor gamma

PPARδ, Peroxisome proliferation-activated receptor delta

PPP, Pentose phosphate pathway

PRC, Peroxisome proliferation-activated receptor gamma co-activator 1 related co-activator

PTP1B, Protein phosphatase 1B

QH2, Ubiquinol

ROS, Reactive oxygen species

rRNA, ribosomal RNA

RSV, Resveratrol

RT-PCR, Real-time polymerase chain reaction

SDHb, Succinate dehydrogenase iron-sulfur subunit

Sir2, Silent information regulator 2

SIRT1 KO, Adult-inducible SIRT1 knockout mice

SIRT1 Tg, SIRT1 overexpressor mice

SIRT1, Sirtuin 1

SIRT2, Sirtuin 2

SIRT3, Sirtuin 3

SIRT4, Sirtuin 4

SIRT5, Sirtuin 5

SIRT6, Sirtuin 6

SIRT7, Sirtuin 7

SREBP, Sterol regulatory element-binding protein

STACs, Sirtuin activating compounds

TCA cycle, tricarboxylic cycle

TFAM, Mitochondrial transcription factor A

TFB1M, Mitochondrial transcription factor B1

TFB2M, Mitochondrial transcription factor B2

TMPD, Tetramethylphenylene-diamide

TMRM, Tetramethylrhodamine methyl ester

tRNA, transfer RNA

Uqcrc1, Ubiquinol-cytochrome c redutase core protein 1

Uqcrc2, Cytochrome b-c1 complex subunit 2

VDAC, Voltage dependent anion channel

VEGFa, Vascular endothelial growth factor a

VHL, Von-Hippel Lindau protein

WAT, White adipose tissue

YY1, Initiation element binding factor

Chapter 1- General Introduction

1.1. Mitochondria, the Powerhouses of the Cell

Mitochondria are highly dynamic organelles and the power generator of the cell, as they convert intermediates derived from nutrients into energy in the presence of oxygen in a process named aerobic respiration. As such, mitochondria are extremely important in the maintenance of cellular homeostasis (Wenz, 2009). The number of mitochondria present in each cell greatly depends upon their metabolic requirements, and may range from hundreds to thousands. For instance high metabolic tissues, like skeletal muscle, have more mitochondria than tissues with less metabolic requirements (Johannsen et al., 2009). Since its first description by R. A. Von Kölliker in 1857 (Liesa et al., 2009), our understanding of mitochondria has also tremendously evolved regarding the dynamic nature of this organelle. Indeed, the static bean-shaped view of isolated mitochondria, while still observed in many textbooks, has now been replaced by a highly branched and dynamic network. Mitochondria move throughout the cell and undergo structural transitions, changing the length, morphology/shape, and size depending on the particular cell type. Interestingly, mitochondria were reported to exist in greater number around the endoplasmatic reticulum (Collins et al., 2002), further demonstrating dynamic nature of mitochondria and the importance of its interaction with other organelles.

1.1.1. Mitochondrial Alterations as a Cause of Disease

Energetic demands vary not only among cell types but also in different physiological states. Thus, the ability of the cell to adapt and maintain an optimal range of conditions in mitochondria, which enables them to preserve their function (mitochondrial homeostasis), plays an important role in such physiological transitions. The maintenance of mitochondrial homeostasis is, therefore, an important task for the cell and needs to be tightly regulated through gene expression programs, and impairments in these programs are often associated with mitochondrial dysfunction and disease states.

Pointing out the importance of mitochondrial homeostasis for disease states, are the mitochondrial genetic disorders. Mutations in genes encoding mitochondrial components cause mitochondrial dysfunction and result in the development of multisystemic diseases like myoclonic epilepsy and ragged red fiber (MERRF), mitochondrial encephalomyopathy, lactic acidosis, stroke-like episode syndrome

(MELAS) and Leigh Syndrome, which ultimately lead to early mortality (Wallace et al., 2010b).

Mitochondrial dysfunction is also related with the development of metabolic disorders like insulin resistance and diabetes. The fact that high fat-loads are correlated, in some circumstances, with reduction in mitochondrial mass suggests that the onset of insulin resistance might be caused by a decrease in mitochondrial quantity (Rolo et al., 2011). Indeed, reduced expression of genes related with mitochondrial biogenesis, the tricarboxylic acid cycle (TCA cycle) and the mitochondrial electron transport chain (ETC), declines in mitochondrial DNA (mtDNA) and in mitochondrial area and number have been shown to contribute to deregulation of fuel metabolism creating an energy-deficient state in the muscle of pre-diabetic and diabetic patients as well as in mouse models of obesity (Crunkhorn et al., 2007; Kelley et al., 2002; Koves et al., 2008; Lowell and Shulman, 2005; Mootha et al., 2003; Patti et al., 2003; Rolo and Palmeira, 2006). Moreover, the development of insulin resistance has also been associated with β-cell dysfunction caused by impairments in mitochondrial function (Wiederkehr and Wollheim, 2006), giving further strength to the key role of mitochondrial dysfunction in the development of diabetes.

Mitochondria have also been suggested to play a key role in the aging process and age-related diseases (Figueiredo et al., 2008). Indeed, not only do the number of mitochondria decrease in post mitotic tissues like heart, skeletal muscle and brain during aging (Samorajski et al., 1971; Tate and Herbener, 1976), but a number of age-associated structural changes of mitochondria have been reported as well (Tate and Herbener, 1976). Moreover, reductions in the expression of genes encoding mitochondrial proteins, as well as proteins that regulate mitochondrial homeostasis, have been found in several organs of aged mice (Linford et al., 2007; Liu et al., 2004; Melov et al., 2007). Interestingly, restriction of caloric intact by 30-40%, caloric restriction (CR), is the only known non-pharmacological intervention that extents lifespan, and has been shown to induce mitochondrial biogenesis and prevent age-related mitochondrial decline (Gredilla et al., 2001; Lopez-Lluch et al., 2006a; Sanz et al., 2005). Importantly, and giving strength to the importance of mitochondria in the aging process, a mouse model that accumulates high levels of point mutations due to a proofreading deficiency of the mitochondrial polymerase gamma (Polγ) (mtDNA mutator mouse) display a range of premature aging phenotypes starting at 6 months of age and has a dramatic lifespan reduction compared to wild type mice (Kujoth et al., 2005; Trifunovic et al., 2004).

1.1.2. Mitochondrial Structure

Mitochondria have an elaborate structure, essential to the functioning of the organelle. They possess a double membrane structure, with inner membrane invaginations towards the internal matrix, designated by cristae (Figure 1.1).

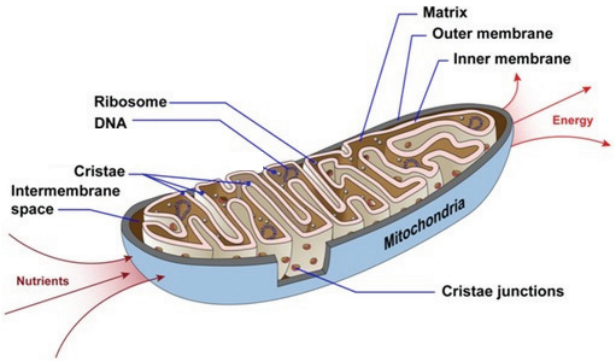


Figure 1.1 - Schematic representation of the mitochondrial structure. (From Hiebert et al., 2012).

Mitochondrial structure has been revisited from what was initially established, as a pioneer study revealed that mitochondrial cristae are not just invaginations of the inner membrane, but independent tubular structures that can sometimes be completely detached from the inner membrane (Figure 1.2). Importantly, these studies also revealed that tissues with high energetic demands mitochondria have more cristae, which increases membrane area and maximize their efficiency (Frey and Mannella, 2000; Mannella, 1998, 2000).

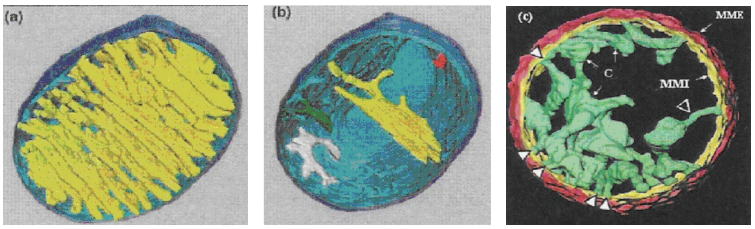


Figure 1.2 - The mitochondrion, the new model. (a) Computer- generated tomographical model of a chicken cerebellum mitochondrion with the cristae in yellow, outer membrane in dark blue, and inner membrane in light blue; (b) Representation of the same model, with both membranes and 4 individual cristae marked with different colors; (c) Computer-generated tomographical model of a rat liver mitochondrion. MMI - Inner mitochondrial membrane; MME - Outer mitochondrial membrane; C - Cristae. (From Frey and Mannella, 2000).

The outer mitochondrial membrane is smooth and it contains many proteins like hexokinase, creatine kinase and the benzodiazepines peripheral receptor, among others. It also contains several protein channels, like the voltage dependent anionic channel (VDAC) that allow the traffic of molecules of 5000 Da or less. Importantly, this membrane is very permeable to most ions, nutrients and small molecules. The inner mitochondrial membrane is less permeable as it allows the passage of oxygen, carbon dioxide and water, but is impermeable to most ions and polar molecules (Crompton, 1999). Therefore, the movement of solutes across this membrane occurs through highly regulated and selective channels. Indeed, about 80% of the dry weight of the inner membrane is proteins (Darnell et al., 1990). Among them are several elements of the various metabolic pathways that take place in the mitochondria, from the complexes of the ETC, the di and tricarboxylic acid transporters, the ATP synthase complex, components of fatty acid oxidation and transport system, to the pyruvate dehydrogenase and the uncoupling proteins (Caldwell et al., 2004; Paradies et al., 1998). Importantly, the inner membrane has high content of cardiolipin which plays an important role in the maintenance of mitochondrial function, through greatly reducing the permeability of the membrane to protons and regulating mitochondrial proteins like cytochrome c oxidase (Complex IV, COX) and the adenine nucleotide translocator (ANT) (Hoch, 1992; Paradies et al., 1998). In addition, there are also multimeric complexes in both membranes that allow the import of peptides inside the mitochondria, the TIM/TOM complex (inner membrane transporter/outer membrane transporter) (Paschen and Neupert, 2001).

Present in the mitochondrial matrix are enzymes responsible for processes like amino acid catabolism, TCA cycle, urea cycle, and heme synthesis. Importantly, the mitochondrial matrix also contains several copies of mtDNA and the machinery needed for its replication, transcription and translation, which confers to this organelle the unique capacity for self-replication (Anderson, 1981).

1.1.3. Oxidative Phosphorylation and Electron Transport Chain

Among the many biochemical processes that occur in the mitochondria, oxidative phosphorylation assumes a key role for cellular homeostasis, as it is the most efficient process to produce energy. In the mitochondria, the energy contained in carbohydrates, fatty acids and proteins is used to produce acetyl-coA that is used to produce reductive equivalents (NADH and $FADH_2$) in the TCA cycle. These reductive equivalents are then

utilized in the oxidative phosphorylation process, which produces up to 38 molecules of adenosine triphosphate (ATP) as energy currency, opposed to the anaerobic glycolytic process that has a very low yield of ATP per molecule of glucose (2 ATP and 2 NADH) and, therefore, is considered much less efficient. As such, mitochondria are responsible for the synthesis of approximately 95% of ATP needed by the cell (Erecinska and Wilson, 1982).

During the oxidative phosphorylation process, electrons are transferred from the reductive equivalents (NADH and $FADH_2$) to the electron final acceptor (oxygen) in redox reactions. These redox reactions are carried out by a series of multimeric protein complexes, that together constitute the ETC (Hatefi, 1985). The ETC is composed of several (around 90) polypeptidic subunits within the mitochondrial inner membrane, and are grouped into four complexes: Complex I or NADH: Ubiquinone Oxiredutase, Complex II or Succinate Dehydrogenase, Complex II or Ubiquinol: Cytochrome c Oxiredutase and Complex IV or Cytochrome c Oxidase. In addition to these, the ETC also has two mobile components, an extrinsic small protein denominated Cytochrome c and a hydrophobic quinone denominated coenzyme Q (coQ) (Figure 1.3).

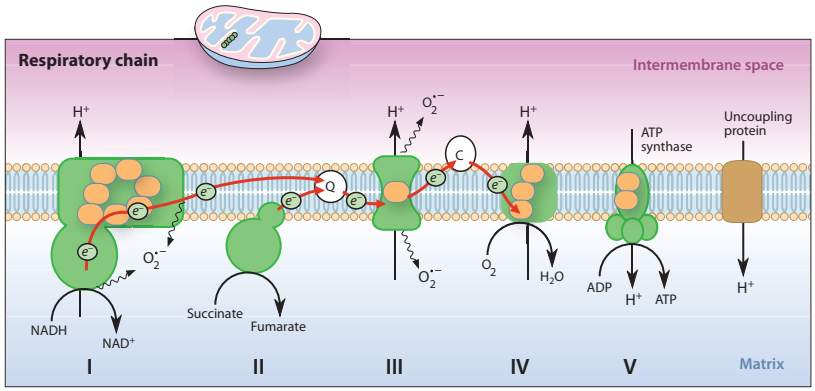


Figure 1.3 - Schematic representation of the mitochondrial electron transport chain and ATP synthase. The red arrows illustrate the pathway of electrons (e^-), from the reductive equivalent (NADH) to the final acceptor, molecular oxygen (O_2). The dark arrows illustrate the protonic (H^+) flow between the matrix and the inter-membrane space, as consequence of the electronic transport and back into the matrix due to ADP phosphorylation (From Larsson, 2010).

These multimeric complexes possess quinoid structures (flavins, quinones) and/or metallic centers (FeS centers, heme groups, Cu centers), which are the main drivers of sequential transfer of electrons to molecular oxygen. Complex I accepts electrons from NADH and transfers them to the CoQ pool. Complex II accepts electrons from $FADH_2$ and also transfers them towards the CoQ pool. This molecule is thus reduced, being named ubiquinol (QH2). The passage from CoQ to QH2 requires the semi-reduction of CoQ by one e^- to semiubiquinone (QH•) and only when another e^- reaches it will it reach the full reduction state, being then named ubiquinol. Ubiquinol, a highly mobile and hydrophobic molecule, will then be able to transfer electrons to Complex III, and from here to Cytochrome c, a mobile protein, which will eventually deliver them to Complex IV. Here, they are transferred to the final electron acceptor, molecular oxygen (O_2) which together with protons generates H_2O. This electronic flux is only possible due to the delicate architecture of the chain, as the electrons flow from complex to complex thanks to their redox potential. As so, CoQ has more affinity for electrons than complexes I and II, Complex III has more affinity for electrons than ubiquinol and so forth. This phenomenon provides the cell with some major advantages: not all energy is released is a single step, but sequentially, which allows for better

harvesting of this energy; the electron flow is carried sequentially, without excessive loss of electrons nor dangerously high energy dissipation; and lastly, the flow of electrons is carried without any energy consumption, and the passage of electrons by the transporter centers causes a conformational change in the structure of the complexes allowing them, in this state of high instability, to eject protons from the matrix to the intermembrane space. The exception is Complex II, due to the fact that it has a redox potential similar to the one of CoQ and, does not reach a proton-ejecting conformational status (Figure 1.4).

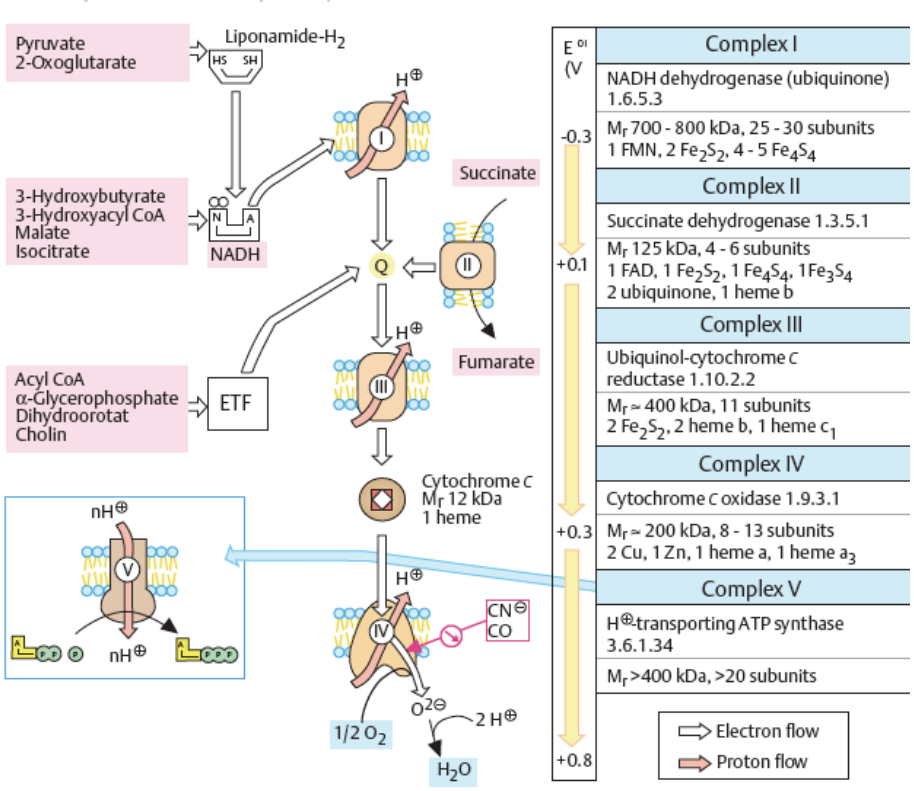


Figure 1.4 - Sequential flow of electrons throughout the mitochondrial electron transport chain, according to the redox potential and protonic ejection. The mitochondrial respiratory chain is responsible for the vectorial ejection of protons from the matrix to the inter-membrane space, following electron transport from the reductive equivalents to molecular oxygen. Ejected protons through Complexes I, III and IV of the electron transport chain are then utilized by the ATPsynthase or Complex V to drive ATP synthesis (from Koolman and Röhm, 1996).

As the electrons flow down the ETC, the energy released is used by complexes I, III and IV to pump protons out across the mitochondrial inner membrane to the intermembrane space. This creates a proton electrochemical gradient (constituted by a pH and electric gradients), a capacitor that is acidic and positive in the intermembrane space and negative and alkaline on the matrix side. The potential energy stored in this electrochemical gradient is used for multiple purposes: (a) to import proteins and Ca^{2+} into the mitochondrion, (b) to generate heat, and (c) to synthesize ATP within the mitochondrial matrix. The energy to convert ADP + Pi to ATP comes from the flow of protons through the ATP synthase (complex V) back into the matrix. Matrix ATP is then exchanged for cytosolic adenosine diphosphate (ADP) by the inner membrane ANT and used for cellular processes.

1.1.4. Reactive Oxygen Species and Oxidative Damage

Reactive oxygen species (ROS) are chemically reactive molecules that contain oxygen, and are highly reactive due to the presence of unpaired valence electrons. ROS are formed as a natural byproduct of the oxygen metabolism, and have important roles in cell signaling and homeostasis. The majority of oxygen consumption in the cell is through oxidative phosphorylation, therefore, mitochondria are the primary source of ROS in the cell. Under normal physiological conditions, ROS production is highly regulated, at least in part, by complex I and complex III (Evans et al., 2000; Hansen et al., 2006; Jones, 2006; Kelley and Parsons, 2001; McCord, 2000). However, when the ETC is inhibited by mutation in an ETC-related gene or becomes highly reduced from excessive calorie consumption relative to exercise level, the ETC electron carriers accumulate excessive electrons, which can be passed directly to O_2 to generate superoxide anion ($O_2^{\cdot -}$). The $O_2^{\cdot -}$ generated by complex I is released into the mitochondrial matrix, where it is converted to hydrogen peroxide (H_2O_2) by the Manganese Superoxide Dismutase (MnSOD; Sod2). The $O_2^{\cdot -}$ generated from complex III is released into the mitochondrial intermembrane space, where it is converted to H_2O_2 by copper/zinc superoxide dismutase (Cu/ZnSOD; Sod1) located in the intermembrane space and cytosol. H_2O_2 can then be converted to H_2O and back to O_2 by the antioxidant enzyme, catalase. Alternatively, mitochondrial H_2O_2 can diffuse into the nucleus-cytosol. If H_2O_2 encounters a reduced transition metal or is mixed with $O_2^{\cdot -}$, it can be further reduced to hydroxyl radical ($OH^{\cdot}$), the most potent oxidizing agent of the ROS. ROS can damage cellular proteins, lipids, and nucleic acids. Hence, excessive mitochondrial ROS

production can exceed the antioxidant defenses of the cell, generating damage to cellular components and this cumulative damage can ultimately destroy the cell by necrosis or apoptosis.

1.1.5. Mitochondrial Biogenesis

According to the endosymbiont hypothesis, mitochondria arose from the engulfment of aerobic eubacteria by a primordial anaerobic eukaryote, an event that was possibly coincident with the origin of the eucariotic cells (Gray et al., 1999). Thus, as mentioned before, this organelle has its own genetic system. Interestingly, most of the mitochondrial genes are now encoded in the nucleus leaving the organelle with the capacity to encode only 13 proteins, as well as, 22 tranfer RNAs (tRNAs) and 2 ribossomal RNAs (rRNAs) required for their translation within the mitochondrial matrix (Taanman, 1999; Wallace, 2005). Because the coding capacity of the mitochondrial DNA is limited, nuclear genes specify most of the numerous gene products required for the molecular architecture and biochemical functions of the organelle (Kelly and Scarpulla, 2004; Scarpulla, 2006). These include the majority of respiratory proteins, the constituents of the translational system and all the gene products required for the transcription and replication of mtDNA (Figure 1.5).

Mitochondria are highly dynamic organelles being constantly eliminated and regenerated. The generation of new mitochondria is called mitochondrial biogenesis and involves multiple processes, including: formation of double membrane boundary from phospholipids, which are either imported from other organelles or synthesized locally (Horibata and Sugimoto, 2010; Potting et al., 2010); import of mitochondrial proteins witch are encoded by nuclear genes; synthesis of the fundamental mtDNA encoded protein components in the mitochondrial matrix (Calvo and Mootha, 2010); and replication of the mtDNA and translational machinery (Wallace, 2007). These four processes are inherently interrelated in order to achieve a balanced assembly of the organelle (Scarpulla, 2006).

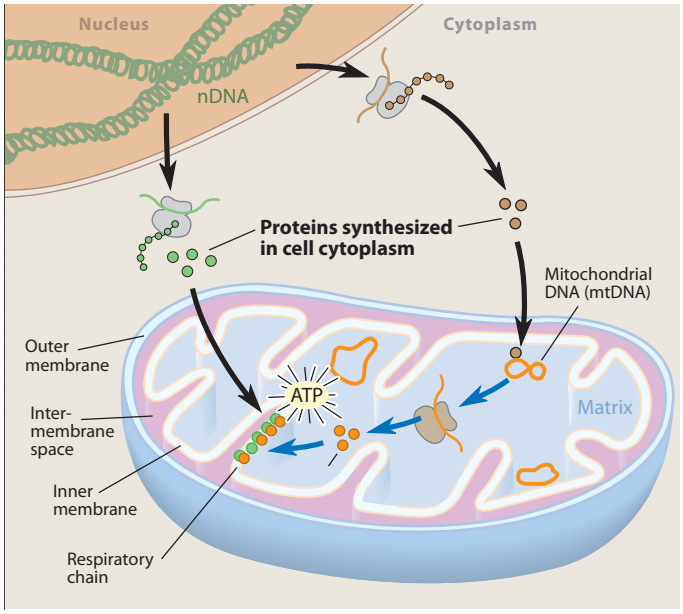


Figure 1.5 - Schematic representation of the mitochondrial electron transport chain biogenesis. The respiratory chain is situated in the inner mitochondrial membrane, and its protein components are encoded by either mtDNA or nuclear DNA. Transcription of mtDNA produces 13 mRNAs, which all encode oxidative phosphorylation system subunits as well as all RNA components (22 tRNAs and 2 rRNAs) needed for translation of these mRNAs on the mitochondrial ribosomes. The mRNAs produced by transcription of nuclear genes are exported to the cytoplasm and translated on cytoplasmic ribosomes, which are distinct from the mitochondrial ribosomes. The nucleus-encoded mitochondrial proteins are imported into mitochondria and constitute the vast majority of the ~1000 proteins present in mammalian mitochondria (From Larsson, 2010).

1.1.5.1 Genetics of the Mitochondrial Electron Transport Chain

The ETC is assembled by polypeptides encoded by both the nuclear and the mitochondrial genomes. The nuclear genes are spread out across all chromosomes on both autosomes and sexual chromosomes. Complex I is assembled from 45 polypeptides, of which 7 (ND1, -2, -3, -4, -4l, -5 and -6) are encoded by the mtDNA; Complex II from 4 nuclear DNA polypeptides; Complex III from 11 polypeptides, of which 1 (cytochrome b) is encoded by the mtDNA; Complex IV from 13 polypeptides of which 3 (COX1, -2 and -3) are from the mtDNA; and Complex V from 16 polypeptides, of which 2 (ATP6 and -8) are from the mtDNA (Wallace et al., 2010b). Of the five complexes only complex I, III, IV and V transport protons, and they also retain mtDNA-encoded

polypeptides. Importantly, all the proton-pumping complexes contribute for the generation of the electrochemical gradient. As such, the proton transport of all four complexes must be balanced to avoid one of the complexes negatively impacting the energy-generating capacity of the other complexes. Therefore, the major electrical components of these four proton-pumping complexes must coevolve. This is accomplished through the maintenance of all the genes encoding these proteins in the mtDNA, allowing them to be to be transcribed a single unit and therefore different mtDNA lineages with different coupling efficiencies cannot be mixed by recombination (Wallace et al., 2010b).

	mtDNA	nDNA
Complex I	7	39
Complex II	0	4
Complex III	1	10
Complex IV	3	10
Complex V	2	14

Table 1.1 - Genetic composition of mitochondrial ETC complexes. mtDNA – mitochondrial DNA; nDNA – nuclear DNA.

1.1.5.2. Mitochondrial DNA and its Regulation

The mammalian mtDNA is a circular double-stranded molecule, which is about 16,600 base pairs in humans and 16,300 base pairs in mice and is maternally inherited (Falkenberg., 2007). The two strands of the mtDNA (Figure 1.6) are for historical reasons denoted the heavy (H) and light (L) strands owing to their different base compositions (Falkenberg et al., 2007). The mtDNA is densely packed with genes and contains only one longer noncoding region, the displacement loop (D loop). The D loop contains the promoters for transcription of the L and H strands (LSP and HSP) and the origin of replication of the H strand (O_H) (Figure 1.6). Transcription from the LSP provides primers for the initiation of replication at O_H (Falkenberg et al., 2007).

Importantly, all the proteins involved in the replication and transcription of the mtDNA and mitochondrial translation are encoded by nuclear genes and imported into the mitochondria (Falkenberg et al., 2007).

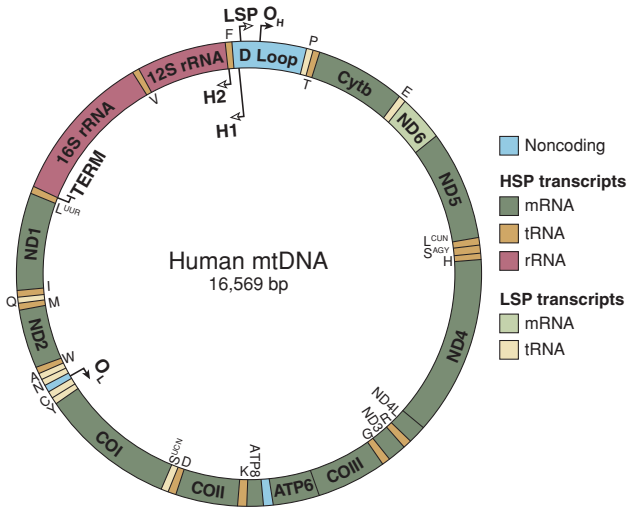


Figure 1.6 - Schematic representation of the mitochondrial DNA molecule. Mammalian mtDNA is a double-stranded circular molecule. The two strands are denoted the heavy (H) and light (L) strand due to different buoyant densities. The only longer noncoding region, the displacement loop (D loop), contains a triplex structure with a nascent H strand. The D loop contains the promoters for transcription of the H and L strand (HSP and LSP) as well as the origin of replication of the leading strand of mtDNA (OH). The origin of replication of the lagging strand (OL) is located in a cluster of tRNA genes. Transcription from HSP produces 2 rRNAs (12S and 16S rRNA), 12 mRNAs (ND1–5, ND4L, Cyt b, COI–III, ATP6, and ATP8), and 14 tRNAs (F, V, L1, I, M, W, D, K, G, R, H, S1, L2, T). Transcription from LSP has a dual function. First, it produces RNA primers needed for initiation of replication at OH. Second, it is needed to produce one mRNA (ND6) and eight tRNAs (P, E, S2, Y, C, N, A, Q) (From Falkenberg et al., 2007).

Transcription of mtDNA is necessary for mtDNA gene expression and also produces the RNA primers necessary for initiation of mtDNA replication at O_H (Falkenberg et al., 2007). The basal mitochondrial transcription machinery consists of the mitochondrial RNA polymerase (POLRMT), mitochondrial transcription factor B2 (TFB2M), and mitochondrial transcription factor A (TFAM) (Falkenberg et al., 2002; Metodiev et al., 2009). These three factors are sufficient and necessary to promote specific initiation of mtDNA transcription in a pure recombinant in vitro system. POLRMT and TFB2M interact in vitro and form a heterodimer (Falkenberg et al., 2002). TFB1M is a paralog of TFB2M, but has no role in mtDNA transcription (Falkenberg et al., 2002; Metodiev et al., 2009). Instead, TFB1M functions as a 12S rRNA methyltransferase essential for the integrity of the small subunit of the mammalian mitochondrial ribosome

(Metodiev et al., 2009). The TFAM protein is absolutely essential for transcription initiation, and there is not even abortive transcription in its absence (Falkenberg et al., 2007). In addition, TFAM also has a direct role in packaging mtDNA (Falkenberg et al., 2002). The activity of the basal mitochondrial transcription machinery is likely modulated by additional factors that affect both transcription initiation and termination. The mitochondrial transcription termination factor 1 (MTERF1) binds downstream of the rRNA genes and has been proposed to have roles in termination of both H and L strand transcription (Fernandez-Silva et al., 1997) (Figure 1.7).

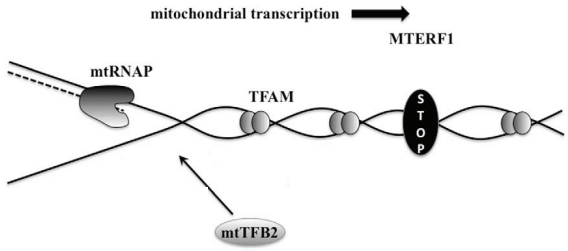


Figure 1.7 - Overview of transcription in animal mitochondria and the proteins involved in the process. The simplified scheme illustrates the mitochondrial transcriptional process and the factors involved in it. The Solid lines represent DNA, and the dashed line represents RNA. mtRNAP- mitochondrial RNA polymerase; TFAM- mitochondrial transcription factor A; mTFB2 – mitochondrial transcription factor B2; MTERF1 – mitochondrial termination factor 1 (From Oliveira et al., 2010).

Replication of the mtDNA is dependent on POLRMT that forms the short RNA primers needed for initiation of mtDNA replication at the replication origin (Clayton, 1991; Fuste et al., 2010). The minimum replicasome need for replication of mtDNA consists of mtDNA Polγ (Falkenberg and Larsson, 2009; Fuste et al., 2010), the twinkle DNA helicase (Spelbrink et al., 2001) and the mitochondrial single-stranded DNA-binding protein (Falkenberg et al., 2007). The mode of mtDNA replication is controversial, as two competing models have been proposed: the strand-asymmetric model, that porposes that the leading strand replication is two thirds complete before lagging strand replication is initiated (Brown et al., 2005; Clayton, 1982); and the strand-coupled replication mode, that argues that the replication of the leading and lagging strands is synchronous (Holt et al., 2000; Yasukawa et al., 2006).

A somatic mammalian cell contains from 1000 to 10,000 copies of mtDNA which are continuously turned over and replicated during the entire cell cycle,

Interestingly, a pathogenic mutation in the mtDNA can be present in all copies (homoplasmy) or only in a fraction of all copies (heteroplasmy). Heteroplasmic mtDNA mutations can be segregated as mtDNA is renewed, generating a mosaic distribution of mutated mtDNA (Larsson, 2010). Importantly, a minimum threshold level of a pathogenic mtDNA mutation must be present in a cell to cause respiratory chain deficiency (Falkenberg et al., 2007).

1.1.5.3. PGC-1α, a Key Regulator of Mitochondrial Biogenesis

The involvement of two distinct genomes creates a demand for elaborate regulatory processes to coordinate gene expression in response to cellular demands. Reflecting the complexity and importance of the control of the mitochondrial biogenesis machinery, expression of the mitochondrial proteome is regulated by a plethora of nuclear factors in a hierarchical manner. Early work identified a set of DNA binding transcriptional factors responsable for the induction of cytochrome c and cytochrome c oxidase subunits, named nuclear respiratory factor 1 and 2 (NRF-1 and -2) (Scarpulla, 2006). In addition, NRFs also indirectly control the expression of mtDNA encoded genes by inducing the transcription of the nuclear encoded TFAM, TFB1M and TFB2M (Scarpulla, 2008). Moreover other transcriptional factors like cAMP response element binding protein (CREB) (Gopalakrishnan and Scarpulla, 1994) and the initiation element binding factor (YY1) (Basu et al., 1997) were also found to regulate mitochondrial genes (Figure 1.8).

Interestingly, most evidence supports a model whereby a relatively small number of nuclear receptors and transcriptional factors serve to coordinate the expression of nuclear and mitochondrial respiratory proteins. This raised the question of how these diverse nuclear receptors and transcription factors are integrated into a program of mitochondrial biogenesis. Investigation into a further level of integration in nuclear transcriptional regulation of mitochondria has been instigated by the discovery of the peroxisome proliferator activated receptor gamma co-activator 1 alpha (PGC-1α), a member of the ever-growing family of nuclear co-regulators. Co-regulators exist and function in large multiprotein complexes, in which rather then binding to DNA, they engage nuclear receptors and transcriptional factors and modulate their transcriptional potency by promoting subsequent biochemical interactions, required for induction or repression of gene transcription. These interactions include allosteric modification of

nuclear receptors and transcriptional factors, chromatin modification and remodeling, initiation of transcription and mRNA editing (O'Malley and Kumar, 2009).

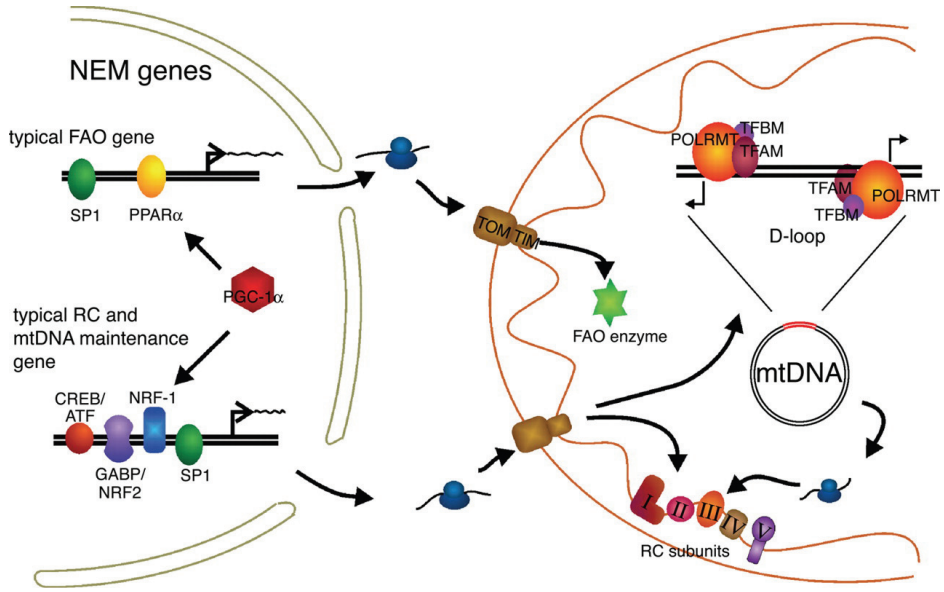


Figure 1.8 - PGC-1α coordination of the two genetic systems in mitochondrial biogenesis. The simplified scheme illustrates two major classes of NEM genes regulated by different DNA-binding transcription factors, probably coordinated by PGC-1α. Nuclear-encoded mitochondrial proteins are imported into the organelles via translocator tomplexes of the outer and inner Membrane (TOM and TIM, respectively), and are distributed to the matrix, membranes and the mtDNA expression and maintenance machinery. NEM – Nuclear encoded mitochondrial genes (From Goffart et al., 2004).

PGC-1α was first cloned in the mouse (Puigserver et al., 1998), and subsequently in humans (Esterbauer et al., 1999), as a co-regulator of the nuclear receptors peroxisome proliferator activated receptor gamma (PPARγ) and thyroid hormone driving adaptive thermogenesis in brown adipocytes, and playing a role in he differentiation of this cell type (Kajimura et al., 2010). This seminal finding was soon followed by the identification of a series of transcriptional factors and nuclear receptors involved in the transcription of the ETC and fatty acid oxidation co-regulated by PGC-1α like, the nuclear respiratory factor 1 and 2 (NRF-1 and -2) and YY1 acting directly on the ETC genes; the sterol regulatory element binding protein (SREBP) family and peroxisome proliferator activated receptor alpha (PPARα) controlling lipid metabolism;

and the estrogen related receptor alpha (ERRα), thus establishing the role of PGC-1α as the 'master regulator' of mitochondrial biogenesis (Wu et al., 1999) (Figure 1.9).

Additional members of a small family of structurally related transcriptional coactivators have also been cloned via their homology to PGC-1α, named PGC-1β (Kressler et al., 2002; Lin et al., 2002) and PGC-1 related coactivator (PRC) (Andersson and Scarpulla, 2001). Genetic approaches have established the complimentary function of PGC-1α and PGC-1β in maintaining an appropriate mitochondrial density in tissues with high energy demand. Individual knockout of PGC-1α or β results in mild phenotypes, while the recently created double knockout mice show markedly reduced mitochondrial number and size in brown adipose tissue, skeletal muscle and cardiac muscle, associated with cardiac failure soon after birth (Lai et al., 2008; Zechner et al., 2010).

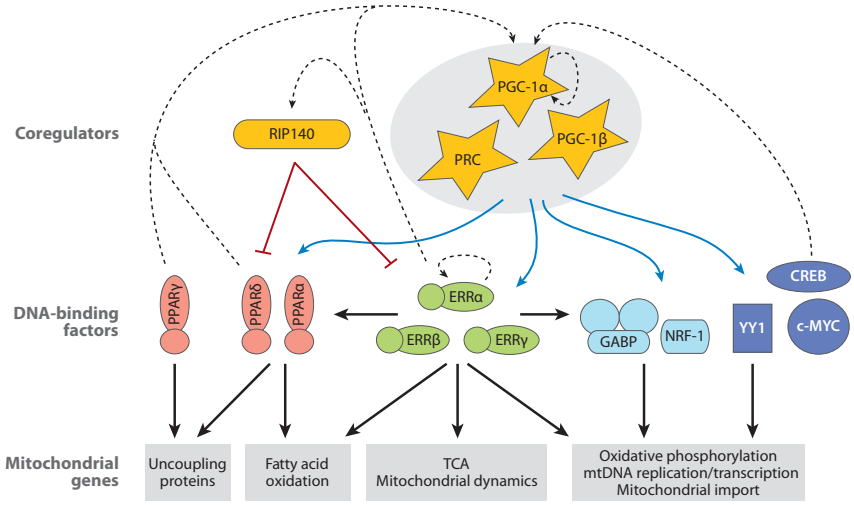


Figure 1.9 - The transcriptional network that controls mitochondrial gene expression. DNA-binding factors regulate overlapping but distinct classes of mitochondrial genes. Co-regulators (PGC-1α, PGC-1β, PRC, and RIP140) interact with multiple DNA-binding factors to coordinate the regulation of multiple classes of mitochondrial genes. The relative levels and activities of these co-regulators are major determinants of mitochondrial biogenesis. Several feed-forward and feedback loops control the activity of this network. DNA-binding factors regulate expression of themselves. These regulatory loops are likely tissue- and signal-specific (From Hock and Kralli, 2009).

Whilst the principal function of the PGC-1 family of co-activators as master regulators of mitochondrial biogenesis is well established, recent evidence has shown that the PGC-1 family have a much broader spectrum of cellular activities. Indeed, the PGC-1 family engages in interactions with other nuclear co-activators and co-repressors, representing a further level of complexity in the regulation of cellular functions in a tissue-specific manner, including the regulation of insulin-dependent glucose disposal in heart, brown adipose tissue and skeletal muscle (Mootha et al., 2003), hepatic gluconeogenesis, fatty acid oxidation (Ling et al., 2004), among others. Importantly, and adding complexity to this regulatory network, the PGC-1 family is modulated by a range of fundamental cellular signaling pathways that can regulate the activity of PGC-1 members either by regulation of their expression levels or through post-translational modifications (Hock and Kralli, 2009; Lin et al., 2005; Scarpulla, 2011a). Energy deprivation, that decreases intracellular ATP/AMP and NADH/NAD^+ ratios, activates PGC-1α both by phosphorylation through the AMP activated protein kinase (AMPK) pathway (Canto et al., 2009) an by sirtuin 1 (SIRT1) mediated deacetylation (Gerhart-Hines et al., 2007; Rodgers et al., 2005). Cellular energy status also signals to PGC-1α through the mammalian target of rapamycin (mTOR) and YY1 (Cunningham et al., 2007), and the regulation of PGC-1 activity by exercise and cold exposure is also under the control of stress signaling through cellular Ca^{2+} and cAMP signaling (Hock and Kralli, 2009) (Figure 1.10).

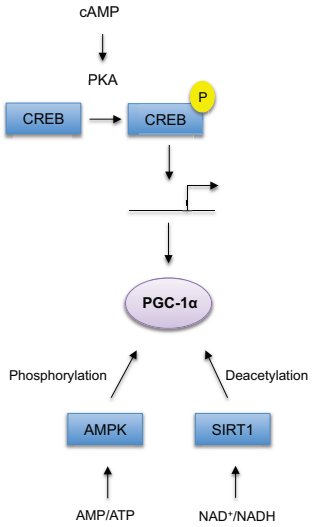


Figure 1.10 - Regulatory network governing PGC-1α. The diagram summarizes the regulation of PGC-1α by transcriptional and posttranslational pathways and its interactions with some of its target transcription factors involved in metabolic regulation (Adapted from Scarpulla, 2011a).

1.1.5.4. PGC-1-independent Pathways Involved the Regulation of Mitochondrial Biogenesis

As addressed before, the PGC-1 family is generally accepted to be the master regulator of mitochondrial biogenesis. Nevertheless, recent reports suggested that, despite its critical role, PGC-1 and its targets are not the only regulators of mitochondrial biogenesis. Indeed, the addition of exogenous pyruvate to muscle cells was sufficient to induce mitochondrial biogenesis in the absence PGC-1α (Wilson et al., 2007), suggesting the existence of a novel energy sensing pathway regulating oxidative capacity. Interestingly, pathways initially discovered as playing a pivotal role in the cellular reprogramming induced by oncogenesis, have recently being shown to also regulate mitochondrial biogenesis. For instance, the Wnt signaling pathway was identified in a large-scale RNAi screen as a regulator of mitochondrial biogenesis (Yoon et al., 2010). Importantly, the Wnt pathway seems to regulate mitochondrial biogenesis through regulation of the proto-oncogene c-Myc (myelocytomatosis viral oncogene homolog) (Yoon et al., 2010).

c-Myc is a helix-loop-helix leucine zipper transcription factor that heterodimerizes with Max to regulate transcription and accelerate the entry of cells into S-phase of the cell cycle, and one of the most frequently activated genes in human cancers. Initial evidence of a relationship between c-Myc and mitochondrial biogenesis came from microarray studies, in which gene ontology analysis revealed that approximately 198 genes from the 1600 genes induced by c-Myc are involved in mitochondrial function and biogenesis (Li et al., 2005). Moreover, c-Myc was also shown to bind to the TFAM promoter (Li et al., 2005), opening the possibility for regulation of mitochondrial-encoded genes independently of PGC-1α. Moreover, using a combination of chromatin immunoprecipitation and promoter microarrays was shown that c-Myc regulates several other nuclear encoded genes involved in mitochondrial biogenesis (Kim et al., 2008a). In fact, c-Myc null fibroblasts present abnormal mitochondria (Graves et al., 2012; Li et al., 2005). *In vivo*, c-Myc activation in the myocardium of adult mice led to an increase in mitochondrial biogenesis. Interestingly, this study showed that c-Myc activation also lead to a reduction in the expression of PGC-1α (Ahuja et al., 2010), revealing that increases in mitochondrial biogenesis can occur even in conditions where PGC-1α is downregulated. These findings give further strength to the idea of a mitochondrial biogenesis pathway independent of PGC-1α, where c-Myc might play a pivotal role under certain conditions.

1.2. Sirtuins

Sirtuins have received significant attention since the discovery that the yeast sirtuin, silent information regulator 2 (Sir2), extends lifespan (Kaeberlein et al., 1999). Sir2 was originally described as a regulator of transcriptional silencing of mating-type loci, telomeres and ribosomal DNA (Guarente, 2011; Haigis and Sinclair, 2010). However, after the discovery of its role in the regulation of yeast lifespan, Sir2 was soon found to be an NAD^+-dependent histone deacetylase (Imai et al., 2000), indicating that sirtuins can serve both as energy sensors and as transcriptional effectors by controlling the acetylation state of histones.

1.2.1. Mammalian Sirtuins: Subcellular Localizations and Enzymatic Activities

There are seven sirtuins in mammals (SIRT1-7). These proteins share an evolutionary conserved catalytic core domain but show little similarity in other regions (Figure 1.11).

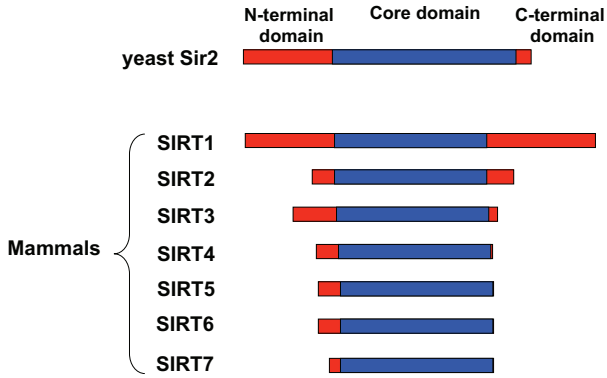


Figure 1.11 - Schematic representation of the seven mammalian sirtuins.

Mammalian sirtuins also vary in their pattern of subcellular localization. SIRT1 is mainly localized in the nucleus but is also present in the cytosol. Its nuclear export signal allows shuttling to the cytosol under specific circumstances. Although the physiological relevance of this shuttling is unclear, it is possible that either cytosolic targets could be deacetylated or that shuttling is another level of control of nuclear target proteins. SIRT2 is considered to be primarily cytosolic, but is also present in the nucleus in the transition of G2 to M phase of the cell cycle (Vaquero et al., 2006). SIRT3, SIRT4 and SIRT5 are localized in the mitochondria, although SIRT3 and 5 can be found in other subcellular compartments under certain conditions (Verdin et al., 2010). SIRT6 is predominantly nuclear (Mostoslavsky et al., 2006) and SIRT7 resides inside of the nucleolus (Ford et al., 2006) (Figure 1.12).

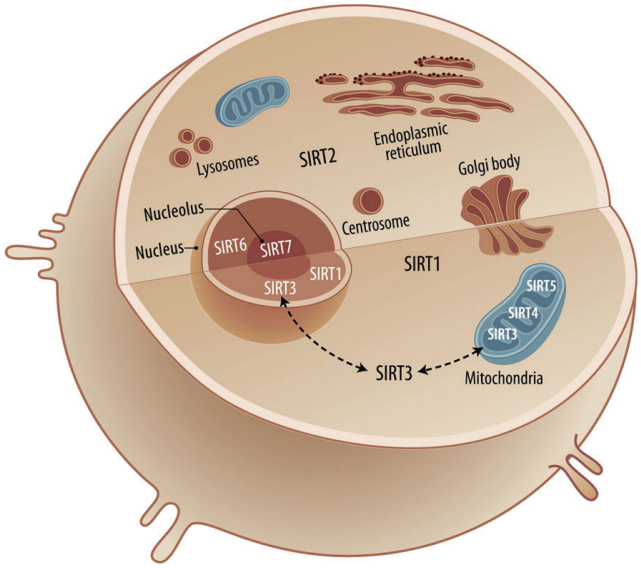


Figure 1.12 - Schematic representation of the subcellular localization of the mammalian sirtuins. The diagram summarizes the subcellular localization of the mammalian sirtuins. In the nucleus are present SIRT1, SIRT3, and SIRT6. SIRT7 is present in the nucleolus. SIRT3, SIRT4 and SIRT5 are present inside of the mitochondria and SIRT2 is mainly present in the cytosol. (From Alhazzazi et al., 2011).

Interestingly, mammalian sirtuins, especially SIRT1, SIRT2 and SIRT3, not only target histones but also deacetylate a wide range of proteins in the different subcellular compartments. In addition, SIRT4 (Haigis et al., 2006) and SIRT6 (Liszt et al., 2005) were reported to function as ADP-ribosyltransferases, even though SIRT6 also can act as a deacetylase (Michishita et al., 2008; Zhong et al., 2010). SIRT5 was initially reported as a deacetylase (Nakagawa et al., 2009), but was recently shown to primarily demalonylate and desuccinylate proteins (Peng et al., 2011). SIRT7 activity was for a long time unknown, however it was recently reported that SIRT7 can act as an NAD^+-dependent histone deacetylase, important for oncogenic transformation (Barber et al., 2012).

The enzymatic reaction catalyzed by sirtuins requires NAD^+ as a substrate, the concentration of which is determined by the nutritional state of the cell (Houtkooper et al., 2010). As such, NAD^+ is well positioned to control adaptive responses to energy stress by modulating the activity of sirtuins. Sirtuins convert NAD^+ to nicotinamide, which

at higher concentrations can non-competitively bind and inhibit sirtuin activity (Anderson et al., 2003; Bitterman et al., 2002). The other by-product of sirtuin deacetylase reaction, O-acetyl-ADP-ribose, was also reported to be a signaling molecule (Kustatscher et al., 2005; Liou et al., 2005) (Figure 1.13).

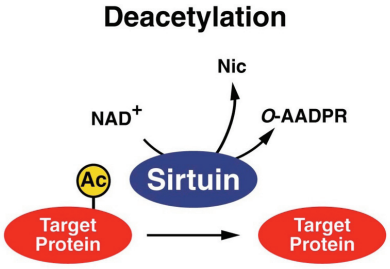


Figure 1.13 - Schematic representation of the deacetylation activity catalyzed by sirtuins. NAD^+–dependent lysine deacetylation of a protein substrate by sirtuins, in which nicotinamide and O-acetyl-ADP ribose are also generated. NAD^+ - Nicotinamide adenine dinucleotide Nic – Nicotinamide; *O*-AADPR - O-acetyl-ADP ribose.

1.2.2. Regulation of SIRT1 Activity

SIRT1, the closest orthologue to yeast Sir2 among the mammalian sirtuins, can be regulated at various levels. Indeed, SIRT1 activity can be regulated by transcriptional and post-translational modifications, by complex formation, and through NAD^+ availability.

SIRT1 expression changes under various physiological conditions, resulting in induction during low energy status and repression during energy excess. For instance, FOXO1 (Nemoto et al., 2004), PPARα (Hayashida et al., 2010), PPARδ (Okazaki et al., 2010) and CREB (Noriega et al., 2011) increase SIRT1 levels, whereas PPARγ (Han et al., 2010a) and chREBP (Noriega et al., 2011) repress SIRT1 expression. Finally, poly(ADP-ribose) polymerase 2 (PARP2), which belongs to a family of nuclear enzymes involved in DNA repair and when activated by DNA damage, catalyse the transfer of ADP-ribose units from NAD^+ to substrate proteins to form branched polymers of ADP-ribose and therefore are NAD^+ dependent, binds to and represses the SIRT1 promoter, although the exact mechanism is not yet understood (Bai et al., 2011a). Importantly, CREB, chREBP and PARP2 not only regulate sirtuin expression *in vitro* but also have been shown to control its expression *in vivo*. At a different level, microRNAs (miRNAs)

modulate mRNA levels through the degradation of the primary mRNA transcript or by inhibition of translation. As such, mouse miR-34a (Yamakuchi et al., 2008) and miR-199a (Rane et al., 2009) represses SIRT1 expression. Interestingly, miR-34a-mediated SIRT1 repression was increased in diet-induced obesity (Lee et al., 2010), suggesting a physiological relevance for this interaction.

SIRT1 activity can also be regulated by post-translational modifications, such as phosphorylation (Sasaki et al., 2008). SIRT1 is phosphorylated *in vitro* by the cyclin B–CDK1 (cyclin-dependent kinase 1) complex, which binds to SIRT1, and mutation of these phosphorylation sites disturbs normal cell cycle progression (Sasaki et al., 2008). JUN N-terminal kinase (JNK) also phosphorylates SIRT1 at three residues, particularly during oxidative stress. This results in deacetylation of histone H3, but not of p53 (Nasrin et al., 2009), suggesting that phosphorylation directs SIRT1 to specific targets. Recently, cAMP was reported to induce the phosphorylation of a highly conserved serine located in the SIRT1 catalytic domain through activation of protein kinase A (PKA). Phosphorylation at this site by PKA was shown to rapidly enhance intrinsic deacetylase activity of SIRT1 independently of NAD^+ levels (Gerhart-Hines et al., 2011). In addition, SIRT1 has also been shown to be sumoylated, which in cultured cells increases its activity (Yang et al., 2007).

Forming complexes with other proteins can also regulate SIRT1 and, therefore, these proteins might impact the regulation of metabolism and the development of diseases where SIRT1 activity plays a role. AROS (active regulator of SIRT1) is the only protein known to positively regulate SIRT1 following complex formation, which leads to suppression of the SIRT1 target p53 (Kim et al., 2007b). Moreover, deleted in breast cancer 1 (DBC1) binds the SIRT1 catalytic domain and inhibits its activity *in vitro* during genotoxic stress (Kim et al., 2008b; Zhao et al., 2008). Indeed, regulation of SIRT1 through complex formation as been shown to play a role in the development of metabolic syndrome, as DBC1 knockout mice are protected from high fat diet-induced glucose intolerance and fatty liver (Escande et al., 2010).

As mentioned above, SIRT1 enzymatic activity depends on the cofactor NAD^+, as such, the availability of NAD^+ is another point of regulation. For example, relative NAD^+ levels decrease under conditions that stimulate its conversion to its reduced form, NADH (Canto et al., 2009). Specifically, NAD^+ levels rise in muscle, liver and white adipose tissue (WAT) during fasting, caloric restriction and exercise (Canto et al., 2010; Chen et al., 2008), accompanied by SIRT1 activation, whereas high-fat diet in mice

reduces the NAD^+/NADH ratio (Kim et al., 2011b). NAD^+ availability is also regulated at the level of synthesis. NAD^+ is synthesized from three major precursors, tryptophan, nicotinic acid, and nicotinamide (Magni et al., 2004; Revollo et al., 2007). Lower eukaryotes and invertebrates, such as yeast, worms, and flies, use nicotinic acid, a form of vitamin B3, as a major NAD^+ precursor, whereas mammals predominantly use nicotinamide, another form of vitamin B3. In mammals, nicotinamide phosphoribosyltransferase (NAMPT) initiates the major NAD^+ biosynthesis pathway by converting nicotinamide to nicotinamide mononucleotide (NMN), which is the rate-limiting step in this NAD^+ biosynthesis pathway (Garten et al., 2009; Imai, 2009). The second enzyme, nicotinamide/nicotinic acid mononucleotide adenylyltransferase (NMNAT), completes NAD^+ biosynthesis by transferring adenine from ATP to NMN. This biosynthetic pathway is also known as the NAD^+ salvage pathway (Figure 1.14). Interestingly, and giving further strength to the importance of NAD^+ as a regulator of SIRT1 activity, treatment with an NAD^+ precursor, NMN, activates SIRT1 and improves glucose tolerance in mice (Yoshino et al., 2011).

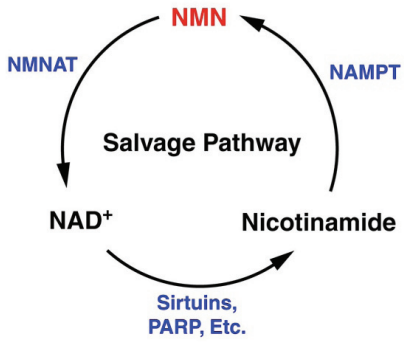


Figure 1.14 - Schematic representation of the NAD^+ salvage pathway. The salvage pathway is critical to mantain intracellular NAD^+ levels. After the conversion of nicotinamide into NMM by NAMPT; NMN is further modified into NAD^+ by Nmnat. NMN – Nicotinamide mononucleotide; NAD^+ - nicotinamide adenine dinucleotide; Nmnat – Nicotinamide mononucleotide adenylyltransferase; NAMPT – nicotinamide phosphoribosyltransferase.

In addition to biosynthesis, manipulating the activities of NAD^+-depleting enzymes also alters NAD^+ levels, thereby regulating SIRT1 activity. PARPs are considered to be the major NAD^+ degrading enzymes (Krishnakumar and Kraus, 2010; Schreiber et al., 2006) and thereby also regulate SIRT1 activity. Indeed, two recent reports revealed that the deletion of PARP1 and PARP2 in mice activates SIRT1,

leading to increased numbers of mitochondria, enhanced energy expenditure and protection from diet-induced obesity (Bai et al., 2011a; Bai et al., 2011b).

1.2.3. SIRT1, a Key Regulator of Metabolism

Maintenance of energy homeostasis requires a coordinated regulation of energy intake, storage and expenditure. This balance is orchestrated by the involvement of inter-organ communication using endocrine and metabolic pathways. Thus, metabolic pathways are designed to sense incoming nutritional and environmental cues and respond appropriately. In mammals, SIRT1 regulates a number of pathways involved in fuel utilization and energy adaptation through deacetylation of key factors in several organs including PGC-1α, PPARα, PPARγ, HIF1 and the forkhead box subgroup O (FOXO) (Haigis and Sinclair, 2010). SIRT1, thus acts as a pleiotropic energy sensor to help mount an appropriate physiological response to changes in nutritional status (Figure 1.15).

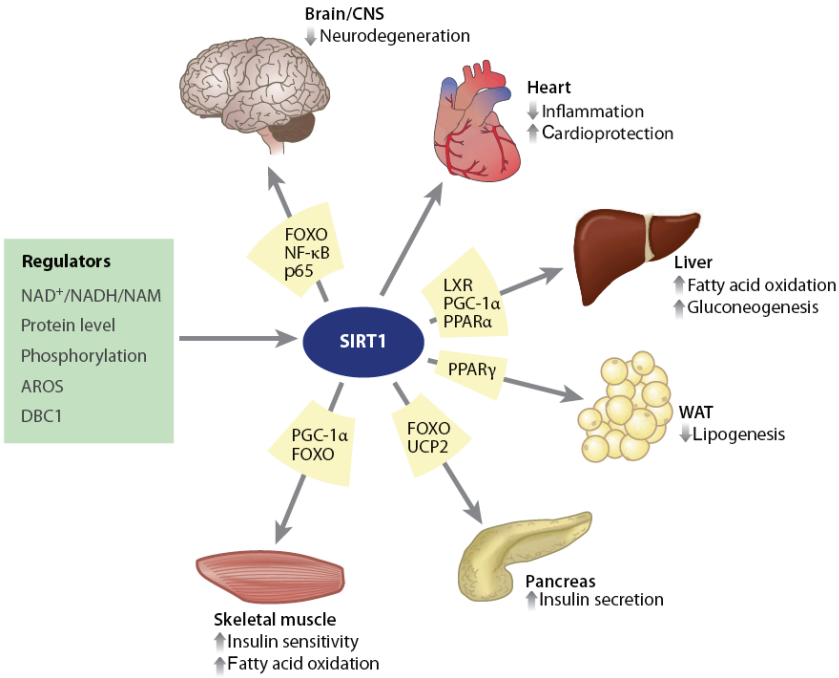


Figure 1.15 - Regulation of metabolism by SIRT1. SIRT1 activity can be regulated through NAD+ and nicotinamide concentrations, by SIRT1 protein level, and by phosphorylation; SIRT1 can be activated by AROS and inhibited by DBC1. SIRT1 activation protects against several age-related and metabolic diseases

by regulation of important cellular processes through deacetylation of several different targets in different organs. NAD^+ - Nicotinamide adenine dinucleotide; NAM – Nicotinamide; DBC1 – deleted in brest cancer1; WAT – White adipose tissue; CNS - central nervous system; FOXO - forkhead box transcription factor, subgroup O; LXR - liver X receptor; NF-κB - nuclear factor kappa B; PGC-1α - peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PPARα - peroxisome proliferator-activated receptor alpha; UCP2 - uncoupling protein 2; AROS – active regulator of SIRT1 (From Haigis and Sinclair, 2010).

1.2.3.1. Glycolysis

Glycolysis is the first step in the metabolic pathway to obtain ATP from glucose. Glucose is imported by glucose transporters (GLUTs) and metabolized through a series of steps to generate pyruvate. Pyruvate can be further oxidized into acetyl-CoA, to enter the TCA cycle in the mitochondria. Under conditions of normal nutrient supply, most of glucose is converted into pyruvate to enter mitochondrial respiration, whereas, under low glucose or low oxygen conditions, pyruvate is diverted to produce lactate through anaerobic glycolysis. Although anaerobic glycolysis is far less efficient, it provides critical intermediate metabolites necessary to maintain macromolecular biosynthesis and thus a shift towards anaerobic glycolysis gives an important advantage to rapidly dividing cells, a phenomenon known as the Warburg effect (Warburg, 1956). SIRT1 is well established to inhibit glycolysis by activating PGC-1α, which among other things attenuates the transcription of glycolytic genes (Rodgers et al., 2005). Additionally, SIRT1 was shown to regulate the glycolytic program by deacetylating the hypoxia-inducible factor 1α, HIF1α, decreasing its activity and consequently repressing glycolysis (Lim et al., 2010) and promoting pyruvate oxidation in the TCA cycle.

1.2.3.2. Fatty Acid Oxidation

Fatty acids are a very important source of energy. Indeed, fatty acids yield more than twice as much energy for the same mass as do carbohydrates or proteins. As such, fatty acid oxidation assumes a pivotal role in the maintenance of energetic homeostasis. Indeed, in conditions where carbohydrates are less abundant, like prolonged fasting, exercise or metabolic stress, fatty acid oxidation assumes a central role as energy source and therefore in the maintenance of cellular homeostasis.

Fatty acid oxidation occurs mainly in the mitochondrial matrix (β-oxidation), therefore long-chain fatty acids need first to be transported from the cytoplasm into

mitochondria, where they undergo β-oxidation to generate acetyl-CoA, which can be used for ATP production via the TCA cycle and oxidative phosphorylation. Thus, β-oxidation defects cause severe metabolic crisis associated with nonketonic hyperglycemia, fatty liver, muscle weakness and cardiomyopathies.

SIRT1 enhances energy expenditure by stimulating fatty acid oxidation, in response to fasting. This is achieved by activating PPARα and one of its key co-activators, PGC-1α (Purushotham et al., 2009), which stimulate the expression of genes involved in fatty acid uptake and/or β-oxidation. In support of this, SIRT1 activation protects mice from diet-induced obesity by increasing the rate of fatty acid oxidation (Feige et al., 2008; Lagouge et al., 2006). These observations confirm the important role of SIRT1 in modulating whole-body energy expenditure. Moreover, and stressing the key role of SIRT1 in the regulation of fatty acid metabolism, liver-specific SIRT1 deletion was found to induce accumulation of fatty acids in the liver (hepatic steatosis), due to, at least in part, a decline in fatty acid oxidation (Purushotham et al., 2009). Accordingly to that, SIRT1 activation has been shown to protect from high fat diet-induced hepatic steatosis (Baur et al., 2006).

1.2.3.3. Mitochondrial Biogenesis and Oxidative Phosphorylation

As discussed before, mitochondrial biogenesis is an essential process for the maintenance of mitochondrial homeostasis and therefore for the development and progression of metabolic disorders. SIRT1 regulates this process by deacetylating and activating PGC-1α (Gerhart-Hines et al., 2007; Rodgers et al., 2005). Indeed, deacetylation of PGC-1α by SIRT1 *in vivo* was shown to increase mitochondrial mass and function in several tissues, as evidenced by overexpression of SIRT1 and treatments with sirtuin activating compounds (STACs) (Baur et al., 2006; Feige et al., 2008; Lagouge et al., 2006; Minor et al., 2011). Importantly, the ability of SIRT1 to regulate PGC-1α and activate mitochondrial biogenesis plays a very important role in the skeletal muscle physiology, as mitochondrial content and activity is one of the hallmarks of fiber type. Muscle fiber type and associated metabolic and functional properties are very important to athletic ability, but its also important for insulin sensitivity in the skeletal muscle. Indeed, insulin-stimulated glucose transport is greater in skeletal muscle enriched with oxidative fibers. Accordingly, obesity-induced insulin resistance and aging are positively correlated with higher abundance of glycolytic fibers. Importantly, STACs

are able to induce a shift from glycolytic to oxidative fibers and therefore maintain muscle physiology and protect from some of the deleterious effects of obesity (Baur et al., 2006; Feige et al., 2008; Lagouge et al., 2006; Minor et al., 2011). Interestingly, CR, in addition to extending lifespan, induces mitochondrial biogenesis and also increases the expression and activity of SIRT1 (Cohen et al., 2004), suggesting that SIRT1 maybe one of the main factors responsible for the improvement in mitochondrial homeostasis observed in CR animals. Moreover, SIRT1 was recently suggested to regulate SIRT3 expression as SIRT3 expression has been linked to the activity of PGC-1α via the ERRa, which binds to the SIRT3 promoter (Giralt et al., 2011; Kong et al., 2010). SIRT3 is known to regulate mitochondrial oxidative phosphorylation through deacetylation of several subunits of the ETC complexes (Bell and Guarente, 2011), suggesting that SIRT1 might regulate mitochondrial OXPHOS through regulation of SIRT3 expression. All together these reports highlight SIRT1 as a major player in the regulation of mitochondrial homeostasis and as such, an important target for drug development for many diseases triggered by mitochondrial dysfunction.

1.2.3.4. Interplay Between SIRT1 and AMPK

AMPK is a serine/threonine kinase that has emerged as a key nutrient sensor with the ability to regulate whole body metabolism. AMPK is activated upon an increase in the AMP/ATP ratio, which reflects the low energy status of the cell, and makes AMPK an energy sensor in the cell. Upon activation, AMPK turns on catabolic pathways to restore ATP levels by promoting mitochondrial biogenesis and fatty acid oxidation (Canto and Auwerx, 2012). Importantly, AMPK is not only activated by AMP but is also regulated by phosphorylation of a threonine residue (thr172) in the catalytic domain, which is required for full enzymatic activity of AMPK. Interestingly, AMPK has many overlapping actions with SIRT1, raising the possibility of an interplay in the integration and cellular response to metabolic cues. Indeed, AMPK was found to induce SIRT1 activity by increasing NAD^+ levels (Canto et al., 2009; Canto et al., 2010). Not surprisingly, SIRT1 also regulates AMPK activity. As mentioned before the phosphorylation status of AMPK is important for its activity and is regulated by several kinases, among them the liver kinase B1 (LKB1), a serine/threonine kinase that was found to directly activate AMPK in response to energy stress (Shaw et al., 2004). SIRT1 deacetylates and activates LKB1 and thereby regulates AMPK activity (Dasgupta and Milbrandt, 2007; Hou et al., 2008; Lan et al., 2008). These findings reveal that a fine-

tuning is exerted by this two metabolic sensors in response to nutritional changes and indicate that one is probably required for the other function, adding an extra layer of regulation in the integration and cellular responses to these stimuli (Figure 1.16).

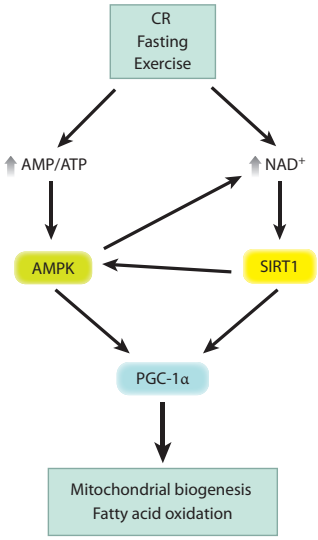


Figure 1.16 - The SIRT1-AMPK interplay. Conditions of perceived energy deprivation, such as fasting, calorie restriction, and exercise, increase the AMP/ATP ratio and activate AMPK. Energy deprivation also increases NAD^+ levels and activates the NAD^+-dependent deacetylase activity of SIRT1. Activated AMPK and SIRT1 converge by activating PGC-1α via phosphorylation and deacetylation, respectively, to induce mitochondrial biogenesis and fatty acid oxidation. Cross talk in this pathway occurs because AMPK activity increases NAD+, and SIRT1 also activates AMPK. CR – caloric restriction; AMP – adenosine monophosphate; ATP – adenosine triphosphate; AMPK – AMP-activated protein kinase; NAD^+ - nicotinamide adenine dinucleotide; PGC-1α - peroxisome proliferator-activated receptor gamma coactivator 1 alpha (From Haigis and Sinclair, 2010).

1.3. Hypoxia-inducible Factors (HIF)

The hypoxia-inducible factors (HIF) are also important regulators of cellular metabolism in response to redox status and oxygen tension. Indeed, the maintenance of oxygen homeostasis is a crucial physiological requirement that involves coordinated regulation of a plethora of genes. HIFs are responsible for a major genomic response to hypoxia, where cellular oxygen demand exceeds supply. The HIF complexes directly regulate the transcription of more than 70 genes involved in cellular processes that act to directly address this deficit by decreasing oxygen dependence and

consumption by cells, and by increasing oxygen delivery. These processes include angiogenesis, metabolism, vasodilation, cell migration and signaling. For that reason the HIF family have been one of the most studied in cancer biology, as it can act as a switch promoting the Warburg effect by reprogramming of cellular metabolism promoting a shift towards non-oxidative forms of carbon metabolism and ATP production such as anaerobic glycolysis (Semenza, 2003). Recently, however, HIFs have been reported to play a role in metabolic tissues like pancreas (Zehetner et al., 2008), adipose tissue (Krishnan et al., 2012) and cardiac tissue (Krishnan et al., 2008; Krishnan et al., 2009), raising the possibility of its involvement in the development of metabolic disorders. Indeed, obesity was shown to cause hypoxia, activating HIF in adipose tissue and regulating several aspects of obesity-induced adipose tissue dysfunction (Krishnan et al., 2012).

1.3.1. HIF Complex

HIFs are basic helix-loop-helix-PER-ARNT-SIM (bHLH-PAS) proteins that form heterodimeric complexes consisting of and O_2-labile α subunit and a stable β subunit (Figure 1.17).

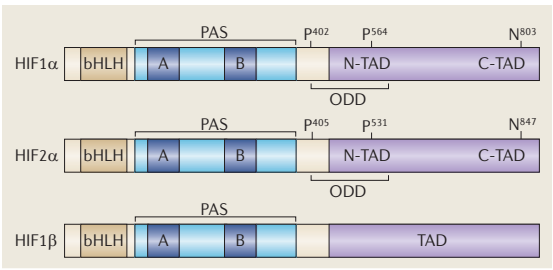


Figure 1.17 - Domain structures of HIF transcription factors. The two HIFα paralogues, HIF1α and HIF2α and common binding partner HIF1β. All contain the bHLH and PAS domain. The α subunits also contain an ODD. HIF1α – Hypoxia-inducible factor 1α; HIF2α – Hypoxia-inducible factor 2α; HIF1β – Hypoxia-inducible factor 1β; ODD – Oxygen dependent degradation domain; C-TAD – C-terminal transactivation domain; N-TAD – N-terminal transactivation domain; bHLH – basic helix-loop-helix; PAS – Per-Arnt-Sim (From Keith et al., 2012).

Mammals possess three isoforms of the HIFα subunit, of which HIF1α and HIF2α are the most structurally similar and best characterized. HIF3α exists as multiple splice variants, some of which inhibit HIF1α and HIF2α activity in a dominant-negative fashion (Kaelin and Ratcliffe, 2008). HIF1α is expressed ubiquitously in all cells,

whereas HIF2α and HIF3α are selectively expressed in certain tissues (Keith et al., 2012). HIFα subunits heterodimerize with the stable HIF1β or ARNT (aryl hydrocarbon receptor nuclear translocator) subunit, and the heterodimers recognize and bind hypoxia response elements (HREs) in the target genes. HREs are similar to enhancer box (E box) motifs and have the consensus sequence G/ACGTG (Lisy and Peet, 2008) (Figure 1.18). Importantly, the HIFα subunit interacts in a complex with the acetyltransferase p300/CBP, which is critical for HIF transcriptional activity (Lisy and Peet, 2008).

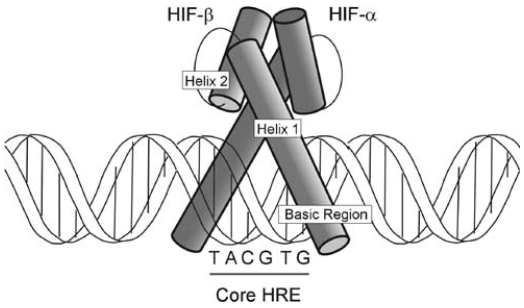


Figure 1.18 - HIF dimerization and DNA binding. Representation of the bHLH domains of the HIFα/β heterodimer contacting DNA. The basic residues near the N-terminus of each protein contact the nucleotides of the core HRE directly to form an a-helix contiguous with helix 1. HIF-α – Hypoxia-inducible factor subunit α; HIF-β – Hypoxia-inducible factor subunit β; HRE – hypoxia-responsive element (From Lisy and Peet, 2008).

Numerous studies have revealed that either HIF1α or HIF2α can regulate the expression of many of the same genes, but that each HIF1α and HIF2α also have unique targets (Keith et al., 2012). For instance, HIF2α specifically regulates particular genes involved in growth and cell cycle, whereas primarily HIF1α regulates genes involved in metabolism (Keith et al., 2012).

1.3.2. HIF1α Regulation

As stated before, HIF1α is recognized as a key modulator of the transcriptional response to hypoxic stress. As such, one of the most prominent ways of regulation of HIF1α occurs through O_2 availability. Importantly, this regulation happens at both protein stability and transcriptional level (Lisy and Peet, 2008).

HIF1α does not directly sense variations in O_2 availability. Therefore, the O_2-mediated regulation of HIF1α is orchestrated by prolyl hydroxylase domain enzymes

(PHD) (Bruick and McKnight, 2001; Epstein et al., 2001; Jaakkola et al., 2001) and asparaginyl hydroxylases (also known as factor inhibiting HIF1α; FIH1) (Lando et al., 2002), as their activity is strictly dependent on the cellular oxygen level. As such, these enzymes are the true cellular O_2-sensing molecules and through their enzymatic activity they control HIF1α transcriptional response to changes in O_2 tension. PDH hydroxylate two prolyl residues in the oxygen-dependent degradation domain (ODDD) of HIF1α. This modification specifies a rapid interaction with the tumor suppressor protein von Hippel-Lindau (VHL), a component of an E3 ubiquitin ligase (Berra et al., 2006; Maxwell et al., 2001). Subsequently, HIF1α subunits become marked with polyubiquitin chains that drive them to proteosomal degradation (Berra et al., 2003; Kallio et al., 1999). FIH1 hydroxylates HIF1α on asparagine residues in the c-teminal transactivation domain. The hydroxylation by FIH1 disrupts a critical interaction between p300/CBP and therefore impairs HIF1α transcriptional activity (Webb et al., 2009) (Figure 1.19).

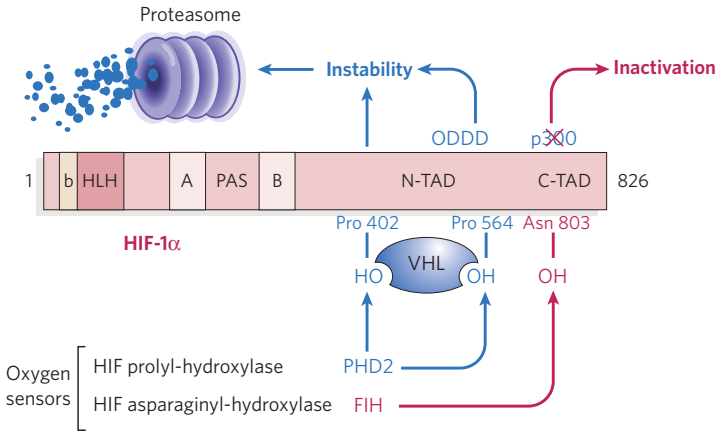


Figure 1.19 - Regulation of HIF1α by oxygen sensors. Two oxygen sensors PHD and FIH determine, respectively, the stability and activity of HIF1α. The PHDs, by hydroxylating two proline residues (402 and 564) in the ODDD, initiate the binding of a component of an E3 ubiquitin ligase, VHL protein, which marks HIF1α for destruction by the proteasome. FIH, by hydroxylating an asparagine residue in the C-TAD of HIF-1α, inhibits the binding of cofactors, such as p300, that are required for the transcription of certain HIF-dependent genes. PHD - Prolyl hydroxylase domain protein; FIH - factor inhibiting HIF1α; ODDD – Oxygen-dependent degradation domain; VHL – von Hippel-Lindau; C-TAD – c-terminal transactivation domain (From Pouyssegur et al, 2006).

Interestingly, mitochondrial metabolism also plays an important role in HIF1α regulation. Indeed, TCA cycle intermediates can modulate PHD activity as α-ketoglutarate is a substrate for PHD and succinate and fumarate can inhibit PHD activity (Boulahbel et al., 2009). Moreover, the mitochondrial ETC also plays a role in PDH regulation through ROS generation (Bell et al., 2008). In fact, production of ROS specifically from complex III of the ETC, the only complex of the ETC that can produce ROS for the intermembrane space and therefore can influence ROS levels in the cytoplasm, was shown to inhibit PHD activity and promote HIF1α stabilization (Bell et al., 2007; Chandel et al., 2000; Guzy et al., 2005) (Figure 1.20).

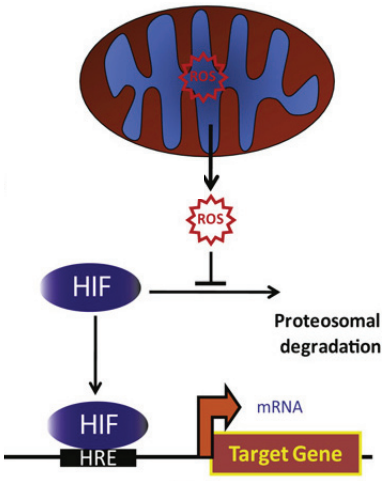


Figure 1.20 - ROS-mediated HIF1α stabilization. Mitochondria release ROS initiating a signaling cascade that results in the stabilization and activation of HIF1α and activation of HIF1α transcriptional targets. ROS – reactive oxygen species; HRE – hypoxia-responsive element; HIF; hypoxia-inducible factor (Adapted from Bell and Guarente, 2011).

O_2 sensing via hydroxylases and mitochondrial metabolism define a core feature of HIF1α regulation. However, a list of additional cues that regulate the HIF1α pathway is growing and range from microRNAs to transcription factors, oncogenic signals and redox sensors (Majmundar et al., 2010). Interestingly, among these new regulators, sirtuins were recently shown to also play a role in HIF1α regulation (Zhong and Mostoslavsky, 2011). Indeed, SIRT1 and SIRT6 were shown to modulate HIF1α transcriptional activity. SIRT1 directly interacts and deacetylates HIF1α, blocking the interaction of HIF1α with p300/CBP and thereby repressing HIF1α transcriptional activity

(Lim et al., 2010) (Figure 1.21). On the other hand, SIRT6 does not directly deacetylate HIF1α, instead SIRT6 deacetylates histones at the promoters of HIF1α target genes and thereby promotes HIF1α co-repression (Zhong et al., 2010). In addition to SIRT1 and SIRT6, SIRT3 was also been shown to regulate HIF1α, but in this case at the protein stability level. In fact, deletion of SIRT3 was shown to induce mitochondrial ROS generation and consequently lead to stabilization of HIF1α (Bell et al., 2011; Finley et al., 2011).

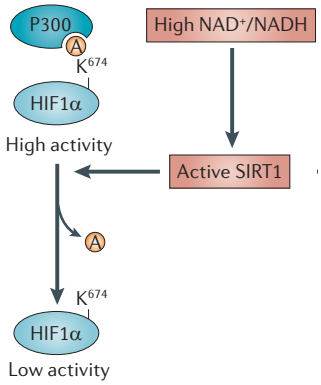


Figure 1.21 - Regulation of HIF1α by SIRT1. High levels of NAD^+ activate SIRT1, which deacetylates HIF1α in lysine 674 and prevents the recruitment of p300/CBP, leading to a decline in HIF1α transcriptional activity. NAD^+ - nicotinamide adenine dinucleotide; HIF1α – hypoxia-inducible factor 1α (Adapted from Keith et al., 2012).

1.3.3. Regulation of Metabolism by HIF1α

As mentioned before, HIF1α is a major regulator of the metabolic reprogramming that induces a shift towards glycolysis. HIF1α guides this shift by promoting the expression of glucose transporters, glycolytic enzymes and lactate dehydrogenase A (LDHA), which replenishes NAD^+ for further glycolysis (Gordan et al., 2007). Moreover, HIF1α also regulates the transcription of pyruvate dehydrogenase kinase 1 (PDK1) and thereby represses the flux of pyruvate into acetyl-CoA diverting carbon away from mitochondria (Gordan et al., 2007) (Figure 1.22).

HIF1α activity was also shown to influence the pentose phosphate pathway (PPP) (Zhao et al., 2010). The PPP converts glycolytic intermediates into ribose-5-phosphate, a substrate for nucleotide biosynthesis (Tong et al., 2009). HIF1α was shown to promote the flux of glucose carbon through a non-oxidative arm of the PPP relative to the oxidative arm (Zhao et al., 2010) and therefore redirect the metabolism of glucose for

use both as an energy source and as a building block for RNA and DNA, facilitating cell growth and survival (Figure 1.22).

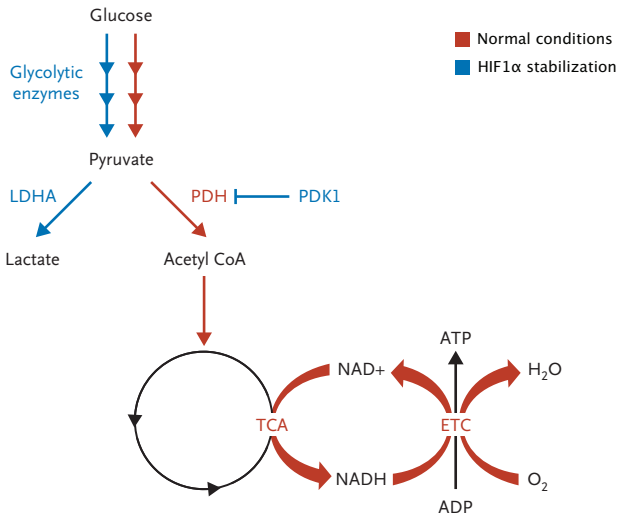


Figure 1.22 - Regulation of glucose metabolism by HIF1α. Glucose is converted to pyruvate by the action of the glycolytic enzymes. In cells under normal conditions (red pathway), pyruvate dehydrogenase converts pyruvate to acetyl CoA, which is oxidized in the mitochondrial tricarboxylic acid cycle, generating electrons that are transported through the electron transport chain and are eventually transferred to oxygen to form water. Under conditions where HIF1α is stabilized (blue pathway), pyruvate dehydrogenase kinase 1 inactivates PDH, and lactate dehydrogenase A converts pyruvate to lactate. The expression of the glycolytic enzymes is also induced to increase flux through the pathway PDH – pyruvate dehydrogenase; TCA – tricarboxylic acid cycle; ETC – electron transport chain; PDK1 – pyruvate dehydrogenase kinase 1; LDHA – lactate dehydrogenase A (Adapted from Semenza, 2011).

In addition to promoting anaerobic glycolysis, HIF1α might also play a role in the regulation of mitochondrial biogenesis through regulation of c-Myc. As discussed above, c-Myc has been recently pointed out as a potential regulator of mitochondrial biogenesis. c-Myc is a transcriptional factor that exerts its functions by forming a heterodimer with MAX and a complex with SP1. HIF1α binds to SP1 and MAX, thus disrupting c-Myc transcriptional complex and transcriptional activity (Gordan et al., 2007) (Figure 1.23). As such, by regulating c-Myc, HIF1α might also regulate mitochondrial biogenesis adding a further step into redirecting the carbon flux away from mitochondria.

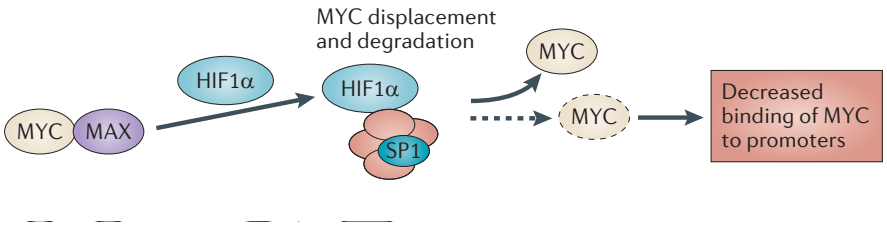


Figure 1.23 - Regulation of c-Myc by HIF1α. HIF1α decreases MYC activity owing to diminished association of MYC with MAX and SP1, and reduced stability of MYC. HIF1α – hypoxia-inducible factor 1α (Adapted from Keith et al., 2012).

1.4. Compounds that Protect from Metabolic Diseases Through Induction of Mitochondria

1.4.1. Resveratrol

Resveratrol (3,5,4'-trihydrosystilbene) (Figure 1.24) is a polyphenol found in grapes (*Vitis vinifera*), a variety of berries, peanuts and medical plants (Baur and Sinclair, 2006).

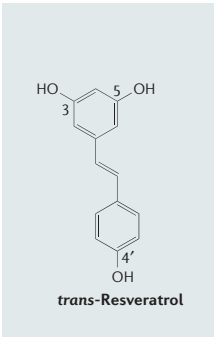


Figure 1.24 - Chemical structure of trans-Resveratrol. (Adapted from Baur and Sinclair, 2006).

Scientific interest in resveratrol has continually gained momentum since it was first demonstrated to prevent carcinogenesis in mice (Jang et al., 1997). More recently, this molecule has received considerable attention for its anti-inflammatory, anti-tumorigenic and antioxidant properties, as well as, its ability to increase lifespan in lower organisms and improve general health in mammals (Baur et al., 2006). Reports of significant life span extension in simpler laboratory organisms, combined with thousands of *in vitro* and *in vivo* studies supporting a role for resveratrol in either prevention or

treatment of chronic diseases, suggest that resveratrol may have the potential to make and impact on human health (Baur and Sinclair, 2006).

1.4.1.1. Mechanisms of Action of Resveratrol

The exact mechanism through which resveratrol exerts its wide range of beneficial effects is currently still unclear (Baur and Sinclair, 2006). Similar to most other polyphenols, resveratrol is suggested to possess intrinsic antioxidant capacity, but also has the ability to induce the expression of a number antioxidant enzymes, like superoxide dismutase and catalase, with probably both mechanisms contributing to an overall reduction in oxidative stress (Halliwell, 2007). The ability of resveratrol to induce expression of antioxidant enzymes is, at least in part, due to resveratrol's ability to activate the NF-E2- related factor 2 (Nrf2), which is a transcription factor that regulates coordinated expression of key antioxidant defenses (Ungvari et al., 2011).

Resveratrol further interacts with a large number of receptors, kinases, and other enzymes that could plausibly make a major contribution to its biological effects. Among them, resveratrol was proposed to activate SIRT1 *in vitro* (Howitz et al., 2003). In addition to that, several subsequent studies also showed that resveratrol induces SIRT1 activity in several species (Baur, 2010). Importantly, and in line with being a SIRT1 activator, resveratrol mimics numerous aspects of CR in all eukaryotes tested to date, and in most of them, the effects appear to be dependent on SIRT1 (Timmer et al., 2012). However, recently some studies reported that the direct activation of SIRT1 by resveratrol reported by Howitz and colleagues, was a result of an *in vitro* artifact of the assay used (Dasgupta and Milbrandt, 2007; Pacholec et al., 2010), and thereby raised the question of whether or not resveratrol is a SIRT1 activator (Borra et al., 2005; Kaeberlein et al., 2005).

Resveratrol was also shown to activate AMPK (Baur et al., 2006; Dasgupta and Milbrandt, 2007) by driving an imbalance in the AMP/ATP as a consequence of interference with the mitochondrial ETC (Hawley et al., 2010). Indeed a growing number of studies in genetic engineered mice and cells have further showed the critical role of AMPK in resveratrol's actions, including the ability of resveratrol to activate SIRT1 (Canto and Auwerx, 2012) (Figure 1.25). However, SIRT1 was also shown to be required for resveratrol-induced activation of AMPK through deacetylation of LKB1 in cell lines (Dasgupta and Milbrandt, 2007; Hou et al., 2008; Lan et al., 2008), suggesting that this two metabolic sensors might coordinate each other for the effects of resveratrol on

metabolism. Nevertheless, and due to the non-existence of an appropriated SIRT1 knockout animal, the role of SIRT1 in resveratrol's effects in vivo remains to be clarified. As such, more studies are necessary to untangle the relationship between AMPK and SIRT1 in resveratrol's effects *in vivo*.

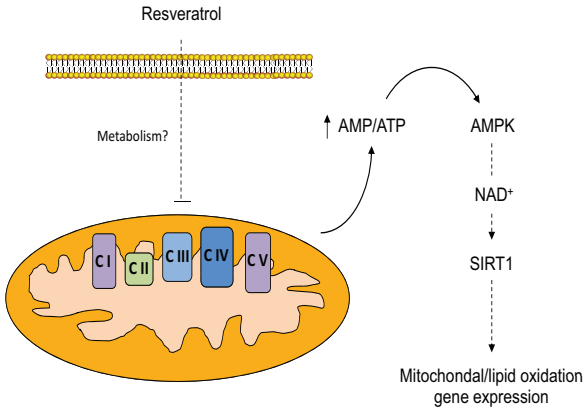


Figure 1.25 - Proposed mechanism of activation of AMPK and SIRT1 and induction of mitochondrial biogenesis by resveratrol. Resveratrol promotes mitochondrial biogenesis and lipid oxidation through indirect AMPK and SIRT1 activation. Although still a matter of debate, most data currently indicate that the metabolic actions of resveratrol or its metabolites may stem from its ability to act as a mild mitochondrial poison, impairing ATP synthesis. The energy stress induced by resveratrol activates AMPK, subsequently stimulating SIRT1 by enhancing NAD^+ levels. Then, SIRT1 activates key down-stream targets through deacetylation, ultimately leading to an adaptive potentiation of mitochondrial biogenesis and lipid oxidation pathways. AMP – adenosine monophosphate; ATP – adenosine triphosphate; AMPK – AMP-activated protein kinase; NAD^+ - nicotinamide adenine dinucleotide; CI-V - mitochondrial electron transport chain complexes I to V. (From Canto and Auwerx, 2011).

1.4.1.2. Regulation of Mitochondria by Resveratrol

As discussed above, mitochondrial biogenesis is likely to be involved in the regulation of cellular metabolism, redox regulation and signal transduction. Therefore, impairment of this process has been described to contribute to cellular imbalance and organ failure in several pathologies. Importantly, the physiological improvements induced by resveratrol in rodent models of aging and metabolic disorders were shown to be accompanied by an increase in mitochondrial content in key metabolic organs like liver, skeletal muscle and brain (Baur et al., 2006; Baur and Sinclair, 2006; Lagouge et al., 2006; Robb et al., 2008). Multiple mechanisms may explain resveratrol-induced

mitochondrial biogenesis and its contribution to maintaining mitochondrial homeostasis under disease states. Among them is the activation of SIRT1 and AMPK by resveratrol, which promotes activation of PGC-1α and therefore induces mitochondrial biogenesis (Figure 1.25).

Oxidative stress in the mitochondria is another factor that can disturb mitochondrial homeostasis. Resveratrol was shown to reduce ROS production and oxidative stress, which can be explained either by its intrinsic antioxidant effect or its ability to activate Nrf2 (Ungvari et al., 2011) and in this way protect mitochondrial homeostasis.

1.4.2. Berberine

Berberine is an isoquinoline alkaloid (Figure 1.26), which is present in many plants including *Berberis vulgaris*, *Hydrastis canadensis*, *Coptis chinensis*, *Arcanglisia flava*, *Berberis aquifolium* and *Berberis aristata* (Imanshahidi and Hosseinzadeh, 2008). This natural compound has been used in traditional chinese medicine for centuries, namely in the treatment of gastrointestinal infections (Vuddanda et al., 2010). Berberine has been reported to have multiple pharmacological effects (Vuddanda et al., 2010) that also turn this natural compound into a promising candidate in the treatment of many age-related disorders such as Alzheimer's disease (Asai et al., 2007; Zhu and Qian, 2006), cancer (Diogo et al., 2011), obesity (Kim et al., 2009; Lee et al., 2006) and diabetes (Yin et al., 2008b; Zhang et al., 2008b). Indeed, berberine is a potent insulin sensitizer and a glucose lowering-agent, providing two reasons why berberine is so efficient in the treatment of rodent models of obesity and diabetes (Lee et al., 2006, Turner et al., 2008).

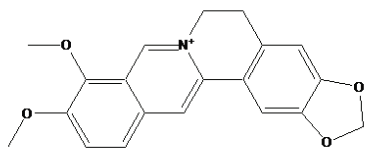


Figure 1.26 – Chemical structure of berberine.

1.4.2.1. Mechanisms of Action of Berberine

The mechanisms by which berberbine exerts its wide range of beneficial effects are broad, as berberine was found to regulate several receptors, kinases and other enzymes that regulate key signaling pathways in the cell. Indeed, berberine was found to influence several key metabolic pathways in the cell, such as: induction of glycolysis (Yin et al., 2008a) and the expression of glucose transporters (GLUT1 and GLUT4); inhibition of gluconeogenesis, at least in part due to inhibition of FOXO1, SREBP1 and chCREBP (Xia et al., 2011b); regulation of the insulin pathway through several mechanisms like increase in the expression of the insulin receptor (Zhang et al., 2010), inhibition of protein phosphatase 1b (PTP1B) (Chen et al., 2010), activation of ERK and P38 MAPK (Cheng et al., 2006; Kim et al., 2007c; Zhou et al., 2007) and mTOR inibition (Liu et al., 2010). Moreover, berberine might also exerts its beneficial effects through regulation of the inflammatory process, as inflammation has been shown to contribute to the development of several diseases like the metabolic syndrome and cancer. Indeed, berberine reduces the translocation of the nuclear factor-kappa B (NF-kB) to the nucleus, thus preventing the induction of the inflammation program that is regulated by the NF-kB (Cui et al., 2007; Lee et al., 2007). In addition, berberine was also found to interfere with the HIF1α pathway as it was found to prevent hypoxia-induced HIF1α protein stabilization (Lin et al., 2004), which may also contribute to the changes in metabolism induced by berberine.

Importantly, berberine is also known to activate AMPK (Lee et al., 2006; Turner et al., 2008; Zhou et al., 2008a), and thereby inducing the activity of a variety of nuclear receptors like PPARα, PPARγ and PGC-1α. Similar to resveratrol, it is proposed that berberine activates AMPK by inhibiting the mitochondrial ETC, more specifically Complex I of the ETC (Turner et al., 2008), leading to a decline in ATP production by the mitochondria and consequent rise in the AMP/ATP ratio.

1.4.2.2. Regulation of Mitochondria by Berberine

As referenced before, berberine is known to induce AMPK activity as well as to induce the expression of PGC-1α (Lee et al., 2006), suggesting that the beneficial effects of berberine against metabolic disorders might be mediated through increase in mitochondrial metabolism.

Interestingly, *in vitro* studies have demonstrated that high doses of berberine resulted in mitochondrial impairment mainly due to inhibition of the ETC (Turner et al.,

2008) and induction of the mitochondrial permeability transition pore (MPTP) through regulation of its constituent, ANT (Pereira et al., 2008). These effects seem to be opposite of the beneficial effect of berberine on induction of mitochondrial biogenesis, but might play a role in the anti-tumorigenic effects of berberine as it leads to mitochondrial-induced programmed cell death (apoptosis) through release of cytochrome c and consequent activation of the apoptotic program (Diogo et al., 2011). Nevertheless, further studies are required to clarify the role of berberine in mitochondrial homeostasis *in vivo* and under different conditions and disease models.

1.5. Objective

Deregulation of mitochondrial function and homeostasis has been for years associated with several metabolic diseases, as well as the aging process. In addition, SIRT1 has been shown to regulate several aspects of the cellular metabolism and protect from several diseases. As such, the idea that both regulation of mitochondrial homeostasis and SIRT1 activity are related have attracted attention. Indeed, SIRT1 is known to regulate mitochondrial biogenesis by deacetylation of PGC-1α, the “master regulator” of mitochondrial biogenesis. However, due to the fact that SIRT1 knockout animals are embryonic lethal and when outcrossed present several developmental abnormalities (Cheng et al., 2003; McBurney et al., 2003; Sequeira et al., 2008), evaluation of the role of SIRT1 in mitochondrial homeostasis *in vivo* has been a challenge. Moreover, growing evidence pointing out the existence of an alternative pathway to PGC-1α that regulates mitochondrial biogenesis lead to the hypothesis that SIRT1 might have a dual role in the regulation of mitochondrial metabolism. As such, the work presented in this thesis was conducted to on one hand, study the role of SIRT1 in mitochondrial homeostasis *in vivo* and the potential regulation of alternative mitochondrial regulatory pathways by SIRT1 (Figure 1.27). On the other hand this work also aimed to determine the importance of SIRT1 for the preservation of mitochondrial homeostasis by two natural compounds with high potential for treatment of metabolic disorders, resveratrol and berberine (Figure 1.27).

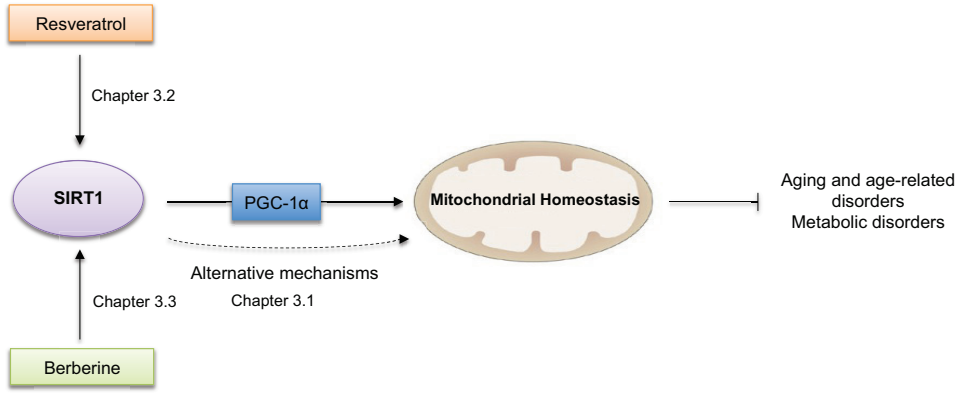


Figure 1.27 – Schematic representation of the proposed work.

Chapter 2- Experimental Procedures

2.1. Animals, Diets and Treatments

All animal studies performed in chapter 3.1 and 3.2 followed the guidelines of and were approved by the Harvard Institutional Animal Care and Use Committee. The animal studies performed in chapter 3.3. followed the guidelines of the European Directive 86/609/CEE and present in the Portuguese law.

2.1.1. Generation of a Whole Body Adult-inducible SIRT1 Knockout Mouse (chapter 3.1 and chapter 3.2)

Mice harboring a Cre-ERT2 fusion protein consisting of Cre recombinase fused to a triple mutant form of the human estrogen receptor; which does not bind its natural ligand (17β-estradiol) at physiological concentrations but binds the synthetic estrogen receptor ligands 4-hydroxytamoxifen and tamoxifen citrate, as described previously (Ruzankina and Brown, 2007). Cre-ERT2 is restricted to the cytoplasm and can only enter the nuclear compartment following treatment with tamoxifen. These mice were then crossed to SIRT1Δex4 mice (Cheng et al., 2003) to generate SIRT1Δex4ERT2 mice in which the catylic region of SIRT1 can be deleted upon treatment with tamoxifen. Cre induction was carried out by a 6 week feeding of a diet containing 360 mg/kg tamoxifen citrate in both SIRT1Δex4ERT2 and control mice. Controls included animals with a WT SIRT1 allele, with ERT2, and SIRT1Δex4 mice lacking ERT2. Following tamoxifen treatment, the efficiency of deletion in DNA from tail samples was determined by PCR and animals were maintained on the experimental diets. Mice were housed under a 12 h light/12 h dark cycle, with lights on at 7 a.m., and lights off at 7 p.m. For the fasting experiments, mice were fasted for 16 hours prior to sacrifice.

2.1.2. Aging Cohorts and NMN Treatments (chapter 3.1)

C57BL/6J mice of 3, 6, 22, 24, or 30 months of age were obtained from the National Institutes of Aging colony. Mice were acclimated for at least one week prior to sacrifice. 3 and 24 old mice were given interperitoneal injections of 500 mg NMN (Sigma-Aldrich) per kilogram body weight per day or the equivalent volume of PBS for 7 consecutive days at 5:00pm and 7:00am on day 8 and sacrificed 4h after last injection.

2.1.3. Adult-inducible SIRT1 KO Mice and Resveratrol Treatments (chapter 3.2)

Adult-inducible SIRT1 KO mice were generated as described in 2.1.1. and were maintained on experimental diets for eight months. Diets included AIN-93G standard

diet (SD), AIN-93G modified to provide 60% of calories from fat (HF), or HF diet with the addition of 0.04% resveratrol (HCR) (Baur et al., 2006), as well as, a HF diet containing 0.4% resveratrol (Lagouge et al., 2006).

2.1.4. Generation of a Whole Body SIRT1 Overexpressor Mouse (chapter 3.2)

A Cre-inducible SIRT1 transgenic mice harboring a transcriptional STOP element flanked with *loxP* sites inserted between a CAGGS promoter and the murine SIRT1 cDNA was described previously (Firestein et al., 2008). To generate constitutive SIRT1 transgenic animals (SIRT1tg), $SIRT1^{STOP}$ mice were crossed with CMV-Cre transgenic mice obtained in the C57/BL6J background from Jackson Labs (Bar Harbor, ME). SIRT1tg;CMV-Cre double transgenics were then backcrossed to C57BL/6J to outcross the CMV-Cre allele.

2.1.5. Berberine Treatments (chapter 3.3)

6-8 weeks old male Sprague Dawley rats were housed under a 12-12h light/dark cycle at 22°C and given free access to water and standard chow (Control group) or high fat diet (HFD) for 12 weeks. After the 12 weeks, a third group of rats was maintained on HFD with a supplement of berberine (100 mg/kg/day) in the drinking water for 4 more weeks (HFD + BBR). Berberine intake was monitored over the course of the study. The diets were purchased from Research Diets, Inc (New Jersey, USA). The diet used to induce obesity (HFD) has 60% kcal from fat, whereas the control diet (Ctl) has 10% kcal from fat.

2.2. Surgical Procedures, Body Composition and Glucose Tolerance Test (chapter 3.3)

Chronic indwelling catheters were implanted and the animals were monitored daily and the catheters flushed with saline. All animals were allowed to recover for 5 days. Experiments were performed on conscious, unrestrained animals fasted for 24 hours. A saline solution enriched to 70% with ^2H_2O was administered via the jugular catheter for 225 minutes to measure total body water and fat-free mass (FFM). ^2H-enrichments of plasma water and infusate precursor were quantified using ^2H NMR as described before (Jones et al., 2006; Rossetti et al., 1997). For determining ^2H-body water enrichment, a blood sample was taken at 225 minutes, or 45 minutes after the infusion was terminated. The body water pool (ml) for each animal was quantified from

the ratio of infusate to plasma body water ^{2}H-enrichment of the 225 minute sample multiplied by the total volume of infused $^{2}H_2O$. Body water mass (grams) was assumed to be equal to body water volume. FFM was calculated by dividing the body water mass by 0.73 (Rossetti et al., 1997) at mass was calculated by subtracting FFM from the total body weight. To determine insulin sensitivity and glucose tolerance a glucose tolerance test was performed, by oral gavage of 1.5 g glucose/ kg body weight. Blood samples were taken from the tail vein at several time points after the bolus injection and the concentration of glucose for each sample was measured.

2.3. Glucose, Triglycerides and Hormone Levels (chapter 3.3)

Plasma glucose was determined by a glucometer (Accu-cheK Aviva - Roche, Portugal) and by a glucose microplate assay kit (Invitrogen, Spain). Plasma insulin levels were determined by ELISA (EZRMI-13K - Millipore, Portugal). Plasma leptin and adiponectin levels were measured using a commercially available kit (Invitrogen, Spain). Triglycerides were extracted using isopropanol and quantified using a colorimetric based commercial available kit (Cromatest, Spain).

2.4. Skeletal Muscle Mitochondria Isolation (chapter 3.1, 3.2 and 3.3)

Mitochondria were isolated from skeletal muscle of both mice (chapter 3.1 and 3.2) and rats (chapter 3.3) as described previously (Frezza et al., 2007), with minor modifications. Briefly, skeletal muscle was minced in isolation buffer (250 mM Sucrose, 10 mM HEPES (pH 7.4), 1.5 mM EGTA). The tissue was then digested with 0.25% trypsin (Cellgro) for 15 minutes. After digestion, trypsin was removed and the tissue was placed back in isolation buffer and homogenized. The homogenate was then centrifuged at 2.500 rpm for 10 min at 4°C. The resulting supernatant was spun at 10.000 rpm for 10 min at 4°C to pellet mitochondria, which were resuspended in washing buffer (250 mM Sucrose, 10 mM HEPES (pH 7.4)). This last step was repeated two more times to obtain an ultra-pure mitochondrial fraction. Protein content was determined by the Bradford method calibrated with bovine serum albumin (Bradford, 1976).

2.5. Oxygen Consumption (chapter 3.2 and 3.3)

Oxygen consumption of isolated mitochondria was polarographically determined with a Clark oxygen electrode (Estabrook, 1967), as previously described (Rolo et al., 2000). Mitochondria (0.5 mg) were suspended under constant stirring, at 25 °C, in 1 mL

of standard respiratory medium (130 mM sucrose, 50 mM KCl, 5 mM MgCl2, 5 mM KH2PO4, 5 mM HEPES (pH 7.4) and 50 µM EDTA) supplemented with 2 µM rotenone. Mitochondria were energized with 5 mM succinate and State 3 respiration was induced by adding 200 nmol ADP. The oxygen consumption was also measured in the presence of 1 µM carbonylcyanide-p- trifluoromethoxyphenylhydrazon (FCCP).

2.6. Mitochondrial Membrane Potential (chapter 3.1, 3.2 and 3.3)

To monitor mitochondrial membrane potential, C2C12 cells (chapter 3.1, 3.2 and 3.3) were loaded with 6.6 µM tetramethylrhodamine methyl ester (TMRM) and incubated for 15 minutes at 37°C, in the dark. After this incubation period, the media was replaced with fresh media without the probe. TMRM is a membrane-permeable cationic fluorophore that accumulates electrophoretically in mitochondria in proportion to their mitochondrial membrane potential (Rolo et al., 2003). Fluorescence was measured for 5 minutes, after which 1 µM FCCP was added and fluorescence was recorded for an additional 5 minutes. Mitochondrial membrane potential was estimated taking into account the complete depolarization caused by FCCP. In chapter 3.1 mitochondrial membrane potential was also evaluated by fluorescence microscopy. Briefly, C2C12 cells were incubated with 100 nM TMRM for 15 minutes in the dark, after which the media was replaced and the images were acquired.

To monitor mitochondrial membrane potential in isolated mitochondria (chapter 3.1 and 3.2), 1 mg of mitochondria was loaded with 5 µM of rhodamine 123(Invitrogen) in standard respiratory medium for 15 minutes at 37°C, in the dark. After this period the media was replaced by fresh respiratory medium without the probe. Rhodamine 123 is also a cationic probe that works similarly to TMRM and is widely used to measure mitochondrial membrane potential in isolated mitochondria (Emaus et al., 1986). Fluorescence was measured for 3 minutes, after which 1 µM FCCP was added and fluorescence was recorded for an additional 3 minutes. Mitochondrial membrane potential was estimated taking into account the complete depolarization caused by FCCP.

2.7. Succinate Dehydrogenase Activity (chapter 3.3)

Succinate dehydrogenase activity was polarographically determined based on the O_2 consumption using phenazine metasulphate (PMS) as an artificial electron acceptor, as previously described (Singer, 1974). The reaction was carried out at 25°C

in 1.3 mL of standard respiratory medium (as in mitochondrial respiration) supplemented with 5 mM succinate, 2 μM rotenone, 0.1 μg antimycin A, 1 mM KCN and 0.3 mg Triton X- 100. After the addition of the sample, the reaction was initiated with 1 mM PMS.

2.8. Cytochrome c Oxidase Activity (chapter 3.1 and 3.3)

Cytochrome c oxidase activity was polarographically determined based on the O_2 consumption upon cytochrome c oxidation, as previously described (Brautigan et al., 1978). The reaction was carried out at 25°C in 1.3 mL of standard respiratory medium (as in mitochondrial respiration) supplemented with 2 μM rotenone, 10 μM oxidized cytochrome c, 0.3 mg TritonX-100. Following addition of the sample, the reaction was initiated by adding 5 mM ascorbate plus 0.25 mM tetramethylphenylene-diamine (TMPD).

2.9. ATPase Activity (chapter 3.3)

ATPase activity of the mitochondrial ATP synthase complex (or complex V) was spectrophotometrically determined at 660 nm, in association with ATP hydrolysis, as previously described (Varela et al., 2008). The reaction was carried out at 37°C, in 2 mL of reaction medium (125 mM sucrose, 65 mM KCl, 2.5 mM MgCl2 and 0.5 mM HEPES, pH 7.4). After the addition of freeze–thawed mitochondria (0.25 mg) the reaction was initiated with the addition of 2 mM Mg^{2+}-ATP, in the presence or absence of oligomycin (1 μg/mg protein). After 10 min, the reaction was stopped by adding 1 mL of 40% trichloroacetic acid and the samples centrifuged for 5 min at 3000 rpm. 2 mL of ammonium molybdate plus 2 mL H_2O were then added to 1mL of supernatant. ATPase activity was calculated as the difference in the activity in presence or absence of oligomycin.

2.10. ATP Content (chapter 3.1, 3.2 and 3.3)

Cellular ATP content in C2C12 cells, as well as, in skeletal muscle from animals used in chapter 3.1 and 3.2, was measured with a commercial kit according to the manufacturer's instructions (Roche). For chapter 3.3, endogenous mitochondrial ATP was extracted using an alkaline extraction procedure. Briefly, 1 mg of isolated mitochondria were lysed with 0.3 M perchloric acid and immediately neutralized with 10 M potassium hydroxide, the adenine nucleotides were then separated by centrifugation

and ATP was measured using a commercially available kit according to the manufacture's instruction (Sigma).

2.11. Citrate Synthase Activity (chapter 3.2 and 3.3)

Citrate synthase activity was measured as previous described (Srere et al., 1963) in both skeletal muscle tissue and C2C12 cells spectrophotometrically based on the formation of DTNB-CoA as a product of citrate synthase reaction, which has a spectrophotometric absorbance at 412 nm. Tissue extracts (20 µg) were added to a buffer containing 10 mM Tris pH8, 200 µM Acetyl CoA and 500 µM DTNB. The reaction was initiated by the addition of 1 mM oxaloacetate.

2.12. C2C12 Cell Culture conditions and treatments

C2C12 cells were used in the myoblast form for all experiments unless otherwise is stated.

2.12.1. C2C12 Cell Culture Conditions and Treatments from Chapter 3.1

C2C12 cell line (ATCC) was cultured in low glucose Dulbecco's modified eagle medium (DMEM) (Invitrogen) supplemented with 10% FBS (Invitrogen) and a mix of antibiotic and antimycotic (Invitrogen). To inhibit SIRT1, cells were treated with vehicle (0.001% DMSO) or 10 µM EX-527 (Tocris Bioscience) for 12h.

2.12.2. C2C12 Cell Culture Conditions and Treatments from Chapter 3.2

C2C12 cell line (ATCC) was cultured in low glucose Dulbecco's modified eagle medium (DMEM) (Invitrogen) supplemented with 10% FBS (Invitrogen) and a mix of antibiotic and antimycotic (Invitrogen). Cells were treated the vehicle (0.001% DMSO) or 25 µM or 50 µM resveratrol for 24h. To investigate the intracellular pathways involved in resveratrol's effects on mitochondrial function and mitochondrial biogenesis, C2C12 cells were treated with vehicle (0.001% DMSO), 1 µM of EX-527 (Tocris Bioscience) or 20 µM of Compound C (Merck) during treatment with Resveratrol.

2.12.3. C2C12 Cell Culture Conditions and Treatments from Chapter 3.3

C2C12 cell line (ATCC) was cultured in low glucose Dulbecco's modified eagle medium (DMEM) (Invitrogen) supplemented with 10% FBS (Invitrogen) and mix of antibiotic and antimycotic (Invitrogen). Differentiation of C2C12 myoblasts to myotubes

was achieved by allowing the cells to reach confluence and then replacing the FBS with 2% Horse Serum (Invitrogen) for 6 days. C2C12 cells were cultured in high glucose media (25 mM) for 96h to mimic hyperglycemic conditions or with 500 μM of Palmitate and Oleate in a ratio of 1:1 (FFAs) (37) to mimic hyperlipidemic conditions. For experiments performed with FFAs, the cells were cultured in medium containing 0.5% BSA (Sigma-Aldrich) (Koves et al., 2008). Cells treated with 25 mM glucose or FFAs were also treated with the vehicle (0.001% DMSO) or 5 μM of Berberine chloride (Sigma-Aldrich) for the entire period of treatment. To investigate the intracellular pathways involved in berberine's effects on mitochondrial function and mitochondrial biogenesis, C2C12 myotubes were treated with the vehicle (0.001% DMSO), 1 μM of EX-527 (Tocris Bioscience), or 10 μM DCHC (Sigma-Aldrich) during the same period of treatment with hyperglycemia or fatty acids. To investigate the short term effects of berberine on mitochondrial function, C2C12 cells were cultured in low glucose DMEM with the vehicle (0.001% DMSO), 5 μM and 20 μM berberine for 6, 12 and 24 hours. To further address the role of NAMPT in berberine mediated effects, C2C12 cells were treated with 5 μM berberine or 5 μM berberine plus 10 nM FK866 for 12h.

2.13. Generation of Primary Myoblasts from SIRT1 KO and PGC-1α/β KO Mice and Treatments (chapter 3.1 and 3.2)

Primary myoblasts were isolated from WT, SIRT1 KO and PGC-1α/β KO mice according to the following methodologies. Skeletal muscle was dissected from the hind limb muscles of 4 months old animals. The tissue was collected in a tube with phosphate buffered saline (Cellgro), minced and enzymatically dissociated using a solution of collagenase (Sigma-Aldrich), dispase (Roche) and calcium chloride for 2 hours at 37°C. After that, it was mechanically triturated and the cell suspension passed through a cell strainer. The filtrate was then centrifuged at 400 xg for 5 minutes, suspended in DMEM and transferred to a culture flask for 30 minutes. The non-adherent cells were removed from the flask and pelleted by centrifugation at 400 xg for 5 minutes. The pellet was suspended in growth media (F-10 nutrient mix supplemented with 2.5 ng/mL bFGF, 10 ng/mL EGF, 1 μg/mL insulin, 0.5 mg/mL fetuin. 0.4 μg/mL dexamethasone, antibiotic and antimycotic mix and 20% FBS) and plated in collagen coated culture flasks. WT and PGC-1α/β KO primary myoblasts were plated and allowed to differentiate into myotubes by replacing the media with low glucose DMEM supplemented with 2% horse serum (Sigma-Aldrich) for 4 days. To investigate the role of HIF1α in chapter 3.1. PGC-

1α/β KO primary myotubes were treated for 12 hours with 1 mM DMOG (Sigma-Aldrich) or 10 μM DFO (Sigma-Aldrich), 24h after infection with empty vector of flag-SIRT1 adenovirus or 12h after treatment with 500 mM NMN (Sigma-Aldrich). To dissect the role of SIRT1 in AMPK activation primary myoblasts from WT and SIRT1 KO mice were treated with the vehicle (0.001% DMSO) or 500 μM AICAR (Sigma-Aldrich) for 24h (chapter 3.2).

2.14. Adenoviral Infections (chapter 3.1 and 3.2)

C2C12 and primary cells were infected with an empty flag-vector or flag-PGC-1α (chapter 3.2), flag-SIRT1 (chapter 3.1 and 3.2) and flag-LKB1 (chapter 3.2) (Lan et al., 2008) adenovirus as described before (Gerhart-Hines et al., 2007). The media was replaced with fresh DMEM for an additional 48h and, after that the cells were treated as described before.

2.15. Gene Silencing in C2C12 Cells

2.15.1. SIRT1 and AMPK (chapter 3.1, 3.2 and 3.3)

SIRT1 shRNA, AMPKα1 shRNA and control shGFP lentivirus were produced by co-transfection of 293T cells with plasmids encoding Vsv-g, ∆8.9 and pLKO.1-shSIRT1 (TRCN0000039296; Open Biosystems), pLKO.1-shAMPKα (TRCN0000024000; Open Biosystems) or pLKO-shGFP using FuGENE HD in accordance with the manufacturer's protocol. Media was changed 24 hours post-transfection. Virus was harvested after 48 hours, filtered, and used to infect C2C12 cells in the presence of 5 μg/mL polybrene via spin infection (2500 rpm, 30 minutes). Selection of resistant colonies was initiated 24 hours later using 2μg/mL puromycin.

2.15.2. c-Myc, HF1α and ARNT (chapter 3.1)

c-Myc shRNA#1 (TRCN0000042517; Open Biosystems) c-Myc shRNA#2 (TRCN0000054885; Open Biosystems), HIF1α shRNA (TRCN0000054450; Open Biosystems), ARNT shRNA#1 and ANRT shRNA#2 (TRCN0000079930 and TRCN0000079931, respectively; Open Biosystems) and control shGFP lentivirus were produced by co-transfection of 293T cells with plasmids encoding psPAX2 (Addgene plasmid 12260), pMD2.G (Addgene plasmid 12259) using X-tremeGENE HP (Roche) in accordance with the manufacturer's protocol. Media was changed 24 hours post-

transfection and the virus was harvested after 48 hours, filtered and used to infect C2C12 cells in the presence of 5 μg/mL polybrene (Sigma-Aldrich) via spin infection (2500 rpm, 30 minutes). Selection of resistant colonies was initiated 24 hours later using 2 μg/mL puromycin (Invivogen).

For silencing c-Myc in HIF1α knockdown cells, non-target or RNAi targeting c-Myc (Dharmacon) was transfected using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. 24 hours after the first transfection, the transfection was repeated, to enhance the knockdown, and after 24h hours the media was replaced and the cells treated as described previously.

2.16. c-Myc Overexpression and HIF1α and HIF2α DPA in C2C12 Cells (chapter 3.1)

pMXsc-Myc (Addgene plasmid 13375) and empty vector (Addgene plasmid 18656), as well as, pBabe empty (Addgene plasmid 1764), HIF1α DPA (Addgene plasmid 19005), and HIF2α DPA (Addgene plasmid 19006) retrovirus were produced by co-transfection of 293T cells with plasmids encoding gagpol (Addgene plasmid 14887) and vsvg (Addgene plasmid 8454) using X-tremeGENE HP (Roche) in accordance with the manufacturer's protocol. Media was changed 24 hours post-transfection and the virus was harvested after 48 hours, filtered and used to infect C2C12 cells in the presence of 5 μg/mL polybrene (Sigma-Aldrich) via spin infection (2500 rpm, 30 minutes). Selection of resistant colonies was initiated 24 hours later using 2 μg/mL puromycin (Invivogen).

2.17. NAD^+ and NADH Measurement (chapter 3.1, 3.2 and 3.3)

NAD^+ and NADH from C2C12 cells were measured with a commercially available kit (BioVision) according to the manufacturer's instructions. For skeletal muscle, the tissue was grinded to powder and then homogenized in lysis buffer and passed trough a 10 Kd filter (BioVision). After, protein in the initial lysate was quantified using the Bradford method (Bradford, 1976) and the equivalent to 30 μg or 70 μg of protein in the filtered lysate was used to measure NAD^+ and NADH, respectively. The quantification was performed using a commercially available kit (BioVision) according to the manufacturer's instructions.

2.18. TFAM Overexpression in C2C12 cells Lacking SIRT1 (chapter 3.1)

To increase expression of TFAM in C2C12 cells lacking SIRT1, mouse TFAM cDNA cloned into the pIRES2-EGFP (Clontech) backbone with the EGFP cassette replaced with a hygromycin resistance cassette, was transfected using Fugene HD (Roche) in accordance with the manufacturer's instructions. Media was changed 24h post-transfection and the selection of resistant colonies was initiated 48h post-transfection using 100 µg/mL hygromycin as well as 2 µg/mL puromycin to maintain SIRT1 silenced. After selection the cells were maintained and treated as described before with the addition of hygromycin and puromycin to the media.

2.19. Electron Microscopy (chapter 3.1 and 3.2)

Skeletal muscle from mice was fixed in 2.5% glutaraldehyde and 2.5% paraformaldehyde in cacodylate buffer (Electron Microscopy Sciences) then was removed, put directly into fixative, embedded and photographed with an electron microscope (Tecnai G^2 Spirit BioTWIN). Mitochondrial area was quantified blindly with Image J software.

2.20. Mitochondrial Mass (chapter 3.3)

Mitochondrial mass was evaluated using the fluorescent probe N-nonyl acridine orange (NAO). Briefly, C2C12 cells were incubated in DMEM containing 10 nM of NAO for 30 min at 37°C in the dark. The cells were then trypsinized and resuspended in DMEM without NAO. The NAO fluorescence intensity was determined by flow cytometry on the FACSCalibur (BD Biosciences) using the 488 nm laser.

2.21. Gene Expression and mtDNA Analysis (chapter 3.1, 3.2 and 3.3)

RNA from skeletal muscle tissue and C2C12 cells were extracted with RNeasy mini kit (Qiagen) according to the instructions and quantified using the NanoDrop 1000 spectrophotometer (Thermo Scientific). cDNA was synthesized with the iSCRIP cDNA synthesis kit (BioRad) using 600 ng of RNA. Quantitative RT-PCR reactions were performed using 1 µM of primers and LightCycler® 480 SYBR Green Master (Roche) on a LightCycler® 480 detection system (Roche). Calculations were performed by a comparative method ($2^{-\Delta CT}$) using 18S as an internal control. For mtDNA analysis, total DNA was extracted with DNeasy blood and tissue kit (Qiagen). mtDNA was amplified using primers specific for the mitochondrial cytochrome c oxidase subunit 2 (COX2)

gene and normalized to genomic DNA by amplification of the ribosomal protein s18 (rps18) nuclear gene. Primers were designed using the IDT software (IDT) and the primer sequences can be found in the table 2.1 and 2.2.

2.22. Analysis of mtDNA Integrity (chapter 3.1)

Total DNA was extracted with DNeasy blood and tissue kit (Qiagen). Integrity of mtDNA was assessed using the long range PCR mediated detection method as described previously (Santos et al., 2006), using the following primer sequences:
Fwd:GCCAGCCTGACCCATAGCCATAATAT
Rev: GAGAGATTTTATGGGTGTAATGCGG

2.23. TFAM Promoter Activity (chapter 3.1)

TFAM promoter activity was evaluated using a TFAM promoter-luciferase plasmid. A fragment of the mouse TFAM promoter (1.4kb upstream of the coding sequence) was cloned into a pGL4.15 vector (Promega). Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) with Renilla as the reference 48h after transfection.

2.24. HRE and c-Myc activity (chapter 3.1)

HIF-mediated transcriptional activity was measured using an HRE-luciferase plasmid (Bell et al., 2011). c-Myc-mediated transcriptional activity was measured using a luciferase plasmid containing CDK4 Myc binding sites (Addgene plasmid 16564) and a mutated version as a negative control (Addgene plasmid 16565). The plasmids were transfected using X-tremeGENE HP (Roche) in accordance with the manufacturer's protocol. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) with Renilla as the reference 48h after transfection.

2.25. Immunoprecipitation (chapter 3.2)

C2C12 cells were lysed in a low-stringency IP buffer (0.05% NP-40, 50 mM NaCl, 0.5 mM EDTA, 50 mM Tris-HCl (pH 7.4), 10 mM nicotinamide, 1 μM trichostatin A, protease Inhibitor cocktail (Roche)). FLAG-tagged proteins were immunoprecipitated from C2C12 protein lysates with anti-FLAG M2-agarose affinity gel (Sigma-Aldrich). Immunoprecipitated material was washed five times for 20 min each in low stringency

lysis buffer, and immune complexes were resuspended in SDS-PAGE buffer (0.25 M Tris-HCl (pH6.8), 8% SDS, 30% glycerol, 0.02% bromophenol blue, 10mM dithiotreitol).

2.26. Immunoblot (chapter 3.1, 3.2 and 3.3)

Protein extracts from skeletal muscle or C2C12 cells were obtained by lysis in ice-cold lysis buffer (150 mM NaCl, 10 mM Tris HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 % Triton X-100, 0.5% NP-40) supplemented with a cocktail of protease and phosphatase inhibitors (Roche). Protein content was determined by the Bradford protein assay (Biorad), and 50 µg of protein was run on SDS-PAGE under reducing conditions. The separated proteins were then electrophoretically transferred to a polyvinylidene difluoride membrane (Perkin-Elmer). Proteins of interest were revealed with specific antibodies: anti-TFAM (1:1000; Aviva biosciences), anti-COX2 (1:500; Mitosciences), anti-COX4 (1:10000; Mitosciences), anti-SIRT1 (1:4000; Sigma-Aldrich), anti-β-tubulin (1:10000; Sigma-Aldrich), anti-HIF1α (1:500; Cayman), anti-HA (1:5000; Covance), anti-c-Myc (1:1000; Cell Signaling) anti-phospho-AMPKα (Thr172) (1:1000; Cell Signaling), anti-AMPKα (1:1000; Cell Signaling), anti-acetyl lysine (1:1000; Cell Signaling), anti-GAPDH (1:5000; Millipore), anti-MyHCIIa (1:1000; Developmental Studies Hybridoma Bank, University of Iowa), anti-MyHCIIb (1:1000; Developmental Studies Hybridoma Bank, University of Iowa), anti-LKB1 (1:1000; Abcam), anti-PGC-1α H-300 (1:500; Santa Cruz), anti-NRF-1 (1:500; Abcam) and anti-β-Actin (1:5000; Sigma-Aldrich) overnight at 4ºC. The immunostaining was detected using horseradish peroxidase–conjugated anti-rabbit or anti-mouse immunoglobulin for 1 h at room temperature. Bands were revealed using Amersham ECL detection system (GE Healthcare) and quantitated by densitometry using Image J.

2.27. Immunohistochemical Analysis (chapter 3.2)

Immunohistochemical analysis was performed on four-micron thick paraffin sections, deparaffinized in histoclear (National Diagnostics, HS-200) followed by rehydration through a graded series of alcohol. Following rehydration, antigen retrieval (microwaving) was done in citrate buffer (pH 6.0) for 10 min followed by cooling for 20 min and blocking of endogenous peroxidase with hydrogen peroxide. Slides were incubated overnight at 4^{o}C with the primary antibody antimyosin type 2A (sc71) (mouse; 1:3, Developmental Studies, Hybridoma Bank, University of Iowa, Department of Biology). Following washing with PBS, slides were incubated with biotinylated

antimouse secondary antibody (Vector Laboratories, BA-2001) at 37^{o} C for 1 hour, washed three times with PBS (5 min each) and further subjected to HRP-conjugated treatment (Vector Laboratories, Vectastain PK6101) for 1 hour at 37^{o}C temperature. Color development was done with diaminobenzidine (Dako K3468) as the chromogen, slides were counterstained with hematoxylin, mounted, coverslipped and examined under the microscope for further analysis.

2.28. Statistical Analysis

Data were analyzed by a two-tailed unpaired Student's t-test. All data are reported as mean ± SEM. Statistical analysis was performed using Excel software.

Table 2.1. Mouse primers used for PCR analysis.

Gene		Primer Sequence	Ta(ºC)
PGC-1α	Forward	CACCAAACCCACAGAAAACAG	60
	Reverse	GGGTCAGAGGAAGAGATAAAGTTG	
PGC-1β	Forward	GGTGTTCGGTGAGATTGTAGAG	60
	Reverse	GTGATAAAACCGTGCTTCTGG	
NRF-1	Forward	AATGTCCGCAGTGATGTCC	60
	Reverse	GCCTGAGTTTGTGTTTGCTG	
NRF-2	Forward	TGAAGTTCGCATTTTGATGGC	60
	Reverse	CTTTGGTCCTGGCATCTCTAC	
TFAM	Forward	CACCCAGATGCAAAACTTTCAG	60
	Reverse	CTGCTCTTTATACTTGCTCACAG	
TFB1M	Forward	ATAGAGCCCAAGATCAAGCAG	60
	Reverse	TGTAACAGCCTTCCAGTGC	
TFB2M	Forward	ACCAAAACCCATCCCGTC	60
	Reverse	TCTGTAAGGGCTCCAAATGTG	
NDUFS8	Forward	GTTCATAGGGTCAGAGGTCAAG	60
	Reverse	TCCATTAAGATGTCCTGTGCG	
SDHb	Forward	ACCCCTTCTCTGTCTACCG	60
	Reverse	AATGCTCGCTTCTCCTTGTAG	
Uqcrc1	Forward	ATCAAGGCACTGTCCAAGG	60
	Reverse	TCATTTTCCTGCATCTCCCG	
COX5b	Forward	ACCCTAATCTAGTCCCGTCC	60
	Reverse	CAGCCAAAACCAGATGACAG	
ATP5a1	Forward	CATTGGTGATGGTATTGCGC	60
	Reverse	TCCCAAACACGACAACTCC	
NDUFAB1	Forward	GGACCGAGTTCTGTATGTCTTG	60
	Reverse	AAACCCAAATTCGTCTTCCATG	
SDHd	Forward	CTTGAATCCCTGCTCTGTGG	60
	Reverse	AAAGCTGAGAGTGCCAAGAG	
Uqcrc2	Forward	TTCCAGTGCAGATGTCCAAG	60
	Reverse	CTGTTGAAGGACGGTAGAAGG	

COX6a1	Forward	GTTCGTTGCCTACCCTCAC	60
	Reverse	TCTCTTTACTCATCTTCATAGCCG	
ATP5b1	Forward	CCGTGAGGGCAATGATTTATAC	60
	Reverse	GTCAAACCAGTCAGAGCTACC	
ND1	Forward	TGCACCTACCCTATCACTCA	60
	Reverse	GGCTCATCCTGATCATAGAATGG	
Ctyb	Forward	CCCACCCCATATTAAACCCG	60
	Reverse	GAGGTATGAAGGAAAGGTATAAGGG	
COX1	Forward	CCCAGATATAGCATTCCCACG	60
	Reverse	ACTGTTCATCCTGTTCCTGC	
ATP6	Forward	TCCCAATCGTTGTAGCCATC	60
	Reverse	TGTTGGAAAGAATGGAGTCGG	
ND2	Forward	ATACTAGCAATTACTTCTATTTTCATAGGG	60
	Reverse	GAGGGATGGGTTGTAAGGAAG	
ND3	Forward	AAGCAAATCCATATGAATGCGG	60
	Reverse	GCTCATGGTAGTGGAAGTAGAAG	
ND4	Forward	CATCACTCCTATTCTGCCTAGC	60
	Reverse	CCAACTCCATAAGCTCCATACC	
ND4l	Forward	CCAACTCCATAAGCTCCATACC	60
	Reverse	GATTTTGGACGTAATCTGTTCCG	
ND5	Forward	ACGAAAATGACCCAGACCTC	60
	Reverse	GAGATGACAAATCCTGCAAAGATG	
ND6	Forward	TGTTGGAGTTATGTTGGAAGGAG	60
	Reverse	CAAAGATCACCCAGCTACTACC	
COX2	Forward	AGTTGATAACCGAGTCGTTCTG	60
	Reverse	CTGTTGCTTGATTTAGTCGGC	
COX3	Forward	CGTGAAGGAACCTACCAAGG	60
	Reverse	CGCTCAGAAGAATCCTGCAA	
ATP8	Forward	GCCACAACTAGATACATCAACATG	60
	Reverse	TGGTTGTTAGTGATTTTGGTGAAG	
HIF1α	Forward	GAACATCAAGTCAGCAACGTG	60
	Reverse	TTTGACGGATGAGGAATGGG	
ARNT	Forward	CGAGAATGGCTGTGGATGAG	60

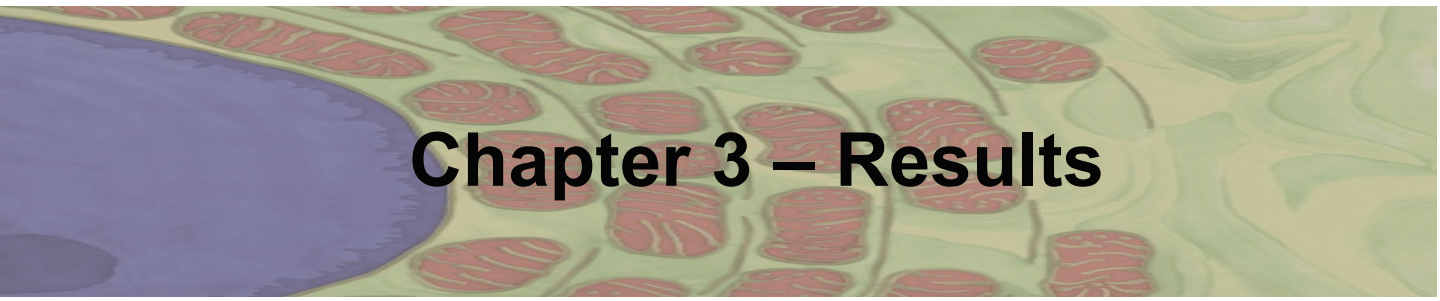
	Reverse	GGATGGTGTTGGACAGTGTAG	
LDHA	Forward	GCTCCCCAGAACAAGATTACAG	60
	Reverse	TCGCCCTTGAGTTTGTCTTC	
HK2	Forward	TCAAAGAGAACAAGGGCGAG	60
	Reverse	AGGAAGCGGACATCACAATC	
GLUT1	Forward	TGCAGCCCAAGGATCTCTCT	60
	Reverse	CGGCTTGCCCGAGATCT	
PKM	Forward	CCATTCTCTACCGTCCTGTTG	60
	Reverse	TCCATGTAAGCGTTGTCCAG	
VEGFa	Forward	GGCAGCTTGAGTTAAACGAAC	60
	Reverse	TGGTGACATGGTTAATCGGTC	
PDK1	Forward	GACTGTGAAGATGAGTGACCG	60
	Reverse	CAATCCGTAACCAAACCCAG	
PGK-1	Forward	AACCTCCGCTTTCATGTAGAG	60
	Reverse	GACATCTCCTAGTTTGGACAGTG	
PFKM	Forward	GATGGCTTTGAGGGTCTGG	60
	Reverse	CTTGGTTATGTTGGCACTGATC	
MCAD	Forward	TGTTAATCGGTGAAGGAGCAG	60
	Reverse	CTATCCAGGGCATACTTCGTG	
LCAD	Forward	GGTGGAAAACGGAATGAAAGG	60
	Reverse	GGCAATCGGACATCTTCAAAG	
CPT1b	Forward	CCTCCGAAAAGCACCAAAAC	60
	Reverse	GCTCCAGGGTTCAGAAAGTAC	
GLUT4	Forward	ACCGGATTCCATCCCACAAG	60
	Reverse	GCTCCAGGGTTCAGAAAGTAC	
RSP18 (mtDNA)	Forward	TGTGTTAGGGGACTGGTGGACA	60
	Reverse	CATCACCCACTTACCCCCAAAA	
COX2 (mtDNA)	Forward	ATAACCGAGTCGTTCTGCCAAT	60
	Reverse	TTTCAGAGCATTGGCCATAGAA	

Ta, Temperature of annealing

Table 2.2. Rat primers used for PCR analysis.

Gene		Primer Sequence	Ta(ºC)
PGC-1α	Forward	CTGCTCTTGAGAATGGATATACTT	58
	Reverse	CATACTTGCTCTTGGTGGAA	
TFAM	Forward	AAATGGCTGAAGTTGGGCGAAGTG	59
	Reverse	AGCTTCTTGGGCGAAGTG	
COX4	Forward	GGCAGAATGTTGGCTACC	59
	Reverse	GCATAGTCTTCACTCTTCACAA	
COX2	Forward	CCAGTATTAGCAGCAGGTATC	58
	Reverse	CCGAAGAATCAGAATAGGTGTT	
Troponin1 fast	Forward	CTGAGGGGCAAGTTCAAGAG	58
	Reverse	ATCTTCTTCCGACCCTCCAT	
Troponin1 slow	Forward	GACTGGAGGAAGAACGTGGA	58
	Reverse	GCTTGAACCCAAGAGAGCTG	
MyHCIIa	Forward	CTGAGGAACAATCCAACACAAA	60
	Reverse	TCTCTTCGGTCATTCTACAGCA	
MyHCIIb	Forward	AGAAAATCAAGAAGCAAATTGACC	58
	Reverse	ACTCCACTACTCTGAGGTGGTTTC	
Actin	Forward	CACCCGCGAGTACAACCTTC	59
	Reverse	CCCATACCCACCATCACACC	

Ta, Temperature of annealing



Chapter 3 – Results

3.1. SIRT1 regulates mitochondrial homeostasis by maintaining the balance between nuclear and mitochondrial-encoded ETC components trough a HIF1α-c-Myc pathway

3.1.1. Summary

Ever since eukaryotes subsumed the bacterial ancestor of modern mitochondria, the nuclear and the mitochondrial genomes have had to coordinate their activities. Hundreds of mitochondrial genes have been transferred to the nuclear genome, where regulation is better integrated. However, the mitochondrial genome still encodes rRNA, tRNA and 13 subunits of the ETC. Maintaining the stoichiometry of ETC subunits is, therefore, a considerable challenge for the cell but one that is essential for health. Interestingly, a decline in mitochondrial function is known to occur as a result of obesity, as well as, aging and may play a prominent role in the development of many age-related diseases including metabolic syndrome and neurodegeneration. However, the precise cause of this dysfunction remains unknown. Using an adult inducible SIRT1 knockout mouse we have shown for the first time that SIRT1 has a dual role in the regulation of mitochondrial homeostasis. As reported, SIRT1 deacetylates and activates PGC-1α, under fasting conditions, and thereby regulates mitochondrial biogenesis. Additionally, we demonstrate that SIRT1 regulates mitochondrial function in a PGC-1α independent manner, through specific regulation of mtDNA and the expression of mitochondrial-encoded genes by regulation of c-Myc-mediated TFAM expression through HIF1α stability. Moreover, we demonstrate that this alternative pathway plays an important role in the disruption of mitochondrial homeostasis with aging and show that increasing NAD^+ levels in old animals, can rescue mitochondrial homeostasis through a SIRT1-HIF1α pathway. These findings emphasize the potential for molecules capable of elevating NAD^+ or activating SIRT1 to treat a number of age related diseases associated with disruption of mitochondrial homeostasis.

3.1.2. Introduction

Aging is the progressive accumulation of changes with time, that are associated and/or responsible for increasing susceptibility to diseases, like Alzheimer's diseases, diabetes, cancer, among others. Interestingly, many of the age-related diseases are indeed metabolic disorders. Therefore, deregulation in metabolic

homeostasis is very likely to underlie the development of the aging process, as well as age-related disorders. Mitochondria play an important role in the aging process because of their ability to respond to cellular nutrient and redox status, and control critical cellular processes including energy production, ROS generation, and induction of apoptosis (Wallace et al., 2010). Mitochondrial function has been found to decline progressively with age in a number of tissues including skeletal muscle. In addition, it has been suggested that the progressive decline of the mitochondrial function with age may play a direct role in the development of cellular dysfunction with age (Figueiredo et al., 2008; Figueiredo et al., 2009; Hartmann et al., 2011; Lanza and Nair, 2010). Consistent with this, impaired mitochondrial function in mice with decreased proofreading capacity of the mitochondrial Polγ results in a premature aging phenotype (Trifunovic et al., 2005; Trifunovic et al., 2004; Vermulst et al., 2008) pointing out the role of mitochondrial metabolism as a key player in the development of the aging process.

Importantly, to maintain mitochondrial homeostasis, the nuclear and mitochondrial genomes have to coordinate their activities due to the fact that the mitochondrial proteome is encoded in both genomes (Wallace, 2010; Scarpulla, 2011b). This assumes particular importance for the ETC, as 13 of its subunits are encoded by the mtDNA. Mantaining the stoichiometry of the ETC complexes is, therefore, a considerable challenge for the cell, but one that is essential for health. Surprisingly, little is known about how the nuclear and mitochondrial genomes communicate and whether this communication breaks down during aging (Finley and Haigis, 2009). Indeed, and despite the interest in mitochondria as a regulator of aging, much remains unknown about how and why mitochondrial function declines with age.

Restriction of caloric intake by 30-40%, the best established intervention to delay aging and age-related disorders, also induces mitochondrial biogenesis (Cerqueira et al., 2011; Choi et al., 2011; Civitarese et al., 2007; Lopez-Lluch et al., 2006b; Nisoli et al., 2005) and prevents age related declines in mitochondrial function (Niemann et al., 2010). While the exact mechanism by which CR extends longevity remains to be determined, many of the beneficial effects are thought to be mediated through several interconnected pathways including sirtuins (Kenyon, 2010). Furthermore, mammalian sirtuins have been shown to protect against several age-related diseases (Haigis and Sinclair, 2010). Indeed, SIRT1 expression is elevated in a number of tissues following CR (Cohen et al., 2004) and overexpression or pharmacological activation of SIRT1 reproduces many of the health benefits of CR, including protection from derangements

in glucose homeostasis (Banks et al., 2008; Baur et al., 2006; Bordone et al., 2007; Lagouge et al., 2006; Milne et al., 2007; Pfluger et al., 2008), cardiovascular disease (Zhang et al., 2008a), cancer (Herranz et al., 2010b; Oberdoerffer et al., 2008) and neurodegeneration (de Oliveira et al., 2010; Donmez et al., 2010; Qin et al., 2006; Srivastava and Haigis, 2011). Consistent with SIRT1 being an important regulator of mitochondrial biogenesis, due to its ability to deacetylate and activate PGC-1α (Rodgers et a, 2005; Gerhart-Hines et al, 2007), pharmacological activators of SIRT1, resveratrol and SRT1720, were found to increase mitochondrial mass (Baur et al., 2006; Lagouge et al., 2006; Minor et al., 2011). These findings suggest that SIRT1 may play an important role in the maintenance of mitochondrial homeostasis in aging and disease.

HIF1α, is a transcription factor which is known to induce a number of important changes necessary for maintaining cellular energy in response to hypoxia (Cadenas et al., 2010) and is regulated in response to redox status and oxygen tension in the cell. HIF1α promotes a reprogramming in metabolism redirecting the cells to non-oxidative forms of carbon metabolism, and therefore, away from mitochondrial oxidative phosphorylation. Interestingly, HIF1α has been shown to be regulated by SIRT1 (Lim et al., 2010). Indeed, SIRT1 was shown to interact with and deacetylate HIF1α, inhibiting HIF1α transcriptional activity in hypoxic conditions (Lim et al., 2010). HIFs have previously been shown to play an important role in regulation of lifespan in C. elegans and may be involved in the effects of CR (Chen et al., 2009; Leiser and Kaeberlein, 2010). In addition, HIF1α was also found to be involved in the development of some diseases like cardiac hypertrophy (Krishnan et al, 2008; Krishnan et al, 2009) and obesity (Krishnan et al, 2012). Importantly, disturbances in mitochondria, as well as decline of SIRT1 activity, are commonly associated with the development of both these disorders and aging, and also play an important role in the beneficial effects of CR. Moreover, HIF1α was also shown to regulate c-Myc transcriptional activity (Koshiji et al., 2004), which as been pointed out as a potential regulator of mitochondrial biogenesis. Together, these reports raise the question of whether HIF1α may be involved in the regulation of mitochondrial homeostasis by SIRT1.

Both SIRT1 activity and mitochondrial homeostasis are critical for the maintenance of cellular function and therefore their declines may play important roles in the development of aging and metabolic diseases. However, due to the poor survival, impaired growth, and developmental abnormalities caused by germline deletion of SIRT1 (Cheng et al., 2003; McBurney et al., 2003; Sequeira et al., 2008), the effect of

loss of SIRT1 on mitochondrial homeostasis *in vivo* remains to be explored. To circumvent these problems, we generated a whole body adult-inducible SIRT1 knockout mouse and used it to study the role of SIRT1 in mitochondrial biology *in vivo*, as well as the underlying mechanisms of regulation.

3.1.3. Results

3.1.3.1. SIRT1 Knockout Induces Mitochondrial Dysfunction and Imbalance Between Nuclear and Mitochondrial-Encoded Genes

SIRT1 has previously been shown to play an important role in the regulation of PGC-1α and induction of mitochondrial biogenesis (Gerhart-Hines et al., 2007; Rodgers et al., 2005). In this study, we sought to determine the role of SIRT1 in the maintenance of mitochondrial function *in vivo* using an adult inducible SIRT1 knockout mouse model (SIRT1 KO). This new animal model was developed by backcrossing the tamoxifen-inducible Cre-ERT2 mice (Ruzankina et al, 2007) with the floxed SIRT1ΔE4 mice (Cheng et al, 2003), creating a mouse that upon tamoxifen treatment has the exon 4 of the SIRT1 gene excised, and therefore has a catalytic inactive form or SIRT1. This mouse model showed efficient excision of the catalytic domain of SIRT1 across a variety of tissues and appears grossly normal and healthy beyond 1 year of age (Figure 3.1.1).

Figure 3.1.1 – Tamoxifen feeding results in efficient deletion of the SIRT1 exon 4 and no phenotypical changes. (A) Photo of wild type and SIRT1 KO mice. (B and C) Representative immunoblot fro SIRT1 and tubulin in (B) skeletal muscle and heart and (C) Liver, white adipose tissue (WAT) and Brain of WT and SIRT1 KO mice.

Mitochondria isolated from skeletal muscle of SIRT1 KO animals showed a decline in mitochondrial oxidative phosphorylation as shown by decreased mitochondrial membrane potential, ATP levels and COX activity (Figure 3.1.2.A-C).

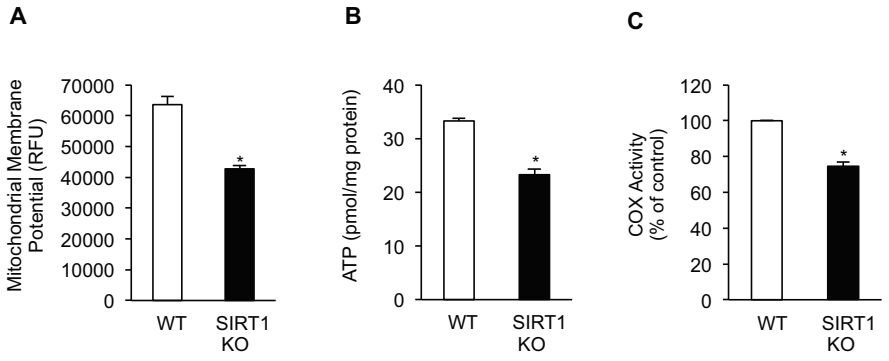


Figure 3.1.2 – Loss of SIRT1 causes a decline in mitochondrial function in skeletal muscle. (A) Mitochondrial membrane potential of isolated mitochondria from skeletal muscle of WT and SIRT1 KO mice (n=4). (B) ATP content from gastrocnemius of WT and SIRT1 KO mice (n=4). (C) Cytochrome c Oxidase (COX) activity in gastrocnemius of WT and SIRT1 KO mice (n=5). Values are expressed as mean ± SEM (*$p<0.05$ versus WT animals).

Surprisingly, this was not correlated with changes in mitochondrial mass, as quantification of EM images did not reveal any differences in the size or number of mitochondria between SIRT1 KO and wildtype (WT) animals (Figure 3.1.3A). Similarly, the expression of nuclear encoded components of the ETC was not altered in SIRT1 KO animals (Figure 3.1.3B).

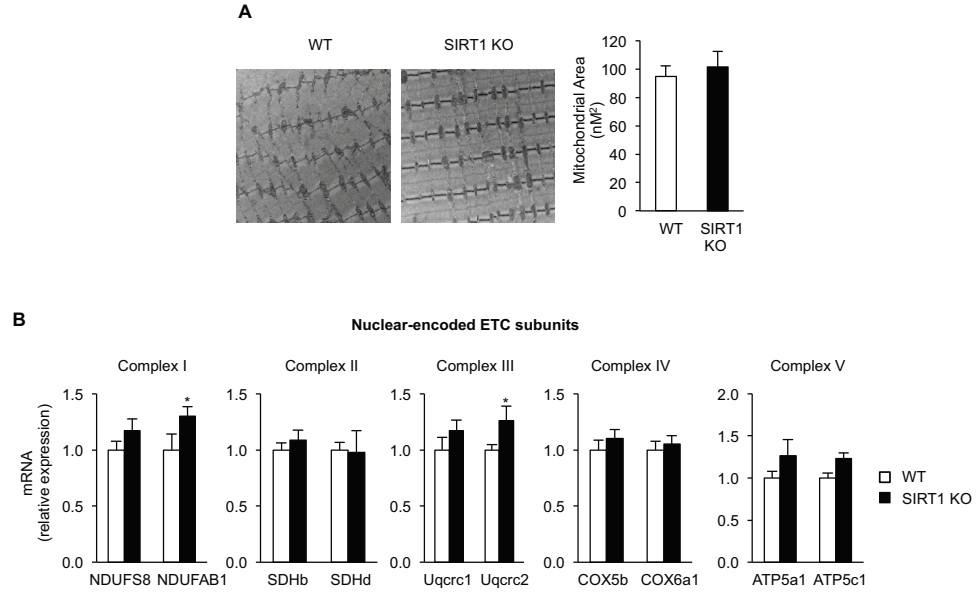


Figure 3.1.3 – Loss of SIRT1 does not affect mitochondrial biogenesis and mass. (A) Electronic microscopy analysis of gastrocnemius from WT and SIRT1 KO mice and the respective mitochondrial area quantification (n=4). (B) NDUFS8, NDUFAB1, SDHb, SDHd, Uqcrc1, Uqcrc2, COX5b, Cox6a1, ATP5a1, ATPb1 mRNA analyzed by qPCR in gastrocnemius of WT and SIRT1 KO mice. Relative expression values were normalized to WT mice (n=4). Values are expressed as mean ± SEM (*p<0.05 versus WT animals).

On the other hand, mtDNA content and the expression of all the 13 genes of the ETC encoded by the mitochondrial DNA was reduced (Figure 3.1.4A and 3.1.4B), generating an imbalance between nuclear and mitochondrial-encoded subunits. In a similar manner, protein content of the nuclear-encoded COX4 was unaltered while the protein content of the mitochondrial-encoded COX2 was reduced in the muscle of animals lacking SIRT1 (Figure 3.1.4C).

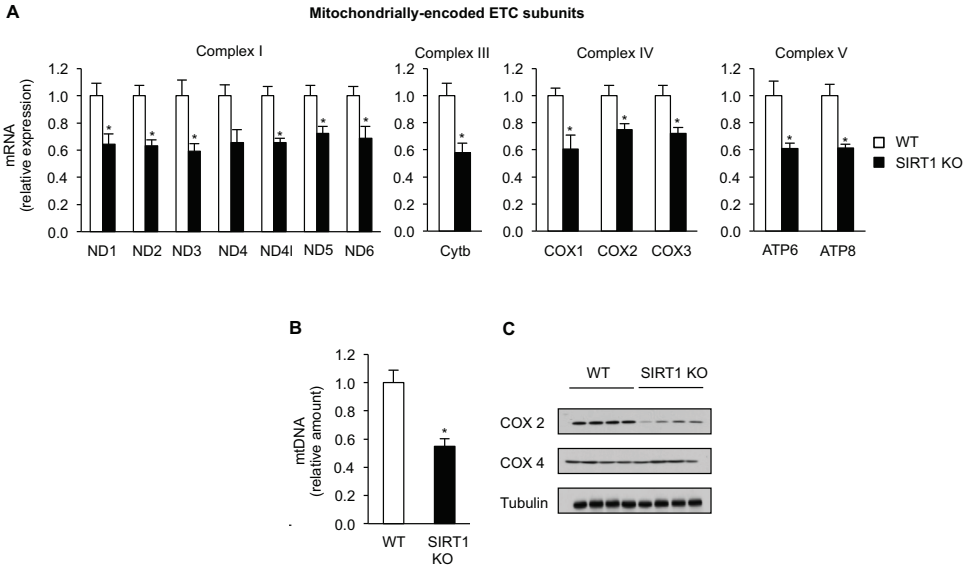


Figure 3.1.4 - Loss of SIRT1 causes a specific decrease of mitochondrial-encoded genes resulting in an imbalance between nuclear-encoded and mitochondrial-encoded ETC subunits. (A) ND1, ND2, ND3, ND4, ND4l, ND5, ND6, Cytb, COX1, COX2, COX3, ATP6, and ATP8 mRNA analyzed by qPCR in gastrocnemius of WT and SIRT1 KO mice. Relative expression values were normalized to WT mice (n=4). (B) Mitochondrial DNA content analyzed by qPCR in gastrocnemius of WT and SIRT1 KO mice Relative amount was normalized to WT mice (n=4). (C) Representative immunoblot for COX2 and COX4 in gastrocnemius of WT and SIRT1 KO mice. Values are expressed as mean ± SEM (*p<0.05 versus WT animals).

3.1.3.2. Aging Induces Mitochondrial Dysfunction and an Imbalance Between Nuclear and Mitochondrial-Encoded Genes Similarly to SIRT1 KO Mice

As SIRT1 has been found to regulate aging in lower organisms and its activity declines in response to reduced NAD^+ levels in a variety of tissues (Braidy et al., 2011), we next sought to determine whether the impaired mitochondrial function apparent in our SIRT1 KO mice was similar to that observed in old animals. Consistent with previous work (Figueiredo et al., 2008), a progressive decline in mitochondrial function was observed with age as evidenced by a decline in mitochondrial membrane potential, ATP content and COX activity evident in 22 month old mice and further exacerbated by 30 months of age (Figure 3.1.5A-C).

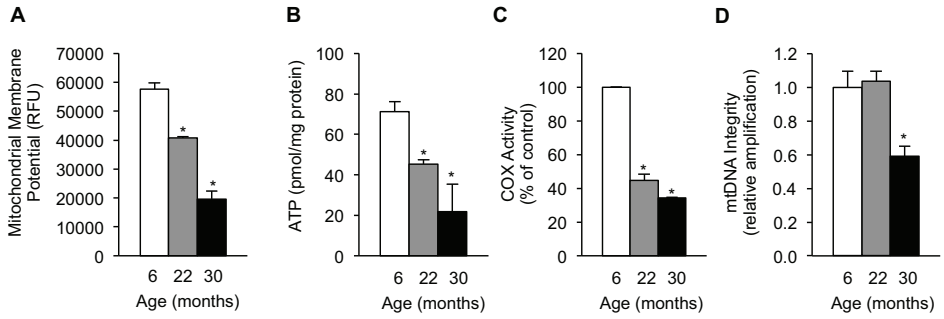


Figure 3.1.5 – Aging leads to a progressive decline in mitochondrial function (A) Mitochondrial membrane potential of isolated mitochondria from skeletal muscle of 6, 22, and 30 month old mice (n=4). (B) ATP content from gastrocnemius of 6, 22, and 30 month old mice (n=5). (C) Cytochrome c Oxidase (COX) activity in gastrocnemius of 6, 22, and 30 month old mice (n=4). (D) Mitochondrial DNA integrity in gastrocnemius of 6, 22, and 30 month old mice. Relative amount was normalized to 6 month old mice (n=5). Values are expressed as mean ± SEM (*$p<0.05$ versus 6 month old animals).

Age-related mitochondrial dysfunction has, for many years, been suggested to be a result primarily of increased in mtDNA damage. However, mtDNA damage was not found to be significantly elevated in 22 month old animals; while at 30 months of age, mtDNA integrity was reduced. (Figure 3.1.5D). This data, together with previous reports (Andziak and Buffenstein, 2006; Andziak et al., 2006; Howes, 2006), suggests that other factors may be primarily responsible for the age-related development of mitochondrial dysfunction.

Interestingly, while basal levels of nuclear-encoded ETC components were not found to be impaired until 30 months of age, mitochondrial-encoded components of the ETC, and mtDNA copy number were reduced by 22 months of age (Figure 3.1.6).

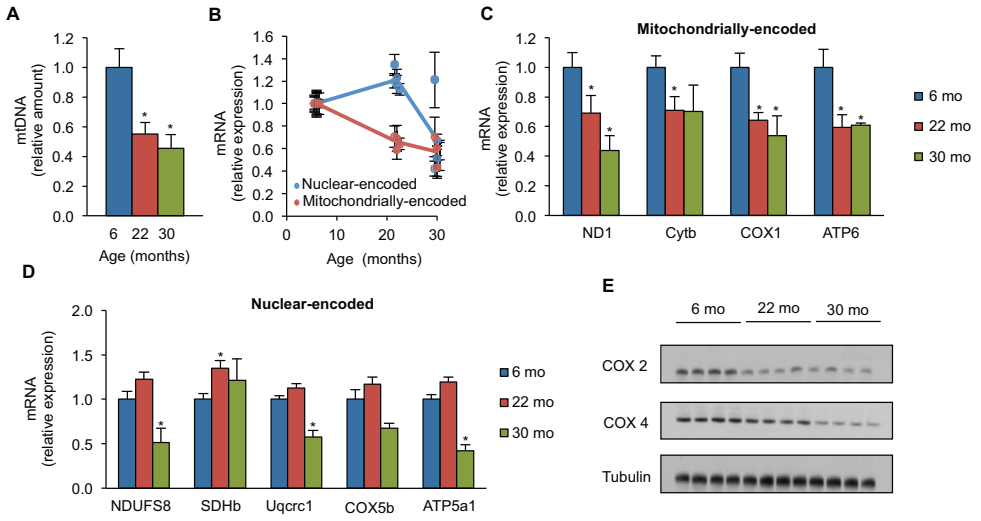


Figure 3.1.6 - Aging leads to imbalance in nuclear and mitochondrial-encoded ETC genes in skeletal muscle. (A) Mitochondrial DNA content analyzed by qPCR in gastrocnemius of of 6, 22, and 30 month old mice. Relative amount was normalized to 6 month old mice (n=5). (B) Relative expression of nuclear-encoded (NDUFS8, SDHb, Uqcrc1, COX5b, ATP5a1) versus mitochondrially-encoded ETC genes (ND1, Cytb, COX1, ATP6) analyzed by qPCR in gastrocnemius of of 6, 22, and 30 month old mice. (C and D) NDUFS8, SDHb, Uqcrc1, COX5b, ATP5a1 (C) ND1, Cytb, COX1, and ATP6 (D) mRNA analyzed by qPCR in gastrocnemius of 6, 22, and 30 month old mice. Relative expression values were normalized to 6 month old mice (n=5). (E) Representative immunoblots for COX2 and COX4 in gastrocnemius of 6, 22, and 30 month old mice. Values are expressed as mean ± SEM (*p<0.05 versus 6 month old animals).

As this data resembled what was observed in the skeletal muscle of the SIRT1 KO mice, we sought to evaluate if SIRT1 expression/activity was altered in old animals. Importantly, although SIRT1 expression levels were not found to change, NAD^+ levels were reduced at 22 and 30 months of age (Figure 3.1.7). This suggests that an imbalance between nuclear and mitochondrial-encoded genes, possibly caused by a decline in SIRT1 activity, might play an important role in the development of age-related mitochondrial dysfunction in skeletal muscle.

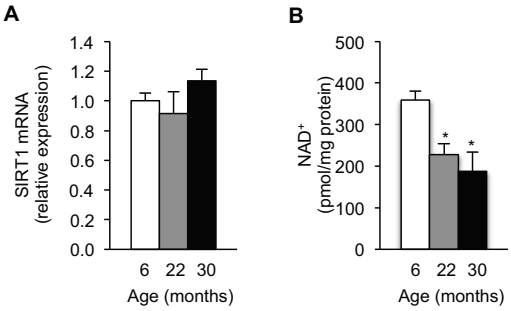


Figure 3.1.7 - Aging leads to a decline in NAD^+ in skeletal muscle. (A) SIRT1 mRNA analyzed by qPCR in gastrocnemius of 6, 22, and 30 month old mice. Relative expression values were normalized to 6 month old mice (n=5). (B) NAD^+ levels in gastrocnemius of 6, 22, and 30 month old mice (n=5). Values are expressed as mean ± SEM (*p<0.05 versus 6 month old animals).

3.1.3.3. SIRT1 Regulates Mitochondrial Homeostasis Through PGC-1α Dependent and Independent Mechanisms

Consistent with previous work showing the role of SIRT1 in regulation of PGC-1α (Gerhart-Hines et al., 2007; Rodgers et al., 2005), the ability to induce expression of PGC-1α and other regulators of mitochondrial biogenesis in response to fasting was lost in animals lacking SIRT1 (Figure 3.1.8A). Accordingly, the expression of both nuclear and mitochondrial-encoded components of the ETC was induced by fasting in WT animals, while SIRT1 KO animals showed no response (Figure 3.1.8B). Importantly, and consistent with our previous findings, under fed conditions, the expression of PGC-1α and most other regulators of mitochondrial biogenesis were increased or unaltered in SIRT1 KO mice (Figure 3.1.8A), demonstrating that SIRT1 regulates mitochondrial metabolism by distinct pathways in the cell, depending on the nutrient availability.

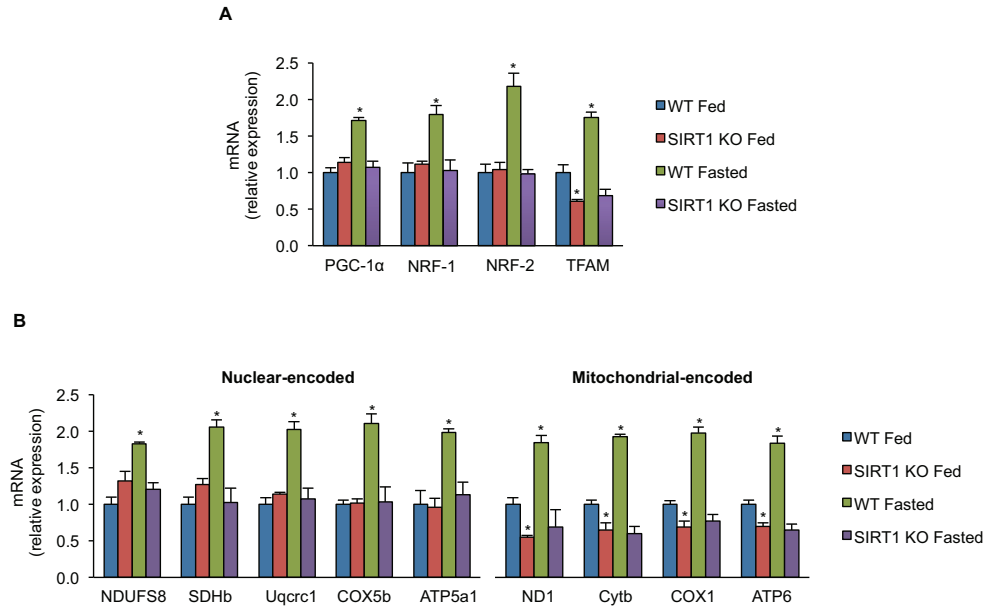


Figure 3.1.8. Loss of SIRT1 leads to impairment in the reprogramming of mitochondrial gene expression induced by fasting. (A) PGC-1α, NRF-1, NRF-2, and TFAM mRNA analyzed by qPCR in gastrocnemius of WT and SIRT1 KO mice. Relative expression values were normalized to WT mice under fed conditions (n=5). (B) NDUFS8, SDHb, Uqcrc1, COX5b, ATP5a1, ND1, Cytb, COX1, and ATP6 mRNA analyzed by qPCR in gastrocnemius of WT and SIRT1 KO mice. Relative expression values were normalized to WT mice under fed conditions (n=5). Values are expressed as mean ± SEM (*p<0.05 versus WT animals).

Confirming that SIRt1 can regulate a PGC-1α independent pathway, overexpression of SIRT1 increased the expression of mitochondrial-encoded components of the ETC in PGC-1α/β knockout primary myotubes (Figure 3.1.9). Interestingly, and in contrast with the other mitochondrial biogenesis regulators that are targets of PGC-1α or its partners, the mitochondrial transcription factor TFAM had reduced expression in animals lacking SIRT1 (Figure 3.1.8A). These findings indicate that SIRT1 can regulate mitochondrial-encoded genes independently of PGC-1α/β and suggest that PGC-1α independent regulation of TFAM might be the mechanism by which SIRT1 promotes functional communication between the nuclear and mitochondrial genomes.

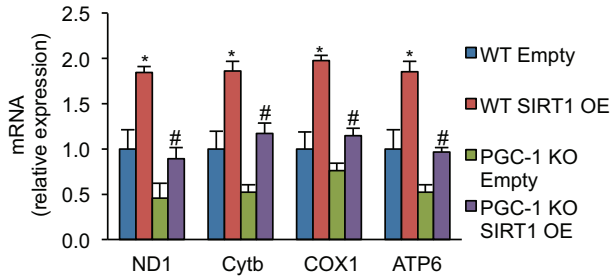


Figure 3.1.9 – SIRT1 regulates the expression of mitochondrial-encoded genes in a PGC-1α independent manner. (A) ND1, Cytb, COX1 and ATP6 mRNA analyzed by qPCR in WT and PGC-1α/β KO myotubes treated with adenovirus overexpressing SIRT1 or empty vector. Relative expression values were normalized to WT control cells (n=4 experiments, *p<0.05 versus WT empty vector, #p<0.05 versus PGC-1α/β KO empty vector). Values are expressed as mean ± SEM.

3.1.3.4. Restoring Mitochondrial-Encoded Genes Prevents Mitochondrial Dysfunction

Consistent with our *in vivo* findings, demonstrating that TFAM expression is decreased in the skeletal muscle of SIRT1 KO animals (Figure 3.1.10A), TFAM promoter activity was also decreased in primary myoblasts isolated from these mice (Figure 3.1.10B). Importantly, restoring mtDNA content and the expression of mitochondrial-encoded genes by overexpression of TFAM (Figure 3.1.10C-E) was sufficient to prevent reductions in ATP levels caused by knockdown of SIRT1 in C2C12 cells (Figure 3.1.10F). Together, these data show that in addition to the known role of SIRT1 in regulation of PGC-1α and induction of mitochondrial biogenesis in response to fasting (Gerhart-Hines et al., 2007; Rodgers et al., 2005), SIRT1 is also important for the maintenance of basal levels of mitochondrial-encoded ETC components and mtDNA, and indicate that, under these experimental conditions, the imbalance between nuclear and mitochondrial-encoded ETC subunits is responsible for the impairment of mitochondrial function.

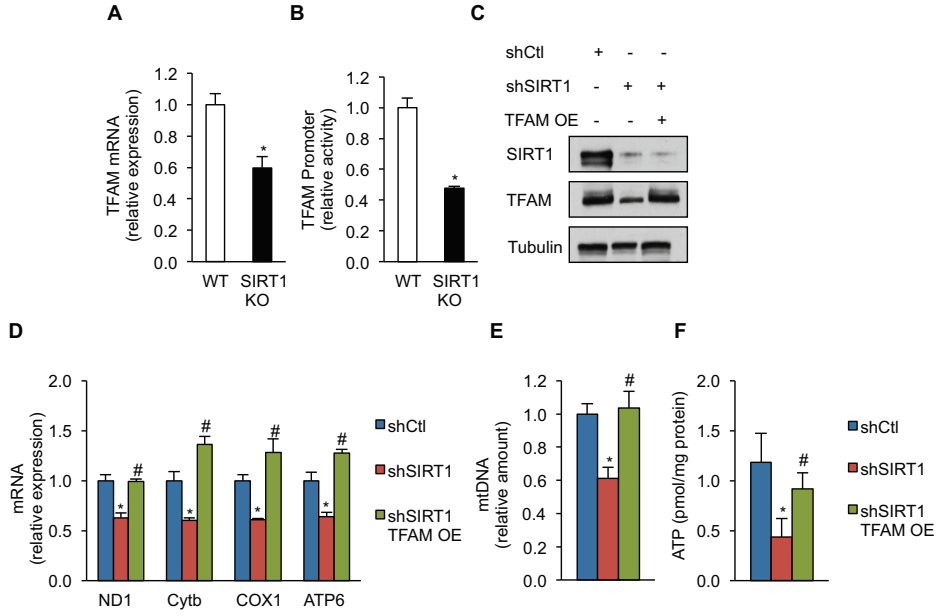


Figure 3.1.10 - SIRT1 regulates mitochondrial function through regulation of TFAM and consequent regulation of mitochondrial DNA and expression of mitochondrial-encoded genes. (A) TFAM mRNA analyzed by qPCR in gastrocnemius of WT and SIRT1 KO animals. Relative expression values were normalized to WT mice (n=4, *p<0.05 versus WT). (B) TFAM promoter activity measured in primary myoblasts extracted from WT and SIRT1 KO mice. Relative luciferase activity was normalized to WT (n=6, *p<0.05 versus control. (C) Representative immunoblot for SIRT1, TFAM and tubulin in C2C12 cells infected with nontargeting or SIRT1 shRNA with or without TFAM overexpression. (D) ND1, Cytb, COX1 and ATP6 mRNA analyzed by qPCR in C2C12 cells infected with nontargeting or SIRT1 shRNA with or without TFAM overexpression. Relative expression values were normalized to control cells (n=4, *p<0.05 versus shCtl, #p<0.05 versus shSIRT1). (E) Mitochondrial DNA content analyzed by qPCR in C2C12 cells infected with nontargeting or SIRT1 shRNA with or without TFAM overexpression. Relative amount was normalized to control cells (n=4, *p<0.05 versus shCtl, #p<0.05 versus shSIRT1). (F) ATP content in C2C12 cells infected with nontargeting or SIRT1 shRNA with or without TFAM overexpression (n=4, *p<0.05 versus shCtl, #p<0.05 versus shSIRT1). Values are expressed as mean ± SEM.

3.1.3.5. Regulation of Mitochondrial-Encoded Genes by SIRT1 is Mediated by c-Myc

c-Myc has previously been suggested to regulate mitochondrial biogenesis and shown to bind directly to the TFAM promoter (Li et al., 2005). Therefore, we hypothesized that the SIRT1-induced PGC-1α independent mechanism of regulation of mitochondrial-encoded genes could be mediated by c-Myc. Indeed, we found that loss of

SIRT1 led to a reduction in c-Myc reporter activity in primary myoblasts isolated from the SIRT1 KO mice (Figure 3.1.11A). Additionally, knockdown of c-Myc in C2C12 myoblasts (Figure 3.1.11B) prevented the increase in mtDNA, the expression of mitochondrial encoded-genes, as well as the TFAM promoter activity induced by SIRT1 overexpression (Figure 3.1.11C-F).

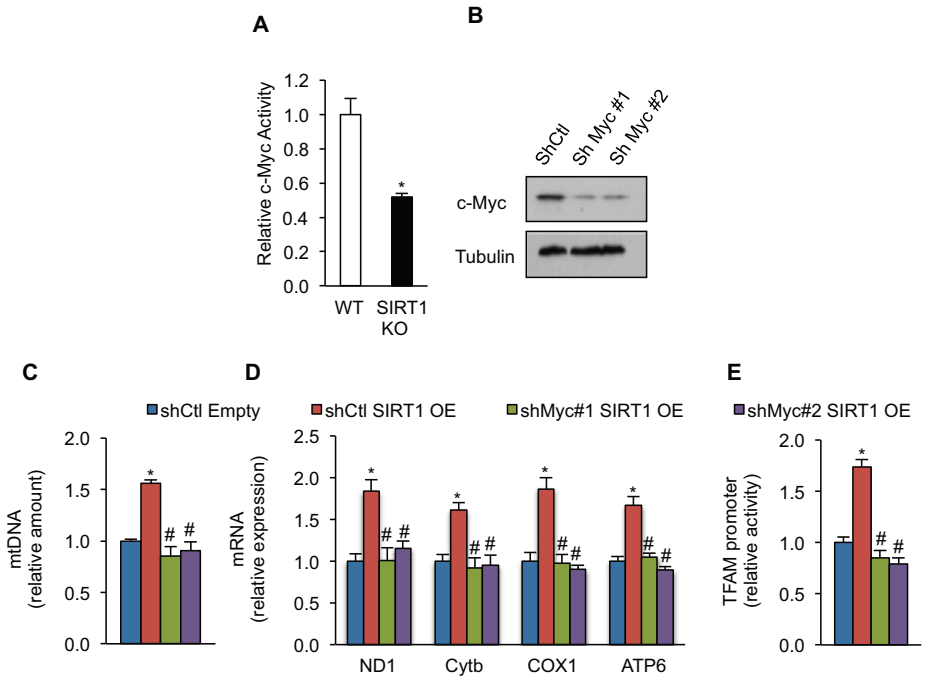


Figure 3.1.11 – Knockdown of c-Myc prevents the increase in TFAM promoter activity and expression of mitochondrial-encoded genes induced by SIRT1. (A) c-Myc activity in primary myoblasts extracted from WT and SIRT1 KO animals. Relative luciferase activity was normalized to WT cells (n=3, *p<0.05 versus control). (B) Representative immunoblot for c-Myc and tubulin in C2C12 cells infected with c-Myc or nontargeting shRNA. (C) Mitochondrial DNA content analyzed by qPCR in C2C12 cells infected with c-Myc or nontargeting shRNA and treated with adenovirus overexpressing SIRT1 or empty vector. Relative amount was normalized to control cells (n=5, *p<0.05 versus empty vector, #p<0.05 versus SIRT1 OE). (D) ND1, Cytb, COX1 and ATP6 mRNA analyzed by qPCR in C2C12 cells infected with c-Myc or nontargeting shRNA and treated with adenovirus overexpressing SIRT1 or empty vector. Relative expression values were normalized to control cells (n=6, *p<0.05 versus empty vector, #p<0.05 versus SIRT1 OE). (E) TFAM promoter activity in C2C12 cells infected with c-Myc or nontargeting shRNA and treated with adenovirus overexpressing SIRT1 or empty vector. Relatiive luciferase activity was normalized to control cells (n=4, *p<0.05 versus empty vector, #p<0.05 versus SIRT1 OE). Values are expressed as mean ± SEM.

Accordingly, overexpression of c-Myc (Figure 3.1.12A) prevented the decrease in mtDNA and TFAM promoter activity in C2C12 cells treated with the specific SIRT1 inhibitor EX-527 (Figure 3.1.12B and 3.1.12C), as well as, the decline in mitochondrial-encoded genes and in cellular ATP levels (Figure 3.1.12D and 3.1.12E). Altogether, these results show that SIRT1 regulates the expression of mitochondrial-encoded genes by a mechanism involving the modulation of the TFAM promoter by c-Myc, which consequently maintains mitochondrial oxidative phosphorylation efficiency.

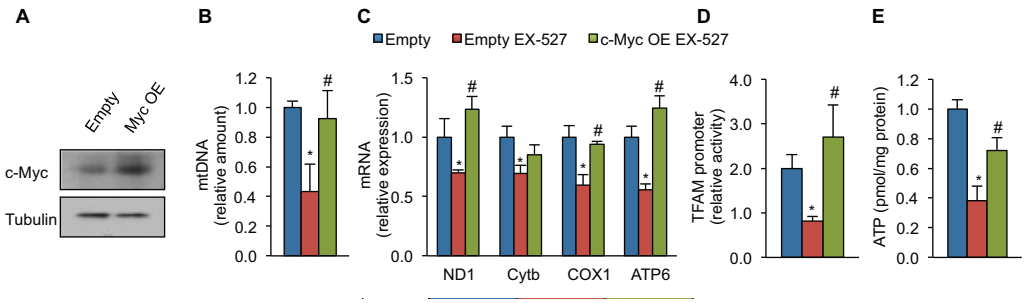


Figure 3.1.12 – Overexpression of c-Myc protects from decline in the TFAM promoter and mitochondrial-encoded gens induced by decline in SIRT1 activity. (A) Representative immunoblot for c-Myc and tubulin in C2C12 cells overexpressing c-Myc. (B) Mitochondrial DNA content analyzed by qPCR in C2C12 cells overexpressing c-Myc. Relative amount was normalized to control cells (n=5, *p<0.05 versus empty vector, #p<0.05 versus c-Myc OE). (C) ND1, Cytb, COX1 and ATP6 mRNA analyzed by qPCR in C2C12 cells overexpressing c-Myc. Relative expression values were normalized to control cells (n=6, *p<0.05 versus empty vector, #p<0.05 versus c-Myc OE). (D) ATP content in C2C12 cells overexpressing c-Myc. (n=6, *p<0.05 versus empty vector, #p<0.05 versus c-Myc OE). (E) TFAM promoter activity in in C2C12 cells overexpressing c-Myc. Relatiive luciferase activity was normalized to control cells (n=6, *p<0.05 versus empty vector, #p<0.05 versus c-Myc OE). Values are expressed as mean ± SEM.

3.1.2.6 Increased HIF1α Induces a Genetic Reprogramming Towards Increased Glycolysis in SIRT1 KO Mice

Interestingly, hypoxia was shown to also lead to a specific decline in mitochondrial-encoded genes of the ETC (Piruat and Lopez-Barneo, 2005; Tello et al., 2011). Since HIFα is known to regulate c-Myc transcriptional activity, we sought to evaluate if HIFα was involved in the phenotypes observed in response to loss of SIRT1 in skeletal muscle. Consistent with this idea, primary myoblasts isolated from the SIRT1 KO animals showed increased HRE activity (Figure 3.1.13A). Accordingly, increased

expression of several different HIF1α targets was also increased in SIRT1 KO animals (Figure 3.1.13B). In addition to increased HIF1α activity, we also observed that protein levels of HIF1α were elevated in skeletal muscle of SIRT1 KO mice, indicating that loss of SIRT leads to HIF1α stabilization (Figure 3.1.13C). Importantly, the expression of genes involved in glycolysis, as well as, the expression of LDHA was increased in muscle of animals lacking SIRT1 (Figure 3.1.13D and 3.1.13E), suggesting that SIRT1 KO mice have increased capacity for anaerobic glycolysis. Together these data indicate that loss of SIRT1 *in vivo* leads to increased stabilization of HIF1α, which then is responsible for an increased in the gene expression of the glycolytic program. This raises the idea that HIF1α stabilization induced by loss of SIRT1 might be involved in the development of the imbalance between nuclear and mitochondrial-encoded gene expression, and impairment in mitochondrial oxidative phosphorylation, thereby leading to disruption of mitochondrial homeostasis observed in the skeletal muscle of the SIRT1 KO animals.

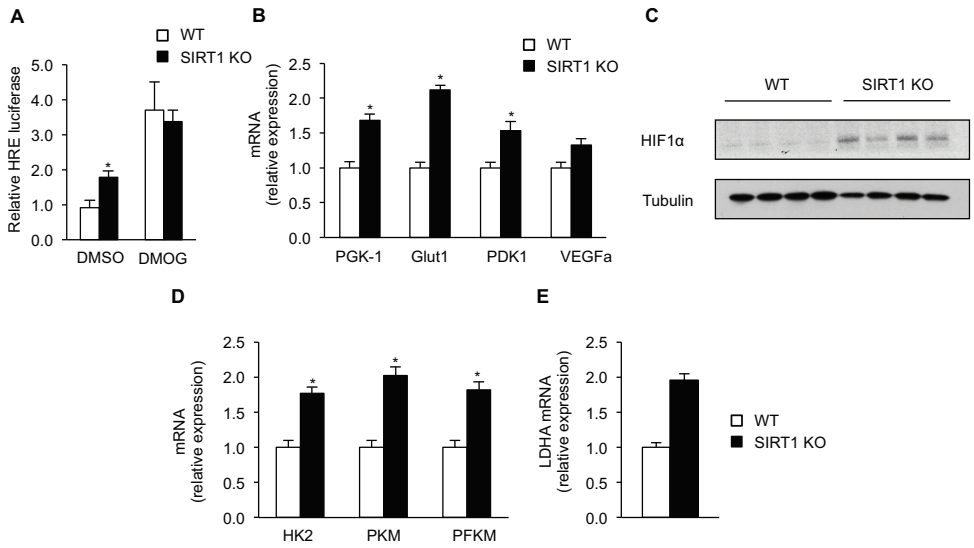


Figure 3.1.13 - Loss of SIRT1 leads to HIF1α stabilization and induces the glycolytic genetic program *in vivo*. (A) Hypoxia response element activity in primary myoblasts isolated from WT and SIRT1 KO mice and treated with or without DMOG. Relative luciferase activity was normalized to WT cells (n=6). (B) PGK-1, Glut1, PKD1, and VEGFa mRNA analyzed by qPCR in gastrocnemius of WT and SIRT1 KO mice. Relative expression values were normalized to WT mice (n=4). (C) Representative immunoblot for HIF1α and tubulin in gastrocnemius of WT and SIRT1 KO mice. (D) HK2, PKM, and PFKM mRNA analyzed by qPCR in gastrocnemius of WT and SIRT1 KO mice. Relative expression values were normalized to WT mice (n=5).

(E) LDHA mRNA analyzed by qPCR in gastrocnemius of WT and SIRT1 KO mice. Relative expression values were normalized to WT mice (n=5). Values are expressed as mean ± SEM (*p<0.05 versus WT).

3.1.3.7. SIRT1 Regulates the c-Myc Activity and Mitochondrial Homeostasis Through HIF1α

To further dissect the possible role of HIF1α in the regulation of the PGC-1α/β independent induction of mitochondrial-encoded genes by SIRT1, HIFα was pharmacologically stabilized in PGC-1α/β primary myotubes. Interestingly, stabilization of HIFα with DMOG and DFO prevented the PGC-1α/β-independent induction of mitochondrial-encoded genes in response to SIRT1 overexpression (Figure 3.1.14), indicating that HIF might indeed be involved in the SIRT1 mediated PGC-1α independent pathway.

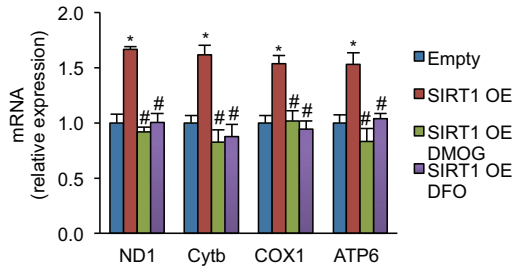


Figure 3.1.14 - HIF1α stabilization prevents the PGC-1α-independent induction of mitochondrial-encoded genes by SIRT1 . ND1, Cytb, COX1 and ATP6 mRNA analyzed by qPCR in PGC-1α/β KO myotubes treated with adenovirus overexpressing SIRT1 or empty vector as well as treatment with DMSO or with HIF stabilizing compounds DMOG and DFO. Relative expression values were normalized to control cells (n=5, *p<0.05 versus empty vector, #p<0.05 versus SIRT1 OE). Values are expressed as mean ± SEM.

Consistent with this, cells expressing a form of HIF1α that is constitutively stabilized due to mutation of key proline residues (DPA), which prevents HIF hydroxylation and consequent proteosomal degradation (Figure 3.1.15A), also showed no induction of mitochondrial-encoded genes or mtDNA content when SIRT1 was overexpressed (Figure 3.1.15B 3.15C). Importantly, HIF2α DPA cells did not show any impairment of the effects induced by SIRT1 overexpression, indicating that this effect is specific to HIF1α (Figure 3.1.15A-C).

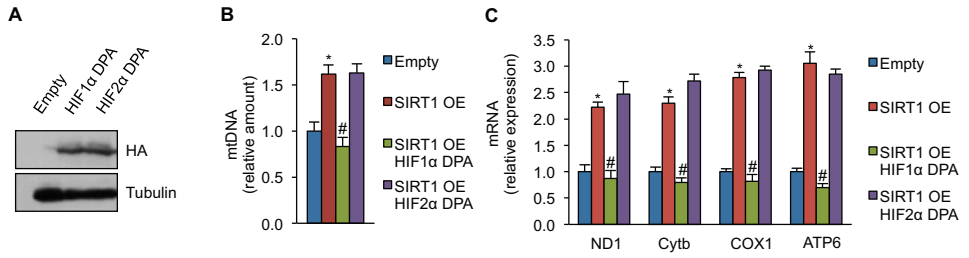


Figure 3.1.15 - HIF1α stabilization, but not HIF2α, prevents SIRT1-induced mitochondrial-encoded genes. (A) Representative immunoblots for HA-tag and tubulin in control C2C12 cells and cells overexpressing either HIF1α or HIF2α with key proline residues mutated (HIF1α DPA; HIF2α DPA). (B) Mitochondrial DNA content analyzed by qPCR in control, HIF1α DPA or HIF2α DPA C2C12 cells treated with adenovirus overexpressing SIRT1 or empty vector. Relative amount was normalized to control cells (n=5, *p<0.05 versus empty vector, #p<0.05 versus SIRT1 OE). (C) ND1, Cytb, COX1, and ATP6 mRNA analyzed by qPCR in control, HIF1α DPA or HIF2α DPA C2C12 cells treated with adenovirus overexpressing SIRT1 or empty vector. Relative expression values were normalized to control cells (n=4, *p<0.05 versus empty vector, #p<0.05 versus SIRT1 OE). Values are expressed as mean ± SEM.

Conversely, knockdown of HIF1α in C2C12 cells (Figure 3.1.16A) largely abrogated the decrease in mitochondrial-encoded genes, mtDNA content, and mitochondrial membrane potential caused by inhibition of SIRT1 with EX-527 (Figure 3.1.16B-D).

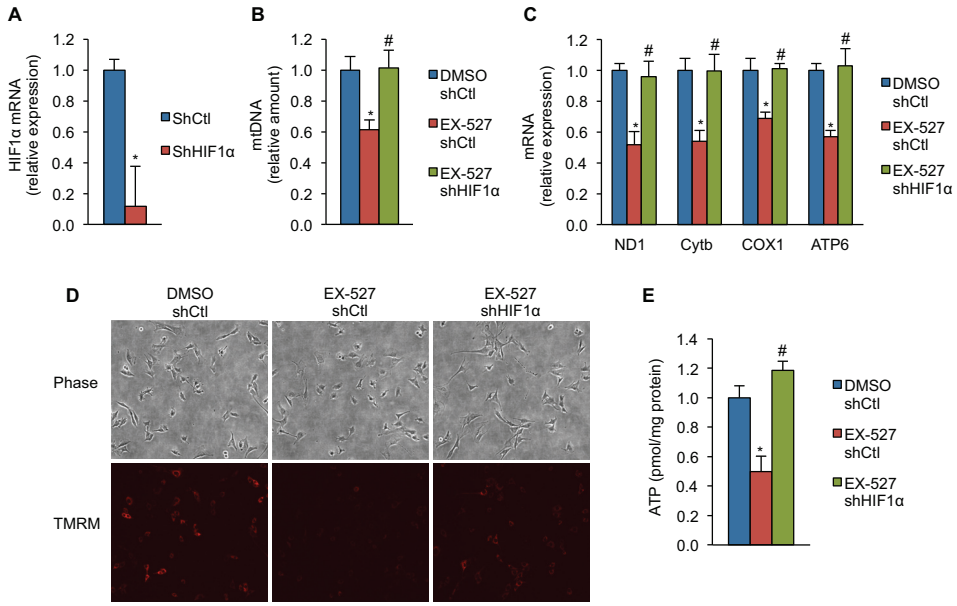


Figure 3.1.16 - HIF1α knockdown mantains mitochondrial homeostasis from decline in SIRT1 activity. (A) HIF1α mRNA analyzed by qPCR in C2C12 cells infected with HIF1α or nontargeting shRNA. Relative expression values were normalized to control cells (n=4, *p<0.05 versus control). (B) Mitochondrial DNA content analyzed by qPCR in C2C12 cells infected with HIF1α or nontargeting shRNA treated with EX-527. Relative amount was normalized to control cells (n=6, *p<0.05 versus control, #p<0.05 versus control EX-527). (C) ND1, Cytb, COX1, and ATP6 mRNA analyzed by qPCR in C2C12 cells infected with HIF1α or nontargeting shRNA treated with EX-527. Relative expression values were normalized to control cells (n=6, *p<0.05 versus control, #p<0.05 versus control EX-527). (D) Representative images of mitochondrial membrane potential in C2C12 cells infected with HIF1α or nontargeting shRNA treated with EX-527 and analyzed by fluorescence microscopy. (E) ATP content in C2C12 cells infected with HIF1α or nontargeting shRNA treated with EX-527 (n=4, *p<0.05 versus control, #p<0.05 versus control EX-527). Values are expressed as mean ± SEM.

Moreover, impairment of the transcriptional activity of the HIF complex by knockdown of its transcriptional partner, ARNT, was not found to protect from the effects of SIRT1 inhibition with EX-527 (Figure 3.1.17), indicating that the effect of HIF1α on mitochondrial homeostasis in response to SIRT1 is not mediated by HIF1α transcriptional activity.

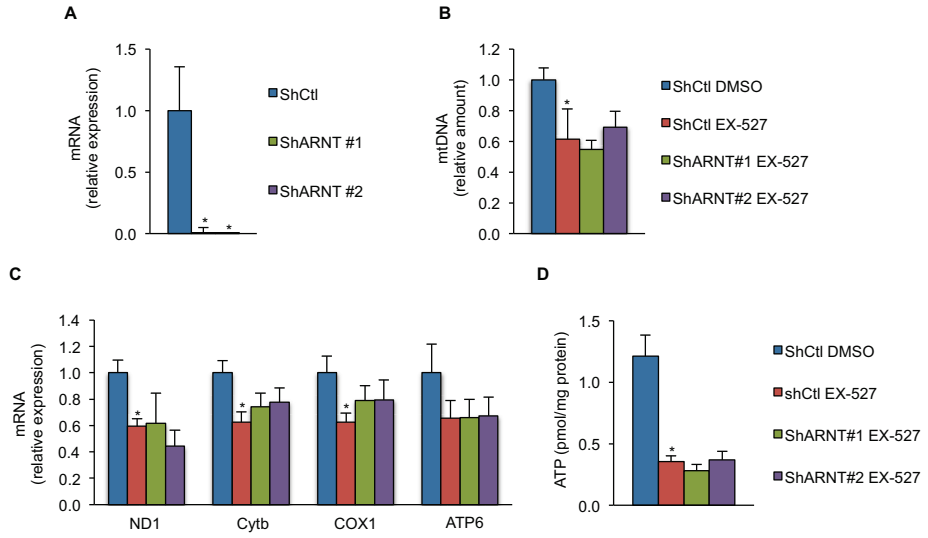


Figure 3.1.17 - Regulation of mitochondrial homeostasis by HIF1α does not require its transcriptional activity. (A) ARNT mRNA analyzed by qPCR in C2C12 cells infected with ARNT or nontargeting shRNA. Relative expression values were normalized to control cells (n=4, *p<0.05 versus control). (B) Mitochondrial DNA content analyzed by qPCR in C2C12 cells infected with ARNT or nontargeting shRNA. Relative amount was normalized to control cells (n=5, *p<0.05 versus control). (C) ND1, Cytb, COX1 and ATP6 mRNA analyzed by qPCR in C2C12 cells infected with ARNT or nontargeting shRNA. Relative levels were normalized to control cells (n=6, *p<0.05 versus control). (D) ATP content in C2C12 cells infected with ARNT or nontargeting shRNA (n=4, *p<0.05 versus control). Values are expressed as mean ± SEM.

HIF1α was previously shown to regulate c-Myc transcriptional activity by binding to c-Myc transcriptional partners, Max and Sp1, and disrupting the c-Myc transcriptional complex (Koshiji et al., 2004; Koshiji et al., 2005), suggesting that HIF1α might be the mechanistic link between SIRT1 and c-Myc. To pursue that hypothesis, we investigated whether the effect of SIRT1 in TFAM promoter required HIF1α. Genetic stabilization of HIF1α abrogated SIRT1-induced TFAM promoter activity, suggesting that HIF1α might regulate TFAM promoter activity through c-Myc (Figure 3.1.18A). Consistent with this, deletion of HIF1α rescued the decline in TFAM promoter activity induced by inhibition of SIRT1 (Figure 3.1.18B). Importantly, and showing that SIRT1 regulates the activity of TFAM promoter through HIF1α-c-Myc, loss of c-Myc blocked the rescue of TFAM promoter activity caused by HIF1α knockdown (Figure 3.1.18B). Together, these data show that SIRT1 regulates TFAM promoter through HIF1α regulation of c-Myc, and that impairment of this regulatory network leads to

miscommunication between the nuclear and mitochondrial genome and mitochondrial dysfunction.

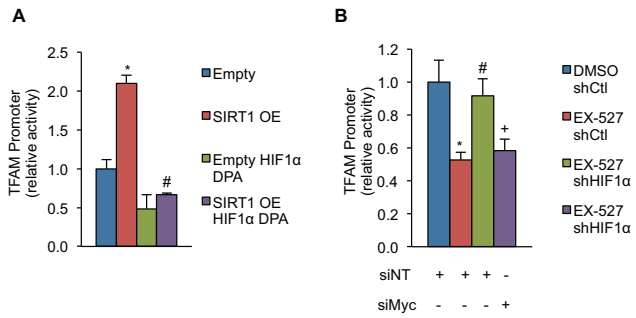


Figure 3.1.18 – The effect of SIRT1 in TFAM promoter requires both HIF1α and c-Myc. (A) TFAM promoter activity in control or HIF1α DPA C2C12 cells treated with adenovirus overexpressing SIRT1 or empty vector. Relative luciferase activity was normalized to control cells (n=6, *p<0.05 versus empty vector, #p<0.05 versus SIRT1 OE). (B) TFAM promoter activity in C2C12 cells infected with HIF1α or nontargeting shRNA treated with EX-527 and c-Myc siRNA. Relative luciferase activity was normalized to control cells (n=6, *p<0.05 versus DMSO, #p<0.05 versus Ex-527, +*p<0.05 versus HIF1α KD). Values are expressed as mean ± SEM.

3.1.3.8. Caloric Restriction Protects from Age-Related Mitochondrial Dysfunction by Maintaining Functional Communication Between Nuclear and Mitochondrial Genomes

CR has previously been shown to improve mitochondrial function and prevent many of the deleterious effects of aging, and it has been proposed that SIRT1 plays an important role in mediating these changes (Civitarese et al., 2007; Cohen et al., 2004). In this study, we found that CR prevented the decline in mitochondrial function induced by aging, resulting in improved mitochondrial membrane potential, ATP levels, and COX activity (Figure 3.1.19A-C). CR increased mitochondrial biogenesis and prevented the decrease in mitochondrial-encoded ETC components and mtDNA content observed in 22 month old mice (Figure 3.1.19D-F). Similar to SIRT1 KO mice, 22 month old animals had HIF1α stabilized, which was prevented by CR (Figure 3.1.19H). Consistent with this, CR prevented the upregulation of HIF1α target genes observed in the 22 month old mice (Figure 3.1.19I). We further found that NAD^+ levels were elevated in CR animals (Figure 3.1.19G), indicating that CR may be mediating its beneficial effects by increasing SIRT1 activity and suggests that preventing age related declines in NAD^+ levels may protect

against the imbalance between the expression of genes encoded by the nuclear and mitochondrial genomes and mitochondrial dysfunction.

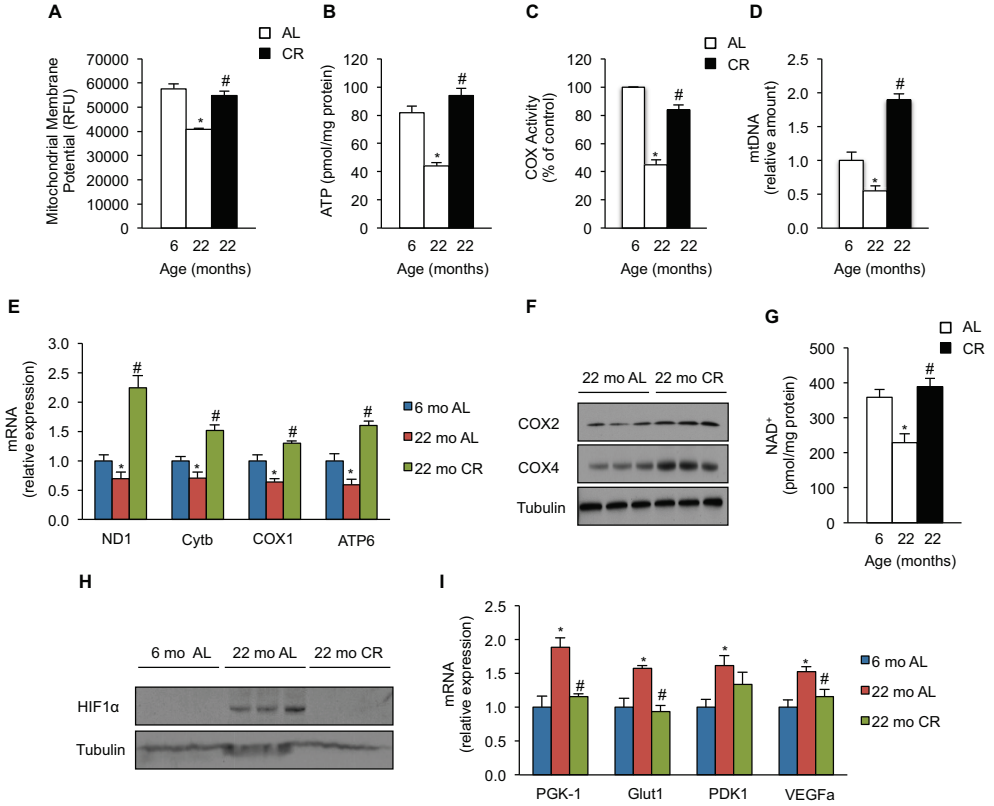


Figure 3.1.19 - Caloric restriction protects from age-related mitochondrial dysfunction in skeletal muscle by preventing HIF1α stabilization and loss of mitochondrial-encoded ETC genes. (A) Mitochondrial membrane potential of isolated mitochondria from skeletal muscle of 6 and 22 month old AL, and 22 month old CR mice (n=5, *p<0.05 versus 6 month old animals #p<0.05 versus 22 month old AL mice). (B) ATP content in skeletal muscle of 6 and 22 month old AL, and 22 month old CR mice (n=5, *p<0.05 versus 6 month old animals #p<0.05 versus 22 month old AL mice). (C) Cytochrome c Oxidase Activity (Cox) activity in skeletal muscle of 6 and 22 month old AL, and 22 month old CR mice (n=4, *p<0.05 versus 6 month old animals #p<0.05 versus 22 month old AL mice). (D) Mitochondrial DNA content analyzed by qPCR in gastrocnemius of 6 and 22 month AL, and 22 month old CR mice. Relative amount was normalized to 6 month old mice (n=5, *p<0.05 versus 6 month old animals #p<0.05 versus 22 month old AL mice). (E) ND1, Cytb, COX1 and ATP6 mRNA analyzed by qPCR in gastrocnemius of 6 and 22 month AL, and 22 month old CR mice. Relative expression values were normalized to 6 month old mice (n=5, *p<0.05 versus 6 month old animals #p<0.05 versus 22 month old AL mice). (F) Representative immunoblots for COX2, COX4, and tubulin in gastrocnemius of 22 month AL and CR mice. (G) NAD^+ levels in gastrocnemius

of 6 and 22 month AL, and 22 month old CR mice (n=5, *p<0.05 versus 6 month old animals #p<0.05 versus 22 month old AL mice).

(H) Representative immunoblots for HIF1α, and tubulin in gastrocnemius of 6 and 22 month AL and 22 month old CR mice. (I) PGK-1, Glut1, PKD1, and VEGFa mRNA analyzed by qPCR in gastrocnemius of 6 and 22 month AL, and 22 month old CR mice. Relative expression values were normalized to 6 month old mice. (n=5, *p<0.05 versus 6 month old animals #p<0.05 versus 22 month old AL mice). Values are expressed as mean ± SEM.

3.1.3.9. NMN Induces NAD^+ Levels in Skeletal Muscle and Reverses Age-Induced Imbalance Between Nuclear and Mitochondrial-encoded genes and Mitochondrial Dysfunction

To determine whether boosting NAD^+ levels is sufficient to prevent the imbalance between nuclear and mitochondrial-encoded genes and restore mitochondrial function in aged animals, we treated 3 and 24 months old animals with the NAD^+ precursor nicotinamide mononucleotide (NMN), which has previously been shown to increase cellular NAD^+ levels in a variety of tissues (Yoshino et al., 2011a). Strikingly, increasing cellular NAD^+ levels in skeletal muscle (Figure 3.1.20A) for just one week by treating animals with NMN was sufficient to restore mitochondrial function in 24 month old animals (Figure 3.1.20B-D) and promote functional communication between nuclear and mitochondrial genomes, by increasing mtDNA content and the expression of mitochondrial-encoded ETC components in 24 month old animals (Figure 3.1.20E-F).

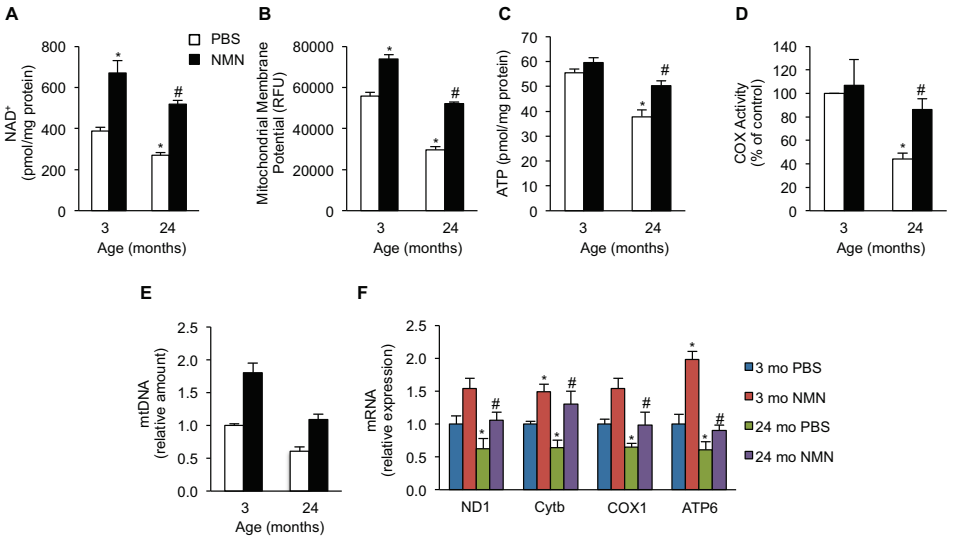


Figure 3.1.20 - Increasing NAD^+ rescues age-related mitochondrial dysfunction and promotes functional communication between nuclear and mitochondrial genomes in skeletal muscle (A) NAD^+

levels in gastrocnemius of 3 and 24 month old mice treated with either the vehicle (PBS) or NMN (n=5, *p<0.05 versus 3 month old PBS animals, #p<0.05 versus 24 month old PBS animals). (B) Mitochondrial membrane potential of isolated mitochondria from skeletal muscle of 3 and 24 month old mice treated with either the vehicle (PBS) or NMN (n=4, *p<0.05 versus 3 month old PBS animals, #p<0.05 versus 24 months old PBS animals). (C) ATP content in skeletal muscle of 3 and 24 month old mice treated with either the vehicle (PBS) or NMN (n=5, *p<0.05 versus 3 month old PBS animals, #p<0.05 versus 24 month old PBS animals). (D) Cytochrome c Oxidase (Cox) activity in skeletal muscle of 3 and 24 month old mice treated with either the vehicle (PBS) or NMN (n=4, *p<0.05 versus 3 month old animals, #p<0.05 versus 24 month old PBS animals). (E) Mitochondrial DNA content analyzed by qPCR in gastrocnemius of 3 and 24 month old mice treated with either the vehicle (PBS) or NMN. Relative amount was normalized to 3 month old PBS animals (n=5, *p<0.05 versus 3 month old PBS animals, #p<0.05 versus 24 month old PBS animals). (F) ND1, Cytb, COX1, and ATP6 mRNA analyzed by qPCR in gastrocnemius of 3, and 24 month old mice treated with either the vehicle (PBS) or NMN. Relative expression values were normalized to 3 month old PBS animals (n=5, *p<0.05 versus 3 month old PBS animals, #p<0.05 versus 24 month old PBS animals). Values were normalized to PBS treated cells. (n=6, *p<0.05 versus PBS, #p<0.05 versus NMN). Values are expressed as mean ± SEM.

Importantly, the ability of NMN treatment to induce mitochondrial-encoded genes and improve mitochondrial function was lost in animals lacking SIRT1 (Figure 3.1.21A and 3.1.21B), indicating that NMN's ability to restore mitochondrial function and expression of mitochondrial-encoded genes is SIRT1-dependent.

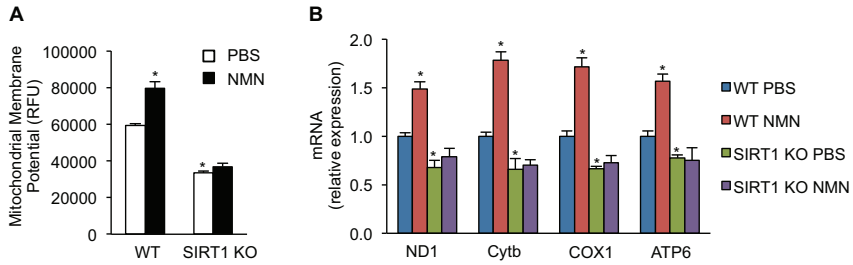


Figure 3.1.21 - Increasing NAD^+ increases mitochondrial function and mitochondrial-encoded genes in a SIRT1-dependent manner in skeletal muscle (A) Mitochondrial membrane potential of isolated mitochondria from skeletal muscle of WT and SIRT1 KO mice treated with either the vehicle (PBS) or NMN (n=4, *p<0.05 versus WT PBS animals). (H) ND1, Cytb, COX1 and ATP6 mRNA analyzed by qPCR in gastrocnemius of WT and SIRT1 KO mice treated with either the vehicle (PBS) or NMN (n=4, *p<0.05 versus WT untreated animals). Values are expressed as mean ± SEM.

Moreover, NMN's effects seem to be also mediated by HIF1α as NMN treatment prevented the age-induced HIF1α stabilization and increase in HIF1α target

genes, similar to effects seen in CR animals (Figure 3.2.22A and 3.2.22B). In support of this, HIFα pharmacologic stabilization abrogated the PGC-1α independent effects of NMN on the expression of mitochondrial-encoded genes (Figure 3.2.22C). Together, these results demonstrate that boosting NAD^+ levels is sufficient to restore mitochondrial function in aged animals and that the induction of mitochondrial-encoded genes as well as improvements in mitochondrial function in NMN treated animals are mediated, at least in part, through a SIRT1-HIFα pathway.

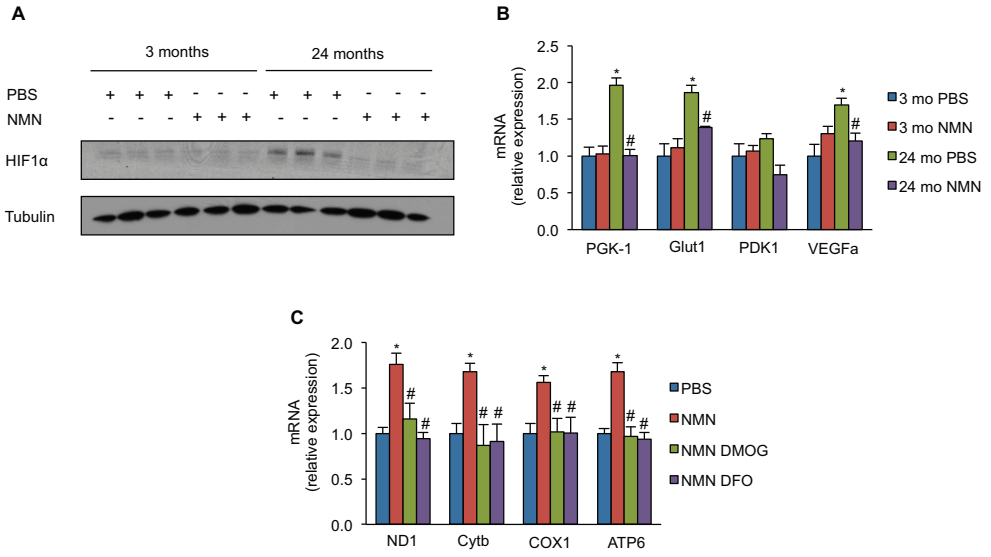


Figure 3.1.22 – Increasing NAD^+ rescues HIF1α age-related stabilization of HIF1α. (A) Representative immunoblot for HIF1α, and tubulin in gastrocnemius of 3 and 24 month old mice treated with either the vehicle (PBS) or NMN. (B) PGK-1, Glut1, PKD1, and VEGFa mRNA analyzed by qPCR in gastrocnemius of 3 and 24 month old mice treated with either the vehicle (PBS) or NMN. Relative expression values were normalized to 3 month old PBS animals. (n=5, *p<0.05 versus 3 month old PBS animals, #p<0.05 versus 24 month old PBS animals). (C) ND1, Cytb, COX1 and ATP6 mRNA analyzed by qPCR in PGC-1α/β KO myotubes treated with PBS or NMN as well as treatment with DMSO or with DMOG or DFO. Relative expression values were normalized to PBS treated cells. (n=6, *p<0.05 versus PBS, #p<0.05 versus NMN). Values are expressed as mean ± SEM.

3.1.4. Discussion

Mitochondrial dysfunction is believed to play an important role in the development and progression of many age related diseases (Coskun et al., 2011; de Moura et al., 2010; Figueiredo et al., 2009; Schulz et al., 2007; Wallace, 2005). Furthermore, genetic disorders involving mitochondrial dysfunction result in rapid onset

of symptoms commonly seen in the elderly, such as insulin resistance, muscle loss, and neurodegeneration (Finsterer, 2004; Sahin et al., 2011; Wallace, 2010; Wallace et al., 2010a). Despite the importance of mitochondrial dysfunction in the development of age-related disease, there is still a great deal of controversy over how and why mitochondrial function declines with age. While findings showing that impaired mitochondrial function can extend lifespan in *C. elegans* (Dillin et al., 2002) and may prevent insulin resistance in skeletal muscle (Pospisilik et al., 2007; Wredenberg et al., 2006) demonstrate that impaired respiratory capacity may have beneficial effects in specific tissues or during specific stages of development, the majority of studies show that genetic induction of mitochondrial deficiencies results in the development of disease phenotypes and early mortality (Akman et al., 2008; Bourdon et al., 2007; Cerritelli et al., 2003; Chen and Chan, 2010; Ekstrand et al., 2007; Fan et al., 2008; Hance et al., 2005; Inoue et al., 2000; Kasahara et al., 2006; Kimura et al., 2003; Kujoth et al., 2005; Larsson et al., 1998; Marchington et al., 1999; Silva et al., 2000; Sligh et al., 2000; Sorensen et al., 2001; Srivastava and Moraes, 2005; Trifunovic et al., 2004; Tyynismaa et al., 2005; Wang et al., 1999; Zhou et al., 2008b).

CR or activation of SIRT1 has been shown to delay the development of a number of age related diseases, possibly due to an induction of mitochondrial biogenesis and improvements in mitochondrial function, thus maintaining mitochondrial homeostasis. Due to its ability to deacetylate and activate PGC-1α, SIRT1 has been established as an important regulator of mitochondrial biogenesis (Gerhart-Hines et al., 2007; Rodgers et al., 2005). Consistent with this, we demonstrate that the ability to activate PGC-1α and induce mitochondrial biogenesis in response to fasting is lost in animals lacking SIRT1. Moreover, we show for the first time that SIRT1 plays a dual role in regulating the expression of TFAM, mtDNA and mitochondrial-encoded genes through both PGC-1α dependent and independent mechanisms, depending on the nutrient status of the cells. Indeed, we show that loss of SIRT1, in a fed state, results in impaired ability to regulate expression of mitochondrial-encoded genes leading to an imbalance between nuclear and mitochondrial-encoded subunits of the ETC. We further demonstrate that this imbalance plays a direct role in the mitochondrial dysfunction observed upon loss of SIRT1, as restoring mtDNA content and the expression of mitochondrial-encoded genes was sufficient prevent the mitochondrial dysfunction. Our results suggest that SIRT1 is involved in fine-tuning the regulation of mitochondrial metabolism. Indeed, the data presented in this chapter suggest that under conditions of

normal nutrient availability, SIRT1 mainly regulates mitochondrial homeostasis through a PGC-1α-independent regulation of HIF1α/c-Myc. However, when nutrient availability is reduced, like in fasting conditions, SIRT1 also activates PGC-1α to promote mitochondrial biogenesis, as well as, inducing fatty acids oxidation (Gerhart-Hines et al., 2007) and thereby adjusts mitochondrial metabolism to maintain mitochondrial homeostasis.

Interestingly, our findings indicate that the ability of SIRT1 to regulate the expression of mitochondrial-encoded genes independently of PGC1α is, at least in part, due to its ability to prevent the stabilization of HIF1α. SIRT1 has previously been shown to deacetylate HIF1α, decreasing its activity under hypoxic conditions (Lim et al., 2010). Importantly, we observe that loss of SIRT1 leads not only to an increase in HIF1α transcriptional activity, but also to HIF1α stabilization in skeletal muscle, adding another layer of regulation of HIF1α by SIRT1. This may play an important role in metabolic regulation *in vivo*, as skeletal muscle from SIRT1 KO animals underwent a shift toward increased glycolytic muscle fibers (chapter 3.2). Indeed, loss of HIF1α prevented the decline in mitochondrial-encoded genes and mitochondrial dysfunction when SIRT1 activity was inhibited. On the other hand, the ability of SIRT1 to induce expression of mitochondrial-encoded genes was lost under conditions where HIF1α was stabilized. These findings are especially interesting since loss of HIF1α has previously been shown to increase lifespan in *C. elegans* and possibly mediate some of the pro-longevity effects of CR (Chen et al., 2009).

In addition to regulating HIF1α transcriptional activity (Lim et al., 2010), SIRT1 has also previously reported to regulate HIF2α (Dioum et al., 2009). HIF1α and HIF2α are known to have both redundant and non-redundant activities in the cell (Keith et al., 2012). Interestingly, the expression of HIF2α, but not HIF1α, was previously shown to be regulated by PGC-1α and to play an important role in fiber type switch mediated by PGC-1α in skeletal muscle (Rasbach et al., 2010). Importantly, we show that HIF1α, but not HIF2α, mediates the SIRT1 induced this PGC-1α independent pathway as specific stabilization of HIF2α did not alter the ability of SIRT1 to regulate expression of mitochondrial genes nor mtDNA content. Moreover, the disturbances in mitochondrial homeostasis caused by HIF1α stabilization are not due to its transcriptional activity, as loss of HIF1α transcriptional partner ARNT did not alter the effects caused by the decline in SIRT1 activity. Interestingly, HIF1α was previously associated with changes in mitochondrial biogenesis under conditions of obesity. Indeed, high fat diet feeding was

shown to induce the expression of HIF1α as well as to increase mtDNA in liver (Carabelli et al., 2011). HIF1α was also reported to be stabilized in white adipose tissue in animal models of obesity, but upregulation of HIF1α was found to be correlated with a decline in mitochondrial related genes in this tissue (Krishnan et al., 2012), suggesting that the role of HIF1α in the regulation of mitochondrial metabolism is tissue specific, possibly acting in accordance to the metabolic specificities of each tissue.

c-Myc has been pointed out as a potential regulator of mitochondrial biogenesis and shown to directly bind to TFAM promoter (Li et al., 2005), suggesting that it may directly regulate TFAM expression. Importantly, c-Myc transcriptional activity was previously demonstrated to be regulated by interacting with HIF1α. HIF1α decreases c-Myc transcriptional activity through interacting and disrupting the c-Myc/Max/Sp1 transcriptional complex (Koshiji et al., 2004; Koshiji et al., 2005), and therefore does not require HIF1α transcriptional activity. Moreover, c-Myc is differentially regulated by HIF1α and HIF2α (Keith et al., 2012), which is consistent with our results, as HIF2α is not involved in SIRT1-mediated regulation of mitochondrial homeostasis. Consistent with this, our data show that loss of SIRT1 leads to a decrease in c-Myc reporter activity. Importantly, recent reports have shown that c-Myc and SIRT1 regulate each other via feedback loops. Whether these are positive or negative loops is still a question of debate as different groups have reached different conclusions (Mao et al., 2011; Marshall et al., 2011; Menssen et al., 2012; Yuan et al., 2009). Moreover, SIRT1 has been shown to directly regulate c-Myc transcriptional activity in cancer cells, either by deacetylation of c-Myc (Menssen et al., 2012) or by binding to c-Myc and promoting its association with Max (Mao et al., 2011). Importantly, we show that under these experimental conditions, the c-Myc mediated effect of SIRT1 on the TFAM promoter requires HIF1α, as when HIF1α is stabilized it blocks the increase in TFAM promoter activity induced by SIRT1. Conversely, loss of HIF1α prevented the decrease in TFAM promoter activity mediated by inhibition of SIRT1. Together, these findings clearly show that in addition to its ability to regulate PGC-1α, SIRT1 is an important regulator of mtDNA maintenance and expression mitochondrial-encoded genes through HIF1α stabilization and regulation of c-Myc and TFAM.

Both SIRT1 and HIF1α have been found to be important regulators of longevity in lower organisms (Leiser and Kaeberlein, 2010) and impaired mitochondrial function is believed to play a direct role in the development of cellular dysfunction with age (Figueiredo et al., 2008; Figueiredo et al., 2009; Hartmann et al., 2011; Lanza and Nair,

2010). However, the causes of the age-induced disruption in mitochondrial homeostasis are yet to be understood. The involvement of two different genomes creates a demand for a tight coordination of the expression of genes encoded in both genomes in order to promote functional and efficient mitochondria (Scarpulla, 2011b). The mitochondrial-encoded components of the ETC are important for stabilization of the ETC complexes and absolutely required for proper function of complex I, III, IV and V (Wallace et al., 2010a). Therefore, an impaired ability to induce mitochondrial biogenesis in a coordinated fashion may lead to deficiencies in mitochondrial respiration by limiting basal expression of mitochondrial-encoded components of the ETC. Our data suggest that the imbalance between nuclear and mitochondrial-encoded genes observed in SIRT1 KO animals might play a role in the development of mitochondrial dysfunction that happens with age. This work demonstrates that NAD^+ levels and mtDNA content declines progressively with age, and this corresponds to a decrease in mitochondrial function. Additionally, expression of mitochondrial-encoded ETC components was selectively reduced in 22-24 month old animals, while expression of nuclear-encoded ETC components was either increased or unaltered. This demonstrates that the ability to synchronize the induction of mitochondrial biogenesis in the nucleus and mitochondria is lost with age and suggests that this might be caused by a decline in NAD^+ levels and consequent loss of SIRT1 activity.

Importantly, while mtDNA integrity was found to be decreased in 30 month old animals, 22 month old animals did not show significant changes in mtDNA integrity, indicating that the impaired ability to maintain expression levels of mitochondrial-encoded genes, mtDNA content and therefore the decline in mitochondrial function, precedes the accumulation of mtDNA damage. Indeed, our data suggest that once mitochondrial homeostasis is disrupted due to loss of SIRT1 activity and consequent loss of functional communication between the nuclear and mitochondrial genomes, it further exacerbates mitochondrial dysfunction and leads to increase oxidative damage and cellular decline in older animals.

Consistent with this idea, long-term CR or treatment with NMN caused an increase in NAD^+ in skeletal muscle, which was sufficient to prevent the imbalance between nuclear and mitochondrial-encoded genes and the development of mitochondrial dysfunction in 22-24 month old animals. Importantly, the changes in mitochondria caused by increase in NAD^+ were found to be SIRT1 dependent, as none of the effects observed with NMN treatment were seen in SIRT1 KO animals. Moreover,

and in accordance to the potential role of HIF1α in the regulation of lifespan in lower organisms (Leiser and Kaeberlein, 2010), HIF1α protein levels were shown to increase with age in liver and shown to be prevented by CR (Kang et al., 2005). Consistent with this, our work shows that both CR and boosting NAD^+ levels with NMN treatment can prevent/reverse the stabilization of HIF1α observed in the skeletal muscle of 22-24 month old animals, suggesting that the HIF1α destabilization might underlie the maintenance of the expression of mitochondrial-encoded genes as well as mtDNA amount. Further supporting this idea, the ability of NMN to regulate mitochondrial-encoded genes was lost under conditions where HIFα was stabilized.

Together, our work demonstrates that SIRT1 plays a dual role in regulating mitochondrial function both through deacetylation and activation of PGC-1α in response to fasting and through destabilization of HIF1α thereby maintaining c-Myc activity and TFAM expression, which consequently promotes a functional communication between the nuclear and mitochondrial genomes. We further demonstrate that declining NAD^+ levels promote an imbalance between nuclear and mitochondrial-encoded genes and the development of mitochondrial dysfunction with age, as boosting NAD^+ levels in old animals was sufficient to prevent accumulation of HIF1α, induce expression of mitochondrial-encoded ETC components and restore mitochondrial function in aged mice. Moreover, these results suggest that an impaired ability of SIRT1 to coordinate mitochondrial biogenesis may play a direct role in the development of mitochondrial dysfunction with age, which is further demonstrated by the requirement of SIRT1 for NMN to improve mitochondrial function. These findings highlight the importance of SIRT1 in coordinating mitochondrial homeostasis both by deacetylation and activation of PGC-1α, and by a PGC-1α-independent regulation of mitochondrial-encoded genes through regulation of HIF1α, c-Myc and TFAM, and emphasize the potential for molecules capable of elevating NAD^+ levels or activating SIRT1 to treat a number of age related diseases caused by disruption of mitochondrial homeostasis (Figure 3.2.23).

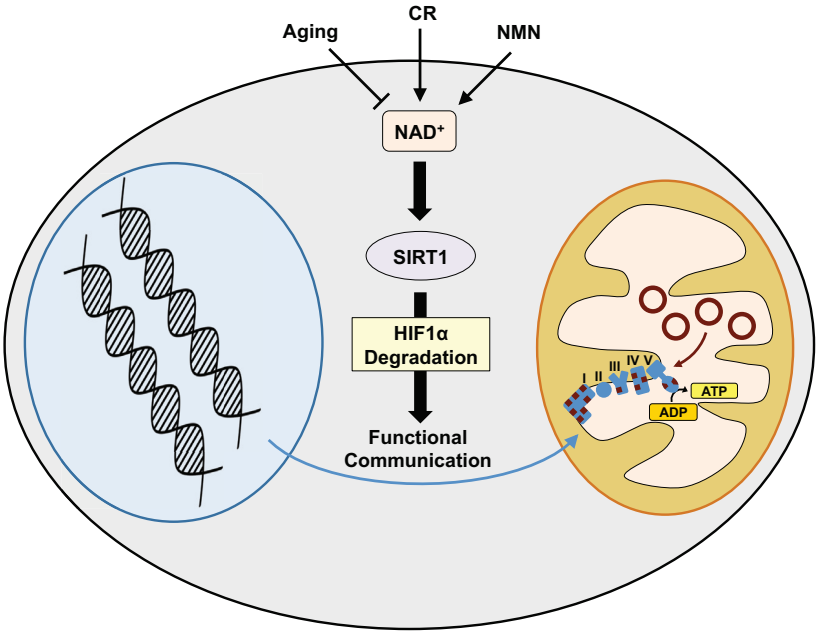


Figure 3.1.23 – Proposed mechanism of regulation of mitochondrial homeostasis by SIRT1 through regulation of HIF1α in response to fluctuations in NAD^+ levels.

3.2. SIRT1 is required for AMPK activation and the beneficial effects of Resveratrol in mitochondrial function

3.2.1. Summary

Resveratrol induces mitochondrial biogenesis and protects against metabolic decline but whether SIRT1 mediates these benefits is still subject of debate. To circumvent the developmental defects of germ-line SIRT1 knockouts, we have developed the first inducible system that permits whole-body deletion of SIRT1 in adult mice. Mice treated with a moderate dose of resveratrol showed increased mitochondrial biogenesis and function, AMPK activation and increased NAD^+ levels in skeletal muscle, whereas SIRT1 knockouts displayed none of these benefits. A mouse overexpressing SIRT1 mimicked these effects. A high dose of resveratrol activated AMPK in a SIRT1-independent manner, demonstrating that resveratrol dosage is a critical factor. Importantly, at both doses of resveratrol no improvements in mitochondrial function were observed in animals lacking SIRT1. Together these data indicate that SIRT1 plays an essential role in the ability of moderate doses of resveratrol to stimulate AMPK and improve mitochondrial function both *in vitro* and *in vivo.*

3.2.2. Introduction

SIRT1 is involved in numerous fundamental cellular processes including gene silencing, DNA repair, and metabolic regulation (Baur, 2010; Donmez and Guarente, 2010; Haigis and Sinclair, 2010). Deletion of SIRT1 in outbred strains of mice abrogates the effect of CR on physical activity (Chen et al., 2005) and lifespan extension (Boily et al., 2008), whereas overexpression of SIRT1 mimics many of the salutary effects of CR, including a reduced incidence of cardiovascular and metabolic diseases (Banks et al., 2008; Bordone et al., 2007; Pfluger et al., 2008), cancer (Herranz et al., 2010b; Oberdoerffer et al., 2008), and neurodegeneration (Donmez et al., 2010; Qin et al., 2006). Recent human genetic studies also support a role for SIRT1 in maintaining human health status with age (Dong et al., 2011; Rutanen et al., 2010).

The polyphenol resveratrol (2,3,4'-trihydroxystilbene) first attracted scientific attention when it was linked to the cardiovascular benefits of red wine and was subsequently found to possess potent anti-tumor activity (Jang et al., 1997). In 2003, a screen for small molecule activators of SIRT1 identified 21 different SIRT1-activating molecules, the most potent of which was resveratrol (Howitz et al., 2003). In the majority

of studies to date, resveratrol has been found to increase lifespan in *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, and *Drosophila melanogaster* in a sirtuin-dependent manner, although the lifespan extension in yeast and flies, and the Sir2-dependence in worms have been challenged (Agarwal and Baur, 2011). In addition, resveratrol extends life and delays the onset of age-related phenotypes in a short-lived species of fish (Valenzano et al., 2006).

In obese rodents, treatment with resveratrol produces a variety of health benefits including improved metabolic and vascular function, decreased hepatic steatosis, reduced inflammation, greater endurance, and a gene expression pattern resembling calorie restriction (Barger et al., 2008a; Barger et al., 2008b; Baur et al., 2006; Lagouge et al., 2006; Pearson et al., 2008; Ramadori et al., 2009). Recent clinical studies show that resveratrol also confers metabolic benefits in humans (Brasnyo et al., 2011; Crandall JP, 2012). Understanding how resveratrol exerts its effects is important, not only for the potential insights into the biological causes of age and metabolic-related diseases, but also to allow the development of more potent and specific molecules.

One of the most robust and reproducible effects of resveratrol treatment is an increase in mitochondrial mass (Baur et al., 2006; Lagouge et al., 2006). Indeed, SIRT1 plays an important role in the maintenance of mitochondrial homeostasis. On one hand promotes mitochondrial biogenesis through deacetylation and activation of PGC-1α (Gerhart-Hines et al., 2007; Rodgers et al., 2005), a master regulator of mitochondrial biogenesis that co-activates NRF-1 and NRF-2, which induce the transcription of genes involved in mitochondrial biogenesis (Scarpulla, 2011a). On the other hand SIRT1 was also found to regulate mitochondrial-encoded genes in a PGC-1α-independent manner through regulation of HIF1α stability (chapter 3.1), pointing out the relevance of SIRT1 for mitochondrial metabolism and for diseases where disturbances in mitochondrial homeostasis play an important role. Interestingly, PGC-1α is not only regulated by SIRT1 but is also activated by AMPK (Jager et al., 2007). Though the effects of resveratrol and SIRT1 on PGC-1α are well established, there is considerable debate about the mechanism by which this regulation is achieved. One school of thought is that the direct activation of SIRT1 by resveratrol is an *in vitro* artifact (Borra et al., 2005; Kaeberlein et al., 2005) (Pacholec et al., 2010) and that resveratrol works primarily by activating AMPK (Canto et al., 2009), potentially by inhibition of phospodiesterases (PDE), ATPase, or complex III (Gledhill et al., 2007; Hawley et al., 2010b; Park et al., 2012; Zini et al., 1999). It has been proposed that AMPK then activates SIRT1 indirectly

by elevating intracellular levels of its co-substrate, NAD^+ (Canto et al., 2009; Fulco et al., 2008). Alternatively, resveratrol may first activate SIRT1 *in vivo*, leading to AMPK activation via deacetylation and activation of the AMPK kinase LKB1 (Hou et al., 2008; Ivanov et al., 2008; Lan et al., 2008).

Unfortunately, studies to date have been unable to determine which model is most relevant under physiological conditions. We and others have shown that resveratrol activates AMPK in cell culture and *in vivo* (Baur et al., 2006; Dasgupta and Milbrandt, 2007) and a study of AMPK knockout mice established that AMPK is required for many of the beneficial effects of resveratrol on metabolic function (Um et al., 2010). On the other hand, recent enzymological studies have presented evidence for direct SIRT1 activation by small molecules (Dai et al., 2010) and there is a growing literature of cell culture studies in which the effects of resveratrol are lost after knocking down or inhibiting SIRT1 (Breen et al., 2008; Csiszar et al., 2009; Fischer-Posovszky et al., 2010; Gracia-Sancho et al., 2010; He et al., 2010; Ivanov et al., 2008; Kao et al., 2010; Kim et al., 2011a; Li et al., 2010; Lin et al., 2010; Ohguchi et al., 2010; Park et al., 2010; Shindler et al., 2010; Sulaiman et al., 2010; Tanno et al., 2010; Ungvari et al., 2009; Vetterli et al., 2011; Xia et al., 2011a; Yang et al., 2010; Yoshizaki et al., 2010). Moreover, resveratrol's central effects on liver gluconeogenesis (Ramadori et al., 2009) are abrogated when SIRT1 activity is impaired in the hypothalamus (Knight et al., 2011), and treatment of mice with a SIRT1 activator that is structurally unrelated to resveratrol, SRT1720, increases mitochondrial capacity in skeletal muscle (Feige et al., 2008) and liver in a SIRT1-dependent manner (Minor et al., 2011), while improving the health and survival of mice on a high fat diet, similar to what has been observed with resveratrol (Minor et al., 2011).

As such, this study was aimed to answer these questions and therefore at testing whether the ability of resveratrol to activate AMPK and increase mitochondrial function requires SIRT1 *in vivo*, and whether SIRT1 overexpression is sufficient to mimic these effects.

3.2.3. Results

3.2.3.1 Resveratrol Improves Mitochondrial Function and Increases Mitochondrial Biogenesis in a SIRT1-Dependent Manner

While both overexpression of SIRT1 and treatment with resveratrol have been shown to increase mitochondrial content via activation of PGC-1α, it remains to be established whether SIRT1 is required for resveratrol to improve mitochondrial function in skeletal muscle. Our initial investigation was performed using C2C12 myoblasts, a murine skeletal muscle cell line. Following treatment with 25 μM resveratrol, C2C12 cells showed a significant increase in mitochondrial membrane potential (Figure 3.2.1A) and cellular ATP content (Figure 3.2.1B). Consistent with the work presented in chapter 3.1, treatment with the SIRT1 inhibitor EX-527 or knockdown of SIRT1 consistently reduced mitochondrial membrane potential and ATP content and completely abolished the ability of resveratrol to improve these parameters (Figure 3.2.1A-E).

Further analysis revealed that resveratrol treatment increased mtDNA copy number in control cells (Figure 3.2.1F and 1G), suggesting that increased mitochondrial biogenesis may underlie the ability of resveratrol to improve mitochondrial function. Similar to the results with mitochondrial membrane potential and ATP, knockdown of SIRT1 or EX-527 treatment completely blocked the ability of resveratrol to increase mtDNA copy number. Immunoprecipitation of the SIRT1 target PGC-1α showed a substantial decrease in PGC-1α acetylation in resveratrol-treated cells, consistent with previous reports (Baur et al., 2006; Lagouge et al., 2006). Importantly, resveratrol treatment had no effect on PGC-1α acetylation in cells lacking SIRT1 (Figure 3.2.1H). Consistent with these findings, resveratrol treatment increased mRNA expression of a number of genes downstream of PGC-1α including transcription factors responsible for stimulating mitochondrial biogenesis (NRF-1, NRF-2) and components of the mitochondrial electron transport chain (NDUFS8, SDHb, Uqcrc1, COX5b, ATP5a1). All of the increases in gene expression were absent in cells in which SIRT1 expression was knocked down (Figure 3.2.1I).

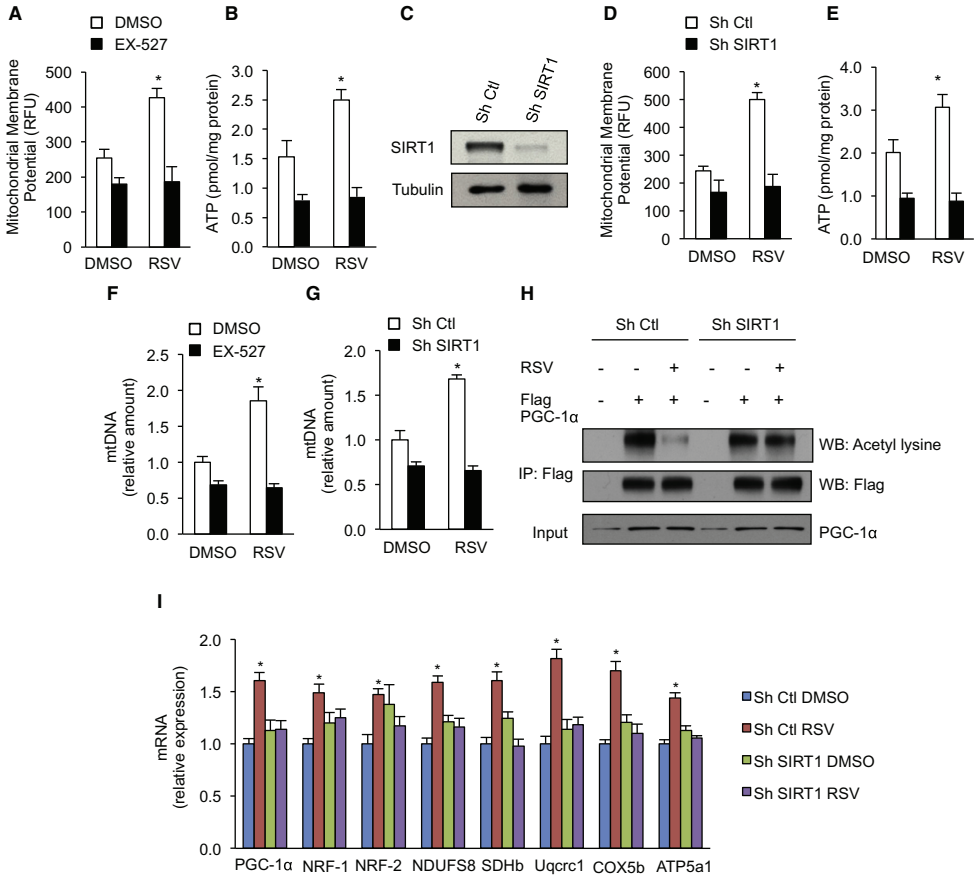


Figure 3.2.1 - Improved mitochondrial function and increased mitochondrial biogenesis in response to resveratrol treatment requires SIRT1. (A) Mitochondrial membrane potential and (B) ATP content in C2C12 cells treated with 25 μM resveratrol and 10 μM EX-527 for 24h. (C) Representative immunoblot for SIRT1 and tubulin in C2C12 cells infected with SIRT1 or nontargeting shRNA. (D) Mitochondrial membrane potential and (E) ATP content in C2C12 cells infected with SIRT1 or nontargeting shRNA and treated with 25 μM resveratrol for 24h. (F) Mitochondrial DNA content analyzed by means of quantitative PCR in C2C12 cells treated with 10 μM EX-527 or (G) infected with SIRT1 or nontargeting shRNA and treated with 25μM resveratrol. Relative expression values were normalized to untreated cells. (H) C2C12 cells infected with SIRT1 or nontargeting shRNA, and expressing Flag-HA-PGC-1α were treated with resveratrol 25 μM for 24h and PGC-1α acetylation was tested in Flag immunoprecipitates. Total PGC-1α was evaluated on total extracts as input. (I) PGC-1α, NRF-1, NDUFS8, SDHb, Uqcrc1, COX5b, ATP5a1 mRNA analyzed by means of quantitative RT-PCR in C2C12 cells infected with SIRT1 or nontargeting shRNA after 24h treatment with 25 μM resveratrol. Relative expression values were normalized to untreated cells. Values are expressed as mean ± SEM. (*$p < 0.05$ versus DMSO).

3.2.3.2. Resveratrol Improves Mitochondrial Function in Skeletal Muscle of WT Mice, but has no Effect on Adult Inducible SIRT1 KO Mice

To test whether these findings were relevant *in vivo*, we used our newly generated adult-inducible SIRT1 KO mice. Following tamoxifen treatment, SIRT1 KO and control mice were placed on one of four different diets: a standard diet (SD), a high fat diet (HF; 60% FDC), a high fat diet supplemented with 400 mg resveratrol/kg of food (HFLR) or a high fat diet supplemented with a high dose of 4 g resveratrol/kg of food (HFHR). The former is a relatively low dose used in our laboratories' previous studies, while the latter dose is similar to the concentrations used by other groups (Lagouge et al., 2006) (Figure 3.2.2). Feeding of these diets resulted in an approximate daily dose of 25-30 mg/kg/day and 215-230 mg/kg of body weight/day, respectively.

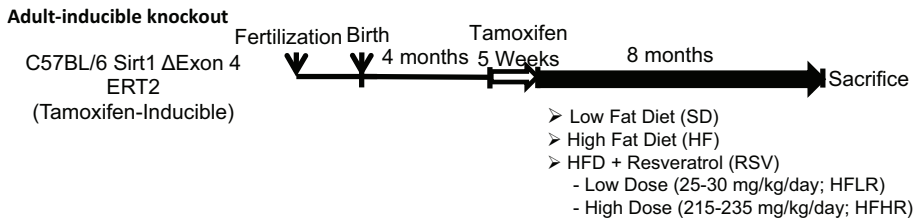


Figure 3.2.2 - Schematic representation of induction of SIRT1 KO and treatment with the different diets.

The function of mitochondria isolated from skeletal muscle was significantly impaired by feeding of a high fat diet, while treatment with both high and low doses of resveratrol prevented these deleterious effects. At both low and high doses, resveratrol produced substantial increases in ADP-induced respiration (State 3), maximal respiration (FCCP-induced), mitochondrial membrane potential, and cellular ATP levels (Figure 3.3.2C-G). Strikingly, none of the significant increases in mitochondrial function seen in the WT mice treated with resveratrol were observed in SIRT1 KO mice (Figure 3.3.2C-G). While the beneficial effects of resveratrol were clearly evident in animals treated with both doses of resveratrol, the variability between animals receiving the higher dose was considerably greater. For this reason, the majority of our subsequent analyses focused on the cohorts receiving the lower dose of resveratrol (i.e. 25-30 mg/kg/day).

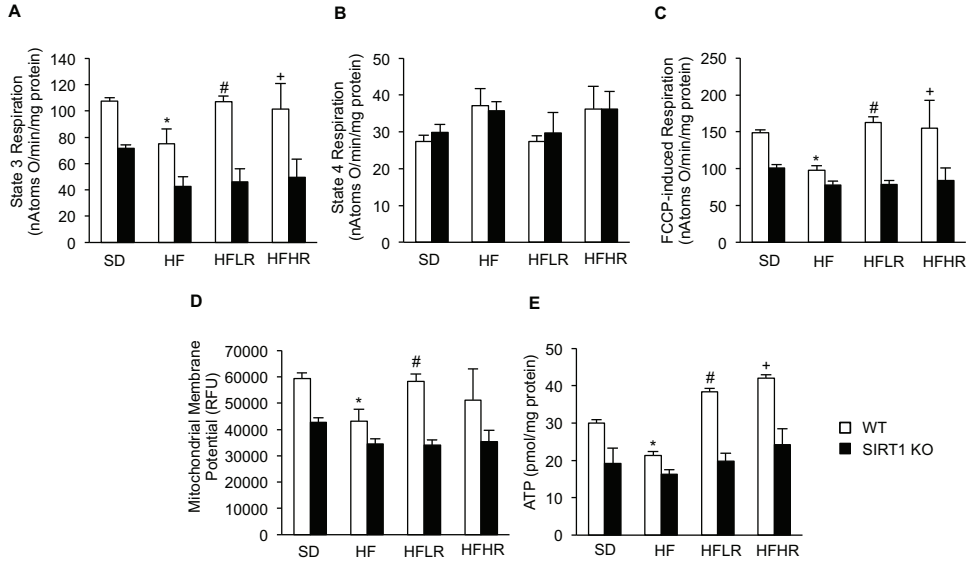


Figure 3.3.2 - Adult inducible SIRT1 KO mice revealed that ability of resveratrol to improve mitochondrial function requires SIRT1 in vivo. (A) State 3 respiration of isolated mitochondria from skeletal muscle of WT and SIRT1 KO mice on experimental diets (n=8) (*p<0.05 versus WT SD, #p<0.05 versus WT HFD). (B) State 4 respiration of isolated mitochondria from skeletal muscle of WT and SIRT1 KO mice on experimental diets (n=8). (C) FCCP-induced respiration of isolated mitochondria from skeletal muscle of WT and SIRT1 KO mice on experimental diets (n=8) (*p<0.05 versus WT SD, #p<0.05 versus WT HFD). (D) Mitochondrial membrane potential of isolated mitochondria from skeletal muscle of WT and SIRT1 KO mice on experimental diets (n=8) (*p<0.05 versus WT SD, #p<0.05 versus WT HFD). (E) Cellular ATP content from gastrocnemius of WT and SIRT1 KO mice on experimental diets (n=8) (*p<0.05 versus WT SD, #p<0.05 versus WT HFD, +p<0.05 versus WT HFD). Values are expressed as mean ± SEM.

3.2.3.3. Treatment with Resveratrol Induces a SIRT1-Dependent Shift Toward more Oxidative Muscle Fibers

In addition to impairment in mitochondrial function, feeding of a high fat diet is known to cause an increased abundance of glycolytic muscle fibers. Thus, we tested whether treatment with resveratrol counteracted these changes and, if so, whether SIRT1 was required. Gene expression analysis of myosin heavy-chain genes from gastrocnemius muscle indicated that while the number of Type I muscle fibers was not altered by resveratrol, the abundance of highly glycolytic fast twitch (Type IIb) muscle fibers was lower, while more oxidative fast twitch (Type IIa and IIx) fibers were more abundant in resveratrol-treated mice (Figure 3.2.3A), consistent with the findings of

Lagouge *et al* (2006). Together, these changes indicate an overall shift toward more oxidative fiber types in response to resveratrol treatment.

Interestingly, as with the measures of mitochondrial function, these changes in muscle type were entirely dependent upon SIRT1 (Figure 3.2.3A). The shift towards more oxidative fibers and the SIRT1-dependence of these effects was not exclusive to gastrocnemius, as similar gene expression changes were observed in the soleus muscle as well (Figure 3.2.3B). This induction of oxidative type II fibers in response to resveratrol treatment in WT but not SIRT1 KO mice was confirmed by Western blot and histological analysis (Figure 3.2.3C and 3.2.3D). These data demonstrate that the ability of resveratrol to induce a shift toward more oxidative muscle fibers, improve mitochondrial function, and increase cellular ATP requires SIRT1. Importantly, an in accordance to the HIF1α stabilization observed in the skeletal muscle of SIRT1 KO animals and its consequent changes in the genetic profile (chapter 3.1), SIRT1 KO animals also have increased more glycolytic muscle fibers (Figure 3.2.3A-C).

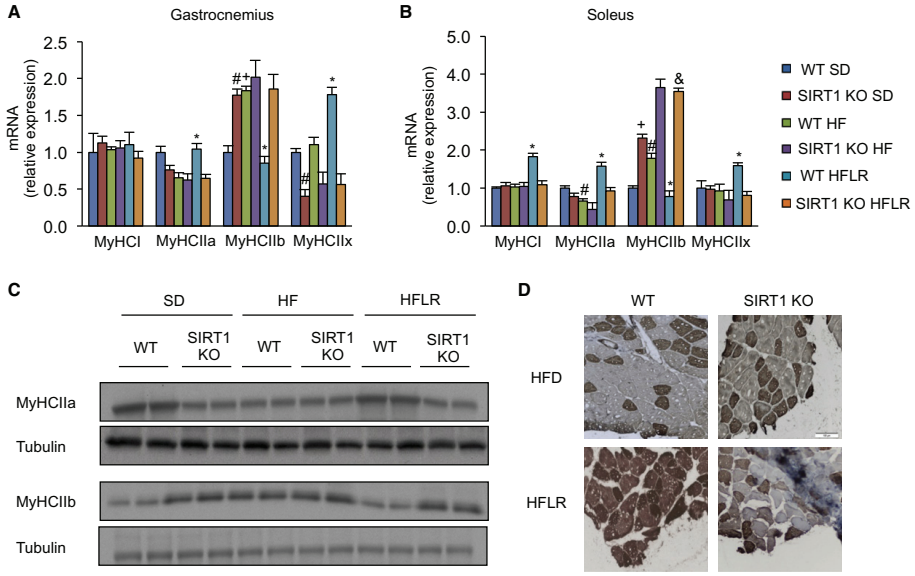


Figure 3.2.3 - Resveratrol induces a shift toward more oxidative fibers in a SIRT1 dependent manner. (A-B) MyHCI, MyHCIIa, MyHCIIb and MyHCIIx mRNA analyzed by quantitative RT-PCR in gastrocnemius (A) and soleus (B) of WT and SIRT1 KO mice on experimental diets. Relative expression values were normalized to WT SD mice (n=5) *p<0.05 versus WT HFD, #p<0.05 versus WT SD, +p<0.05 versus WT SD). (C) Representative immunoblot for MyHCIIa, MyHCIIb and tubulin in gastrocnemius of WT and SIRT1 KO mice on experimental diets. (D) Representative MyHCIIa immunostaining in gastrocnemius of WT and SIRT1 KO mice on experimental diets. Values are expressed as mean ± SEM.

3.2.3.4. Stimulation of Mitochondrial Biogenesis in Skeletal Muscle Requires Functional SIRT1

We next sought to understand more precisely the mechanisms by which resveratrol increases mitochondrial function and consequently ATP production, and to investigate their relationship to SIRT1. Based on previous work (Baur et al, 2006; Lagouge et al, 2006), we expected that resveratrol treatment would increase mitochondrial biogenesis and we hypothesized that these effects would be dependent upon SIRT1. We first assessed citrate synthase activity, a commonly used marker of mitochondrial content. Consistent with the changes observed in fiber type and mitochondrial function, the high fat diet decreased citrate synthase activity in the gastrocnemius of WT mice and resveratrol treatment completely prevented this decrease. Similarly, mRNA levels of components of the mitochondrial ETC were increased by resveratrol treatment. Interestingly, none of these effects were observed in SIRT1 KO mice (Figure 3.2.4).

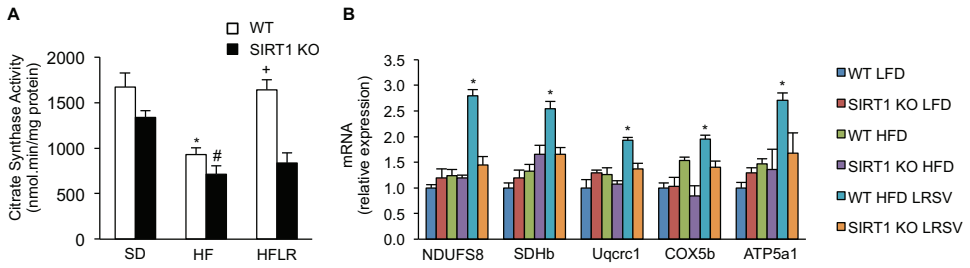


Figure 3.2.4- Resveratrol improves citrate synthase activity and increases ETC components in skeletal muscle of WT but not SIRT1 KO mice. (A) Citrate synthase activity measured in gastrocnemius from WT and SIRT1 KO mice on experimental diets (n=8) (*p<0.05 versus WT SD, #p<0.05 versus SIRT1 KO SD, +p<0.05 versus WT HFD). (B) NDUFS8, SDHb, Uqcrc1, COX5b and ATP5a1 mRNA analyzed by quantitative RT-PCR in gastrocnemius of WT and SIRT1 KO mice on experimental diets. Relative expression values were normalized to WT SD mice. (n=5 experiments *p<0.05 versus WT HFD). Values are expressed as mean ± SEM.

Consistent with the results in C2C12 cells, the decrease in mtDNA copy number in HFD mice was prevented by resveratrol treatment in the gastrocnemius of WT mice, while the SIRT1 KO mice showed no response to resveratrol treatment (Figure 3.2.5A). Measurement and quantification of mitochondrial mass by electron microscopy showed that resveratrol treatment induced an increase in mitochondrial area in WT but not in SIRT1 KO mice (Figure 3.2.5B).

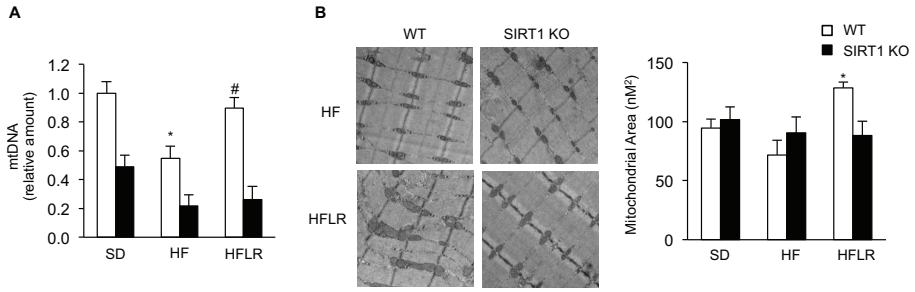


Figure 3.2.5- Resveratrol increases mitochondrial mass in skeletal muscle of WT but not SIRT1 KO mice. (A) Mitochondrial DNA content analyzed by quantitative PCR in gastrocnemius of WT and SIRT1 KO mice on experimental diets. Relative expression values were normalized to WT SD mice. (n=8 experiments *p<0.05 versus WT SD, #p<0.05 versus WT HFD). (B) Electronic microscopy analysis of gastrocnemius from WT and SIRT1 KO mice on experimental diets and the respective mitochondrial area quantification (n=4) (*p<0.05 versus WT HFD). Values are expressed as mean ± SEM.

Additionally, transcript levels of PGC-1α, PGC-1β, and the mitochondrial transcription factors TFAM and TFB2M, were increased in resveratrol treated mice in a SIRT1-dependent manner (Figure 3.2.6). Overall, our findings from adult-inducible SIRT1 KO mice demonstrate that resveratrol increases mitochondrial biogenesis, induces a shift toward more oxidative muscle fibers, and improves mitochondrial function in mice on a high fat diet, and that all of these beneficial effects require SIRT1.

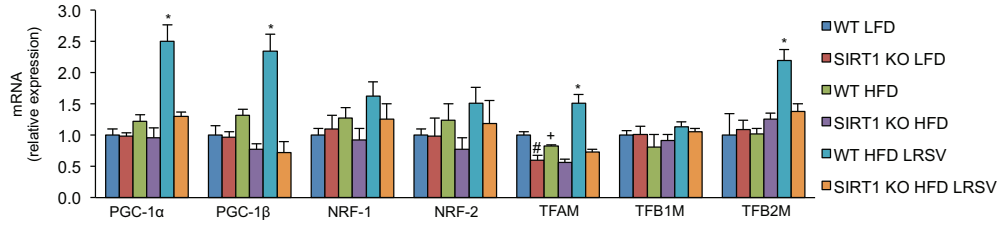


Figure 3.2.6- Resveratrol improves mitochondrial biogenesis in skeletal muscle of WT but not SIRT1 KO mice. PGC-1α, PGC-1β, NRF-1, NRF-2, TFAM, TFB1M and TFB2M mRNA analyzed by means of quantitative RT-PCR in gastrocnemius of WT and SIRT1 KO mice on experimental diets. Relative expression values were normalized to WT SD mice. (n=5) (*p<0.05 versus WT HFD, #p<0.05 versus WT SD, +p<0.05 versus WT SD). Values are expressed as mean ± SEM.

3.2.3.5. Overexpression of SIRT1 Mimics the Effects of Resveratrol Treatment in Skeletal Muscle

These data are consistent with the hypothesis that resveratrol acts via SIRT1 to increase mitochondrial function *in vivo*. This hypothesis also predicts that SIRT1 overexpression should be sufficient to mimic the effects of resveratrol. To test this, we generated a whole body SIRT1 transgenic mouse (SIRT1-Tg) that constitutively expresses high levels of SIRT1 (Figure 3.2.7). This mouse differs from previous whole-body SIRT1 transgenics that either overexpress SIRT1 at lower levels (1.5 – 2 fold) (Banks et al., 2008; Pfluger et al., 2008) or predominately in brain and adipose tissue (Bordone et al., 2007). Interestingly, despite the high levels of SIRT1 expression in at least skeletal muscle, liver and brain (>5X), there was no detectable difference in overall appearance, body weight, or home cage behavior.

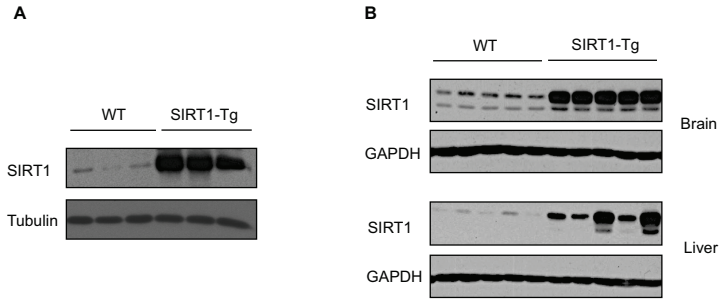


Figure 3.2.7- Expression levels of SIRT1 in a whole body SIRT1 overexpressor mice. (A) Representative immunoblot for SIRT1 and tubulin in gastrocnemius of 6 months old WT and SIRT1 Tg mice. (B) Representative immunoblot for SIRT1 and GAPDH in brain and liver of 6 months old WT and SIRT1 Tg mice.

In a striking recapitulation of the effects of resveratrol treatment, mitochondria isolated from the skeletal muscle of the SIRT1-Tg mice had significantly greater mitochondrial membrane potential, State 3 respiration, and maximal respiration (Figure 3.2.8).

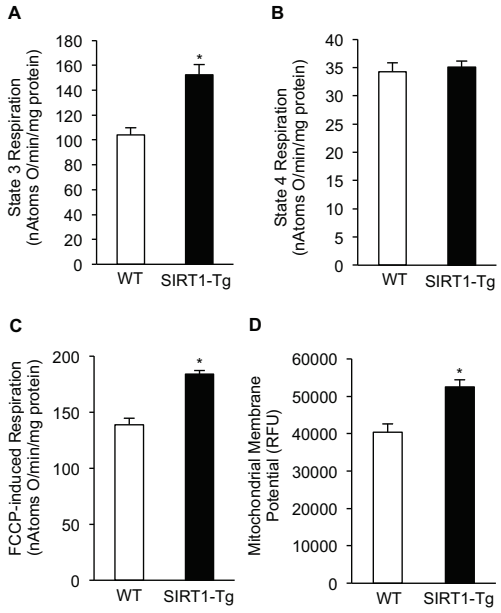


Figure 3.2.8- Mice overexpressing SIRT1 mimic the effects of resveratrol on mitochondrial function. (A) State 3 respiration of isolated mitochondria from skeletal muscle of WT and SIRT1 Tg mice (n=6). (B)

State 4 respiration of isolated mitochondria from skeletal muscle of WT and SIRT1 Tg mice (n=6). (C) FCCP-induced respiration of isolated mitochondria from skeletal muscle of WT and SIRT1 Tg mice (n=6). (D) Mitochondrial membrane potential of isolated mitochondria from skeletal muscle of WT and SIRT1 Tg mice (n=6). Values are expressed as mean ± SEM (*p < 0.05 versus WT).

Additionally, mtDNA copy number (Figure 3.2.9A) as well as mRNA levels of ETC components and regulators of mitochondrial biogenesis that were induced by resveratrol treatment were similarly upregulated in mice overexpressing SIRT1, while those unaffected by resveratrol were also unchanged in SIRT1-Tg mice (Figure 3.2.9B and 3.2.9C). Together these data show for the first time that overexpression of SIRT1 in skeletal muscle is sufficient to induce mitochondrial biogenesis and improve mitochondrial function. When coupled with the SIRT1 KO data establishing that SIRT1 is required for the beneficial effects of resveratrol, these results provide strong evidence that the ability of resveratrol to improve mitochondrial function in skeletal muscle is due to SIRT1-mediated stimulation of mitochondrial biogenesis.

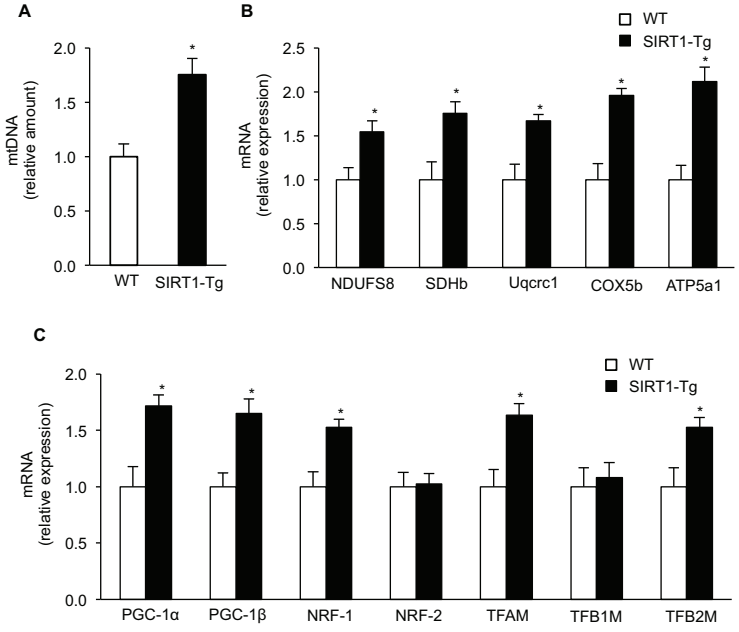


Figure 3.2.9 - Mice overexpressing SIRT1 mimic the effects of resveratrol on mitochondrial biogenesis. (A) Mitochondrial DNA content analyzed by means of quantitative PCR in skeletal muscle of WT and SIRT1 Tg mice. Relative expression values were normalized to WT mice. (n=6). (B) NDUFS8, SDHb, Uqcrc1, COX5b and ATP5a1 mRNA analyzed by quantitative RT-PCR in gastrocnemius of WT and SIRT1 Tg mice. Relative expression values were normalized to WT mice. (n=6). (C) PGC-1α, PGC-1β, NRF-

1, NRF-2, TFAM, TFB1M and TFB2M mRNA analyzed by quantitative RT-PCR in gastrocnemius of WT and SIRT1 Tg mice. Relative expression values were normalized to WT mice. (n=6). Values are expressed as mean ± SEM (*p < 0.05 versus WT).

3.3.2.6. SIRT1 is Required for Activation of AMPK by Resveratrol

Given the complex interplay between SIRT1 and AMPK and their overlapping effects, it has been difficult to untangle their roles in mediating the effects of resveratrol but the inducible SIRT1 KO mouse presented us with an opportunity to test their epistasis. Our previous work showed that resveratrol increases levels of activated (phosphorylated) AMPK *in vivo* (Baur et al., 2006), indicating that AMPK may play a role in mediating resveratrol's benefits. This was supported by a subsequent study demonstrating that AMPK is required for many of the metabolic effects of resveratrol *in vivo* (Um et al., 2010). Consistent with this, the ability of resveratrol and SIRT1 overexpression to boost cellular ATP and mtDNA copy number was prevented by knockdown of the catalytic subunit of AMPK, AMPKα1 (Figure 3.2.10).

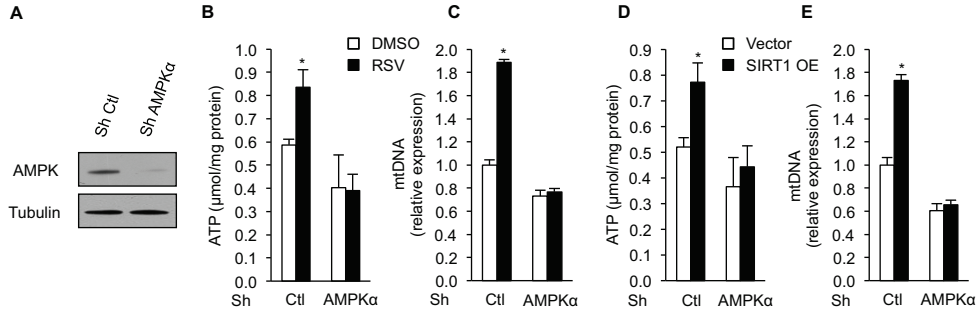


Figure 3.2.10 – AMPK is necessary for induction of mitochondria by both both resveratrol and SIRT1. (A) Representative immunoblot for AMPKα and tubulin in C2C12 cells infected with AMPKα or nontargeting shRNA. (B) ATP content in C2C12 cells infected with AMPKα or nontargeting shRNA.and treated with 25 µM resveratrol for 24h (n=5). (C) Mitochondrial DNA content analyzed by quantitative PCR in C2C12 cells infected with AMPKα or nontargeting shRNA.and treated with 25 µM resveratrol for 24h. Relative expression values were normalized to WT. (n=5). (D) ATP content in C2C12 cells infected with AMPKα or nontargeting shRNA.and treated with adenovirus overexpressing SIRT1 or empty vector (n=5). (E) Mitochondrial DNA content analyzed by quantitative PCR in C2C12 cells infected with AMPKα or nontargeting shRNA.and with adenovirus overexpressing SIRT1 or empty vector. Relative expression values were normalized to control. (n=5). Values are expressed as mean ± SEM (*p<0.05 versus empty DMSO).

Treatment with a moderate dose of resveratrol increased the levels of phosphorylated AMPK and NAD^+ in gastrocnemius of WT mice (Figure 3.2.11). Strikingly, none of these changes were observed in SIRT1 KO mice. Interestingly, at the higher dose of resveratrol, many of these effects were SIRT1-independent, demonstrating that the dose is critical to the outcome (Figure 3.2.11).

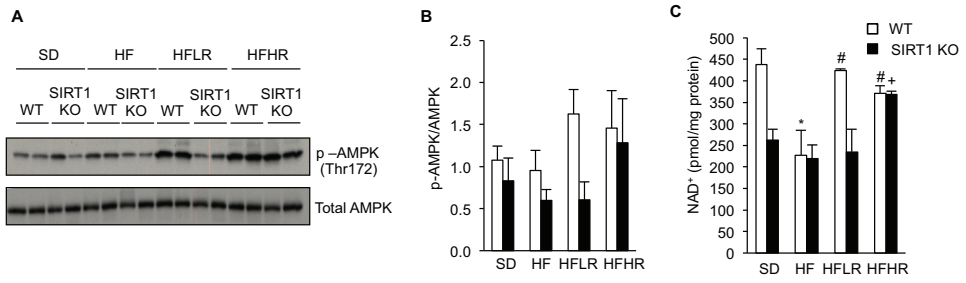


Figure 3.2.11 - Resveratrol activates AMPK through SIRT1 dependent and independent mechanisms depending on dose. (A) Representative immunoblot for p-AMPK (Thr172) and total AMPK in gastrocnemius of WT and SIRT1 KO mice on experimental diets. (B) Quantification of AMPK activity evaluated by the ratio of p-AMPK and AMPK in gastrocnemius of WT and SIRT1 KO mice on experimental diets (n=8). (C) NAD+ content in gastrocnemius of WT and SIRT1 KO mice on experimental diets (n=4) (*p<0.05 versus WT SD, #p<0.05 versus WT HF, +p<0.05 versus SIRT1 KO HF). Values are expressed as mean ± SEM.

Importantly, and giving further strength to the idea that SIRT1 activation can induce AMPK activity, SIRT1 overexpressing mice had significantly increased levels of AMPK phosphorylation (Figure 3.2.12). Together these data demonstrate that SIRT1 is necessary for moderate doses of resveratrol to activate AMPK and increase NAD^+ and that SIRT1 can act upstream of AMPK. Interestingly, despite the different requirements for resveratrol to activate AMPK in the SIRT1 KO mice, neither dose of resveratrol improved mitochondrial function in the absence of SIRT1 (Figure 3.2.3).

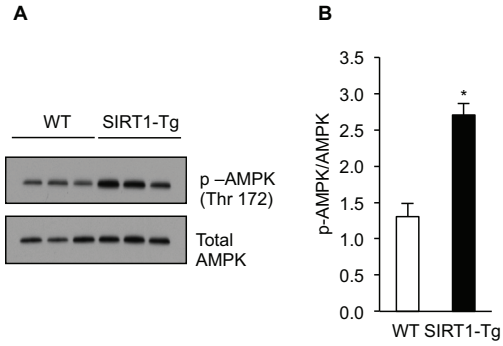


Figure 3.2.12 – SIRT1 overexpression is sufficient to induce AMPK in skeletal muscle. (A) Representative immunoblot for p-AMPK (Thr172), and total AMPK in gastrocnemius of WT and SIRT1 Tg mice. (B) Quantification of AMPK activity evaluated by the ratio of quantification of p-AMPK and AMPK in gastrocnemius of WT and SIRT1 Tg mice (n=6) (*p<0.05 versus WT). Values are expressed as mean ± SEM.

Resveratrol has been implicated in the direct modulation of numerous targets (Baur and Sinclair, 2006), but it has been difficult to discern which of these targets are physiologically relevant. Part of the difficulty has arisen from the fact that doses of resveratrol given to animals are wildly variable and concentrations used on cells vary greatly as well (Kim et al., 2007a; Park et al., 2012). To provide some clarity, we performed a series of dose- and time-course experiments with resveratrol. Treatment of C2C12 cells with a moderate dose of resveratrol (25 μM) activated AMPK in a SIRT1-dependent manner, while at higher dose of resveratrol (50 μM) AMPK was activated in a SIRT1-independent manner (Figure 3.2.13A). Similarly, treatment with the lower dose of resveratrol for 24h mimicked the effects on muscle *in vivo* by increasing ATP and mitochondrial membrane potential. In contrast, the 50 μM dose reduced mitochondrial membrane potential and cellular ATP levels, indicative of mitochondrial dysfunction, an effect of resveratrol that was not observed *in vivo* (Figure 3.2.13B and 3.2.13C).

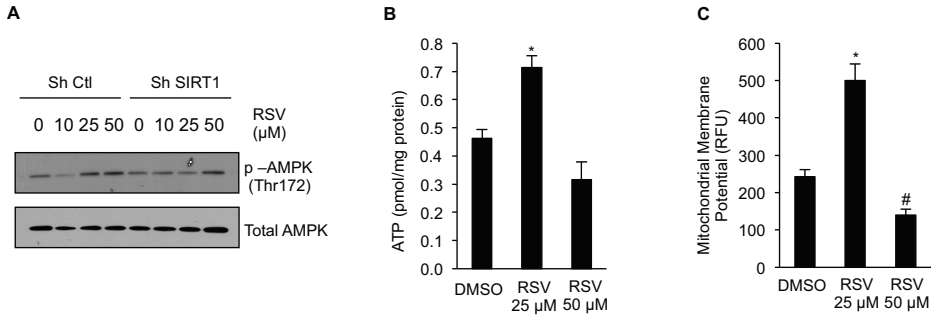


Figure 3.2.13 –Different doses of resveratrol have different effects in mitochondrial metabolism. (A) Representative immunoblot for p-AMPK (Thr172) and total AMPK in C2C12 cells infected with SIRT1 or nontargeting shRNA and treated with 10, 25 or 50 μM resveratrol for 24h. (B) ATP content in C2C12 cells treated with 25 or 50 μM resveratrol for 24h (n=4) (*p<0.05 versus DMSO). (C) Mitochondrial membrane potential in C2C12 cells treated with 25 or 50 μM resveratrol for 24h (n=4) (*p<0.05 versus DMSO). Values are expressed as mean ± SEM.

To give further insight into resveratrol's actions in mitochondrial metabolism and AMPK activation, we performed time-course treatments in C2C12 cells. Treatment with 25 μM resveratrol elevated ATP levels at 4, 6 and 12 hours, consistent with what we observed *in vivo* (Figure 3.2.14A). In contrast, the 50 μM dose significantly decreased ATP levels early as 1 hour after treatment. At the 25 μM dose, activation of AMPK occurred in a SIRT1-dependent manner, while the 50 μM dose activated AMPK independently of SIRT1 (Figure 3.2.14B). Importantly, the increase in ATP was evident eight hours before any changes in cellular NAD^+ levels were detected (Figure 3.2.14C), indicating that improvements in mitochondrial function and elevation of cellular ATP levels are both dependent upon SIRT1 and occur prior to increases in cellular NAD^+.

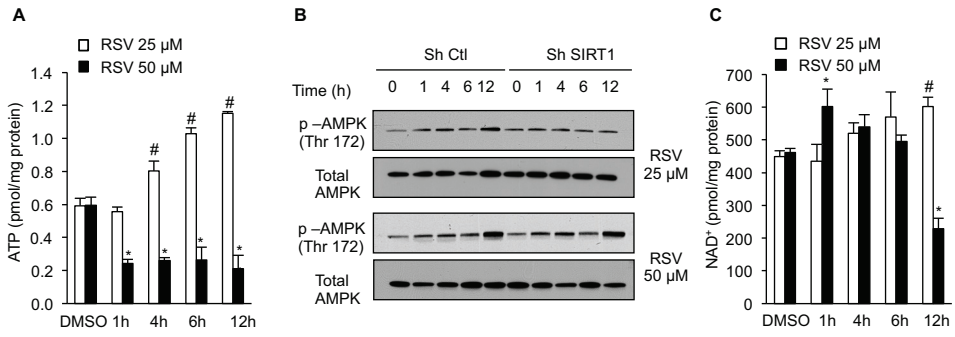


Figure 3.2.14 – Moderate doses of resveratrol activates AMPK in a SIRT1 dependent manner without causing mitochondrial toxicity. (A) ATP content in C2C12 cells treated with 25 or 50 μM resveratrol for 1, 4, 6 and 12h (n=4) (*p<0.05 versus DMSO). (B) Representative immunoblot for for p-AMPK (Thr172), and total AMPK in C2C12 cells treated with 25 or 50 μM resveratrol for 1, 4, 6 and 12h. (C) NAD^+ content in C2C12 cells treated with 25 or 50 μM resveratrol for 1, 4, 6 and 12h (n=4) (*p<0.05 versus 50 μM DMSO, #p<0.05 versus 25 μM DMSO). Values are expressed as mean ± SEM.

To further elucidate the epistasis of SIRT1 and AMPK, we treated primary myoblasts isolated from SIRT1 KO mice with AICAR, an AMP mimetic that directly activates AMPK and promotes its phosphorylation by LKB1. Phosphorylation of AMPK by AICAR was blunted in primary myoblasts lacking SIRT1 (Figure 3.2.15A) as well as the ability of AICAR to increase mitochondrial DNA copy number and ATP (Figure 3.2.15B and 3.2.15C).

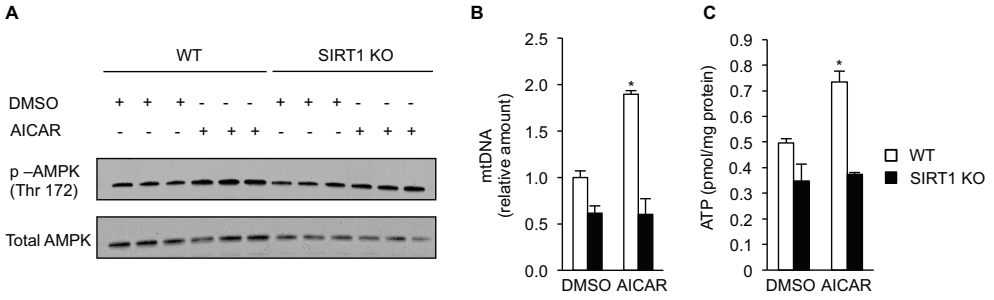


Figure 3.2.15 –SIRT1 is required for induction of AMPK and mitochondria by AICAR. (A) Representative immunoblot for for p-AMPK (Thr172), and total AMPK in primary myoblasts isolated from wild type and SIRT1 knockout mice and treated with 500 μM AICAR for 24h. (B) Mitochondrial DNA content analyzed by quantitative PCR in primary myoblasts isolated from wild type and SIRT1 knockout mice and treated with 500 μM AICAR for 24h. Relative expression values were normalized to control. (n=3) experiments (*p<0.05 versus empty DMSO). (C) ATP content in primary myoblasts isolated from wild type and SIRT1 knockout mice and treated with 500 μM AICAR for 24h (n=3) (*p<0.05 versus DMSO). Values are expressed as mean ± SEM.

In further support of SIRT1 acting upstream of AMPK, treatment of C2C12 cells with 25 μM resveratrol resulted in a SIRT1-dependent decrease in LKB1 acetylation (Figure 3.2.16). These findings are consistent with previous work done in C2C12 cells (Canto et al., 2009) and support previous findings that resveratrol-stimulated, SIRT1-mediated deacetylation of LKB1 plays a direct role in the activation of AMPK (Ivanov et al., 2008; Lan et al., 2008). Taken together, these findings show that treatment of mice on a high fat diet with moderate doses of resveratrol results in increased phosphorylation of AMPK, induction of mitochondrial biogenesis, increased ATP and NAD^+ levels, and a shift toward more oxidative muscle fibers, all of which are SIRT1-dependent effects.

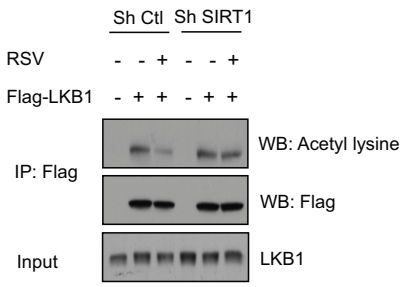


Figure 3.2.16 - Resveratrol activates AMPK in a SIRT1 dependent manner through deacetylation of LKB1. C2C12 cells infected with SIRT1 or nontargeting shRNA, and expressing Flag-LKB1 were treated with resveratrol 25 μM for 24h and LKB1 acetylation was tested in Flag immunoprecipitates. Total LKB1 was evaluated in total extracts as input.

3.3.3. Discussion

Genetic disorders with impaired mitochondrial function are characterized by a rapid onset of symptoms commonly seen in the elderly, such as type II diabetes, muscle loss, and neurodegeneration (Finsterer, 2004; Sahin et al., 2011; Wallace, 2010). Moreover, there is increasing evidence that declining mitochondrial function in normal individuals may underlie a number of common age-related diseases (de Moura et al., 2010; Figueiredo et al., 2009) and that treatments that stimulate mitochondrial function can delay the progression of some of these diseases (Baur et al., 2006; Fillmore et al., 2010; Horvath et al., 2011; Hwang et al., 2009; Lagouge et al., 2006; Wenz et al., 2009). Skeletal muscle is one of the primary tissues responsible for insulin stimulated glucose uptake and reduced mitochondrial function has been shown to play an important role in the development of insulin resistance with obesity (Morino et al., 2006; Wallace, 2005). Therefore stimulation of pathways upstream of mitochondrial biogenesis may prove effective in delaying and treating a variety of rare and common diseases. Treatment of rodents with resveratrol, SRT1720, or overexpression of SIRT1 has been shown to activate PGC-1α and prevent diseases commonly associated with mitochondrial dysfunction and aging (Banks et al., 2008; Baur et al., 2006; Bordone et al., 2007; Herranz et al., 2010a; Lagouge et al., 2006; Pearson et al., 2008). Interestingly, skeletal muscle-restricted overexpression of PGC-1α is sufficient to delay many age-related phenotypes and extend mouse lifespan (Wenz et al., 2009). In cell culture, many of the

effects of resveratrol are no longer observed in cells with impaired SIRT1 activity (Breen et al., 2008; Csiszar et al., 2009; Fischer-Posovszky et al., 2010; Gracia-Sancho et al., 2010; He et al., 2010; Ivanov et al., 2008; Kao et al., 2010; Kim et al., 2011a; Li et al., 2010; Lin et al., 2010; Ohguchi et al., 2010; Park et al., 2010; Shindler et al., 2010; Sulaiman et al., 2010; Tanno et al., 2010; Ungvari et al., 2009; Vetterli et al., 2011; Xia et al., 2011a; Yang et al., 2010; Yoshizaki et al., 2010). However, the ability of resveratrol to elicit cellular changes in a SIRT1-independent manner (Bjorklund et al., 2011; Centeno-Baez et al., 2011; Mader et al., 2010; Zhang, 2006), and the dependency of resveratrol's effects on AMPK *in vivo* (Um et al., 2010), have fueled an active debate about how resveratrol is able to mimic CR, protect against a high fat diet, and improve mitochondrial function *in vivo*.

The adult-inducible SIRT1 knockout mouse strain described here has allowed us to directly assess the effects of resveratrol treatment in otherwise healthy adult animals lacking functional SIRT1. Using this model, we clearly demonstrate that the ability of resveratrol to stimulate mitochondrial biogenesis, increase mitochondrial function, and raise both ATP and NAD^+ levels in skeletal muscle is dependent on SIRT1 *in vivo*. While further work is needed to fully determine the importance of SIRT1 in the ability of resveratrol to prevent metabolic syndrome and other age-related diseases, this study provides the first *in vivo* evidence that beneficial effects of resveratrol on mitochondrial function require SIRT1.

Given our observation that resveratrol increases the transcript levels of PGC-1α, mitochondrial transcription factors, and components of the electron transport chain, the beneficial effects of resveratrol on skeletal muscle are likely due to PGC-1a-mediated increases in mitochondrial biogenesis and a shift toward more oxidative muscle fibers. Consistent with this, overexpression of PGC-1α was recently reported to result in a similar shift toward more oxidative fiber types (Rasbach et al., 2010). This model is also consistent with the data from our SIRT1-Tg mouse, which showed similar changes in mitochondrial biogenesis, mitochondrial function, and cellular energy status as mice treated with resveratrol. This mouse further corroborates the striking parallels between resveratrol treatment, CR, and increased SIRT1 activity (Baur et al., 2006; Cohen et al., 2004; Pearson et al., 2008; Smith et al., 2009).

SIRT1 and AMPK have been shown to play many similar roles, including their ability to respond to stress and nutrient status, induce mitochondrial biogenesis, regulate glucose homeostasis, and control the activity of important transcriptional regulators such

as PGC-1α, FOXO's and p300 (Fulco and Sartorelli, 2008). Similarly, the beneficial effects of both CR and resveratrol have been suggested to involve activation of SIRT1 and AMPK (Boily et al., 2009; Boily et al., 2008; Um et al., 2010). As such it has been difficult to untangle the epistasis of SIRT1 and AMPK. There is clearly a dynamic interaction between these two pathways. AMPK has been shown to activate SIRT1, likely through an indirect increase in cellular NAD^+ levels (Canto et al., 2009), while SIRT1 deacetylates the AMPK kinase LKB1, leading to increased phosphorylation and activation of AMPK (Ivanov et al., 2008; Lan et al., 2008).

In light of the data generated using our adult-inducible SIRT1 KO mice, we can reassess the physiologic relevance of proposed models of resveratrol's action. If resveratrol induces AMPK by acting as an ATPase or Complex III inhibitor (Gledhill et al., 2007; Hawley et al., 2010b; Zini et al., 1999), then ATP levels should be lower in the resveratrol-treated mice, and AMPK activation should occur independently of SIRT1. We do not observe such effects using moderate doses of resveratrol *in vitro* or *in vivo*. In time-course cell culture experiments, ATP levels were not altered after 1 hour and steadily increased at the 4, 6, and 12 hour time points, while NAD^+ levels were not significantly increased until 12 hours of treatment. Moreover, we found no evidence of a decrease in ATP in animals treated with either a low or high dose of resveratrol. Thus, when moderate doses are used, it seems unlikely that resveratrol activates AMPK by altering ATP levels. In contrast, 50 μM resveratrol caused a dramatic decline in mitochondrial membrane potential and ATP levels. Thus, SIRT1-independent activation of AMPK by high concentrations of resveratrol may be secondary to inhibition of mitochondrial respiration and ATP synthesis.

In a recent study, a new model was presented in which resveratrol increases mitochondrial biogenesis by directly inhibiting PDE leading to increased levels of cAMP. This leads an increase in cellular calcium levels, thereby stimulating phosphorylation of AMPK by CamKKβ. As cellular NAD^+ levels were found to be elevated under these conditions, this was proposed as a mechanism by which resveratrol activates SIRT1. This model is not consistent with the effects we have observed with moderate doses of resveratrol *in vitro* or *in vivo*, as increases in p-AMPK, NAD^+, LKB1 acetylation, and mitochondrial function were entirely SIRT1-dependent. Inhibition of PDE may, however, provide an explanation for some of the effects seen in animals treated with a higher dose of resveratrol where phosphorylation of AMPK and increased levels of NAD^+ are observed independently of SIRT1.

In experiments using pharmacological agents, it is well recognized that care should be taken to use the lowest effective dose to minimize the chances of off target effects. Our study exemplifies the risk of using high doses of resveratrol: we clearly show that the ability of resveratrol treatment to increase phosphorylation of AMPK both *in vivo* and *in vitro* was dependent upon SIRT1 but only at moderate doses. Moreover treatment of cells with low doses of resveratrol mimicked the *in vivo* situation but high doses of resveratrol (≥50 μM) resulted not only in SIRT1-independent activation of AMPK but toxic effects that included a dramatic reduction in mitochondrial membrane potential and cellular ATP levels.

In this study we chose to test the SIRT1-dependence of resveratrol in tissues where the beneficial effects of resveratrol treatment are clear, namely skeletal muscle, an organs with high energetic demands requiring efficient mitochondrial function. Using adult-inducible SIRT1 knockout mice we have been able to test for the first time whether beneficial effects of resveratrol treatment on muscle mitochondrial function are dependent upon SIRT1 *in vivo.* Our data clearly show that treatment with moderate doses of resveratrol results in AMPK activation, induction of mitochondrial biogenesis, and improved mitochondrial function in a manner that is dependent upon SIRT1. It is worth noting that even when AMPK was induced in a SIRT1-independent manner *in vivo*, the ability of high doses of resveratrol to improve mitochondrial function still required SIRT1. We favor a model whereby moderate doses of resveratrol first activate SIRT1, which leads to deacetylation of LKB1 and activation of AMPK (Figure 3.2.17). Clearly, SIRT1 and AMPK do not function independently or linearly. The subsequent increase in NAD^+ levels likely contributes to a positive feedback cycle that may serve to sustain the effects of increased SIRT1 activity beyond the activating stimulus. This model supports the enticing possibility of designing and developing potent small molecules that provide the health benefits of resveratrol by activating SIRT1 and downstream pathways to treat metabolic and other age-related diseases.

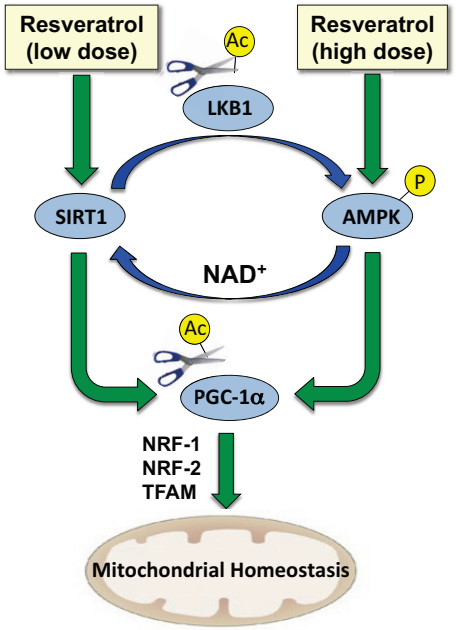


Figure 3.2.17 - Mechanisms of action of different doses of resveratrol in the regulation of mitochondrial homeostasis.

3.3. Berberine protects against high fat diet-induced dysfunction in muscle mitochondria by inducing SIRT1-dependent mitochondrial biogenesis

3.3.1. Summary

Berberine has recently been shown to improve insulin sensitivity in rodent models of insulin resistance. Although this effect was explained partly through an observed activation of AMPK, the upstream and downstream mediators of this phenotype were not explored. Here, we show that treatment with berberine reverts mitochondrial dysfunction induced by High Fat Diet and hyperglycemia in skeletal muscle, in part due to an increase in mitochondrial biogenesis. Furthermore, we observe that the prevention of mitochondrial dysfunction by berberine, the increase in mitochondrial biogenesis, as well as berberine-induced AMPK activation, are blocked in cells in which SIRT1 has been knockeddown. Taken together, these data reveal an important role for SIRT1 and mitochondrial biogenesis in the preventive effects of berberine on diet-induced insulin resistance.

3.3.2. Introduction

The global emergence of obesity as an epidemic has made it a worldwide public health problem, promoted by a sedentary lifestyle and a diet rich in fats and sugar (Haslam and James, 2005; Kopelman, 2000). Indeed, obesity has been linked to numerous health-related pathologies. Visceral obesity is associated with insulin resistance, dyslipidemia, hypertension and increased risk of atherosclerosis, a condition known as metabolic syndrome (Hegarty et al., 2003). Metabolic syndrome results from a positive energy balance, in which caloric intake exceeds oxidation, leading to a deregulation of glucose and lipid metabolism (Muoio and Newgard, 2006).

Skeletal muscle plays an important role in the development of the metabolic syndrome (Hegarty et al., 2003; Krebs and Roden, 2004; Petersen et al., 2007). Since the oxidative capacity of skeletal muscle is predominately dependent on mitochondria, there is growing evidence suggesting that mitochondrial dysfunction, and the associated impairment of fatty acid oxidation, may directly cause or accelerate insulin resistance (Civitarese et al., 2006; Rolo and Palmeira, 2006). This has been shown in patients with insulin resistance and type 2 diabetes (Kelley et al., 2002; Mootha et al., 2003; Patti et al., 2003), as well as in several animal models (Bonnard et al., 2008; Yokota et al., 2009). In skeletal muscle, stimulation of AMPK and SIRT1 has been shown to increase

the expression and activity of PGC-1α, an essential cofactor involved in mitochondrial biogenesis (Gerhart-Hines et al., 2007; Reznick and Shulman, 2006).

SIRT1 is a known regulator of key cellular and physiological processes, including its role in metabolism. SIRT1 acts as a metabolic sensor, playing a very important role in the maintenance of metabolism homeostasis at least in part due to its ability to regulate mitochondrial homeostasis. Indeed, SIRT1 was shown to regulate mitochondrial metabolism by both deacetylate and activate PGC-1α (Gerhart-Hines et al., 2007; Rodgers et al., 2005), but also through a PGC-1α-independent regulation of TFAM mediated by HIF1α and c-Myc (Chapter 3.1). Giving further strength to the idea of SIRT1 as a key player in the maintenance of mitochondrial homeostasis, SIRT1 has been linked to the development and progression of a number of age and metabolic-related disorders (Haigis and Sinclair, 2010), where deregulation of mitochondrial homeostasis play an important role.

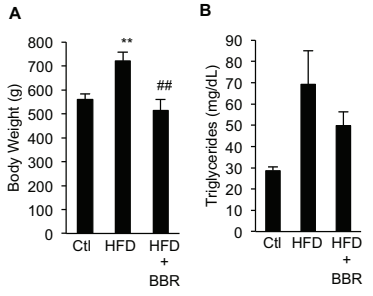
Berberine, [18,5,6-dyhydro-9,10-dimethoxybenzo(g)-1,3benzodioxolo(5.6-a) quinolizinium], is an isoquinoline alkaloid derived from the Berberidacea plant family, which has been used in traditional Chinese medicine for centuries. Multiple pharmacologic effects of berberine have been reported including anti-inflammatory (Jeong et al., 2009), anti-hypertensive (Bova et al., 1992), and anti-proliferative actions (Choi et al., 2008). Moreover, beneficial effects of berberine on insulin sensitivity and glucose tolerance have shown promise in the treatment of metabolic disorders such as hyperglycemia and hyperlipidemia (Brusq et al., 2006; Kim et al., 2009; Lee et al., 2006; Turner et al., 2008). These effects are related, in part, to the ability of berberine to activate AMPK (Cheng et al., 2006; Lee et al., 2006; Turner et al., 2008) and to suppress gluconeogenesis (Xia et al., 2011b).

Since SIRT1 and AMPK activities are intimately related, and SIRT1 activity is crucial to activation of AMPK (chapter 3.2), it is tempting to speculate that the beneficial effects of berberine on metabolism may be mediated in part by SIRT1. Therefore this study was aimed to on one hand test whether the benefical effects of berberine in a rodent model of diet induced obesity were correlated with improved mitochondrial homeostasis, and on the other hand to test whether the ability of berberine to activate AMPK and increase mitochondrial function requires SIRT1.

3.3.3. Results

3.3.3.1. Berberine reverts High fat diet-induced obesity, normalizes hormonal levels and glucose homeostasis

Previous work has demonstrated that berberine protects from HFD-induced obesity and its deleterious effects. Also, berberine has been shown to prevent the natural onset of obesity in several animal models (Brusq et al., 2006; Kim et al., 2009; Lee et al., 2006; Xia et al., 2011b). In this work, we decided to investigate the metabolic effects of berberine on skeletal muscle in a model of diet-induced obesity. We administered berberine (100 mg/kg/day) in the drinking water for 4 weeks to rats previously fed with a HFD for 12 weeks. Body weight measurements at the end of the experimental treatment validated the HFD feeding as a model for obesity, since the HFD rats showed a significant increase in body weight when compared to control rats (on average 160 g more) (Figure 3.2.1A). Moreover, we observed that both fat and lean masses were contributing to this increase (Figure 3.3.1C). This was also correlated with an increase in the triglyceride content in skeletal muscle of HFD fed rats (Figure 3.3.1B). Berberine significantly reduced this increase in body weight to values similar to control rats (Figure 3.3.1A) due to reversion of the increase in both fat and lean mass induced by HFD feeding (Figure 3.3.1C), since weight loss is not purely from adipose mass loss but also includes lean mass loss. Accordingly, berberine treatment also decreased the triglyceride content in the skeletal muscle of these rats (Figure 3.3.1B).



C

Group	Weight (g)	Fat-Free mass (g)	Fat mass (g)	Fat mass (%)	Fasting glucose (mmol/l)	Fasting insulin (ng/ml)
Ctl	560 ± 23	461 ± 35	98 ± 23	18 ± 4	5 ± 1	0.7 ± 0.3
HFD	720 ± 38**	509 ± 49	210 ± 16++	28 ± 6	8.5 ± 1.5	1.2 ± 0.3
HFD + BBR	515 ± 46##	395 ± 26§§	119 ± 35$	23 ± 6	8.1 ± 0.9	0.1 ± 0.1″

Figure 3.3.1 - Berberine Protects from obesity in Sprague-Dawley Rats. (A) Body Weight of rats fed with Chow diet (Ctl), High Fat Diet (HFD) and High Fat Diet supplemented with berberine (HFD + BRR) (n=5 experiments **p<0.01 versus Ctl, ## p<0.01 versus HFD). (B) Triglycerides content expressed in mg/dL in skeletal muscle of rats fed with Chow diet (Ctl), High Fat Diet (HFD) and High Fat Diet supplemented with berberine (HFD + BRR) (n=4 experiments p≥0.05). (C) Comparative whole body characteristics of rats fed with Chow diet (Ctl), High Fat Diet (HFD) and High Fat Diet supplemented with berberine (HFD + BRR) (n=5

experiments **p<0.01 versus Ctl, ## p<0.01 versus HFD, §§ p<0.01 versus HFD, ++ p<0.01 versus Ctl, & p<0.05 versus HFD, "p<0.05 versus HFD). Values are expressed as mean ± SEM.

Hyperglycemia and hyperinsulinemia are common symptoms associated with development of the Metabolic Syndrome (Muoio and Newgard, 2006). Treatment with berberine reduced HFD-induced fasted plasma insulin levels (Figure 3.3.1C), indicating that berberine is able to decrease HFD-induced hyperinsulinemia. However, despite abolishing the HFD-induced hyperinsulinemia, berberine did not effectively change fasting plasma glucose levels (Figure 3.3.1C). To further study the effects of berberine in glucose homeostasis and insulin sensitivity, an oral glucose tolerance test was performed. Concomitant with an increase in body weight, HFD feeding decreased the ability to clear blood glucose, as demonstrated by glucose excursions (Figure 3.3.2A) and AUC (Figure 3.3.2B). Despite the weak effect on fasting glucose plasma levels, berberine was able to significantly improve glucose tolerance and hyperinsulinemia over HFD fed rats to values comparable to the control group (Figure 3.3.1C and Figure 3.3.2). Together this data revel that treatment with berberine has a positive impact on glucose and insulin homeostasis upon HFD feeding.

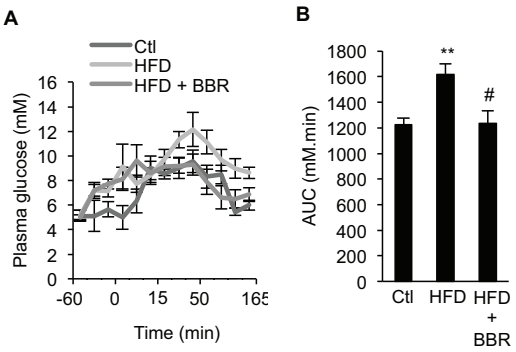
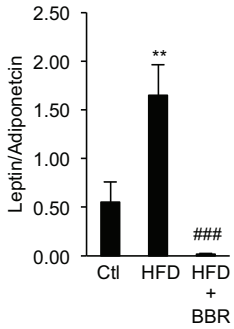


Figure 3.3.2 - Berberine Protects from obesity-induced impairment in glucose homeostasis in Sprague-Dawley Rats. (A and B) Plasma glucose concentrations at the indicated time points after glucose challenge were measured (A) and values for areas under the insulin curve were calculated (B) in rats fed with Chow diet (Ctl), High Fat Diet (HFD) and High Fat Diet supplemented with berberine (HFD + BRR) (n=5 experiments **p<0.01 versus Ctl, # p<0.05 versus HFD). Values are expressed as mean ± SEM.

Leptin and adiponectin levels were also assessed since alterations in the levels of these two adipokines have been extensively correlated with obesity and obesity-related disorders (Muoio and Newgard, 2006). Indeed, adiponectin and leptin are the two main adipokines thought to contribute to systemic abnormalities in both lipid and glucose homeostasis (Ruderman et al., 2003). HFD fed rats presented a marked increase in leptin levels (Figure 3.3.3), which was reverted by berberine (Figure 3.3.3). Moreover, berberine also induces a dramatic increase in the plasmatic levels of adiponectin (Figure 3.3.3), resulting in mitigation of the dramatic increase in the leptin/adiponectin ratio induced by HFD.



Group	Leptin (nmol/l)	Adiponectin (nmol/l)
Ctl	4433 ± 714	10406 ± 2958
HFD	20398 ± 4386***	19713 ± 3079
HFD + BBR	579 ± 78###	31943 ± 3290§§

Figure 3.3.3 - Berberine Protects from obesity-induced abnormalities in plasmatic adipokine levels in Sprague-Dawley Rats. Leptin and Adiponectin content in the plasma of rats fed with Chow diet (Ctl), High Fat Diet (HFD) and High Fat Diet supplemented with berberine (HFD + BRR) (n=4 experiments **p<0.01 ***p<0.001 versus Ctl, ### p<0.001 versus HFD &&& p<0.001 versus HFD). Values are expressed as mean ± SEM.

3.3.3.2. Berberine rescues HFD-induced mitochondrial dysfunction

Mitochondria play an important role in the development of insulin resistance and hyperglycemia (Lowell and Shulman, 2005; Rolo and Palmeira, 2006). Boosting mitochondrial activity provides, at least in part, a potential mechanism by which several therapeutic agents act (Baur et al., 2006; Feige et al., 2008; Lagouge et al., 2006). In order to address whether the beneficial effects of berberine are related to its ability to alter mitochondrial function, we evaluated the function of isolated mitochondria. As reported before, HFD feeding induced mitochondrial dysfunction in skeletal muscle (Figure 3.3.4). This was demonstrated by decrease in the activity of the ETC complexes like SDH (Figure 3.3.4A) and COX (Figure 3.3.4B) and mitochondrial ATPase (Figure 3.3.4C). In accordance with this, mitochondrial ATP content also presented a tendency

to be decreased by HFD feeding (Figure 3.3.4D). Berberine was able to revert mitochondrial dysfunction induced by HFD by restoring ATPase and ETC activities (Figure 3.3.4A-C), as well as, mitochondrial ATP content.

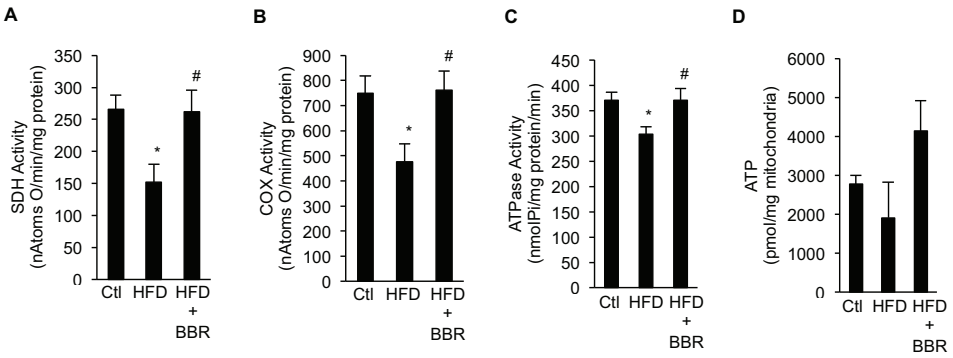


Figure 3.3.4 - Berberine Protects from obesity-induced skeletal muscle mitochondrial dysfunction in Sprague-Dawley Rats. (A and B) Succinate Dehydrogenase (A) and Cytochrome c Oxidase (B) activity in skeletal muscle of rats fed with Chow diet (Ctl), High Fat Diet (HFD) and High Fat Diet supplemented with berberine (HFD + BRR) measured polarographically and expressed in nAtomsO/min/mg protein (n=4 experiments *p<0.05 versus Ctl, ## p<0.01 versus HFD). (C) ATPase activity measured spectrophotometrically in skeletal muscle of rats fed with Chow diet (Ctl), High Fat Diet (HFD) and High Fat Diet supplemented with berberine (HFD + BRR) expressed in nmolPi/mgprotein/min (n=4 experiments *p<0.05 versus Ctl, # p<0.05 versus HFD). (D) ATP content in isolated mitochondria from skeletal muscle of rats fed with Chow diet (Ctl), High Fat Diet (HFD) and High Fat Diet supplemented with berberine (HFD + BRR) (n=5 experiments p≥0.05). Values are expressed as mean ± SEM.

HFD-induced mitochondrial dysfunction was also correlated with decreased oxidative type 1 fibers (Figure 3.3.5A) and a switch towards the glycolytic type II fibers (Figure 3.3.5B), while treatment with berberine increased oxidative fiber type in skeletal muscle (Figure 3.3.5A and 3.3.5B) and slightly decreased glycolytic type IIb fibers (Figure 3.3.5B). Citrate synthase activity in HFD fed rats was also decreased, and this effect was again rescued by berberine (Figure 3.3.5C). These observations led us to speculate that berberine may be rescuing mitochondrial function from HFD damage by increasing mitochondrial biogenesis.

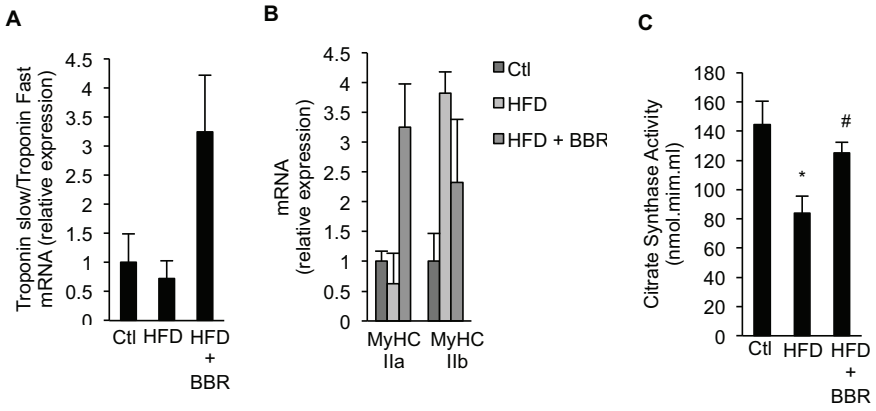


Figure 3.3.5 - Berberine induces a switch towards more oxidative fibers in skeletal muscle of Sprague-Dawley Rats. (A) Amount of oxidative fibers analyzed by the ratio of Troponin 1 slow and Troponin 1 fast mRNA measured by means of quantitative RT-PCR in skeletal muscle of rats fed with Chow diet (Ctl), High Fat Diet (HFD) and High Fat Diet supplemented with berberine (HFD + BRR) (n=4 experiments $p \geq 0.05$). (B) MyHCIIa and MyHCIIb mRNA analyzed by means of quantitative RT-PCR in skeletal muscle of rats fed with Chow diet (Ctl), High Fat Diet (HFD) and High Fat Diet supplemented with berberine (HFD + BRR) (n=4 experiments ** $p<0.01$ versus Ctl, # $p<0.05$ versus HFD). (C) Citrate Synthase activity measured spectrophotometrically in skeletal muscle of rats fed with Chow diet (Ctl), High Fat Diet (HFD) and High Fat Diet supplemented with berberine (HFD + BRR) expressed in nmol.mim.ml (n=4 experiments *$p<0.05$ versus Ctl, # $p<0.05$ versus HFD). Values are expressed as mean ± SEM.

3.3.3.3. Berberine rescues mitochondrial function in a SIRT1-dependent manner

The observations from the *in vivo* studies were extended to cell culture models by investigating potential mechanisms for berberine's beneficial effects on mitochondrial function under conditions of excess of nutrients. SIRT1 has been highlighted as a potential therapeutic target for numerous mitochondrial pathologies (Baur et al., 2006; Feige et al., 2008; Lagouge et al., 2006) and was shown to play a key role in the maintenance of mitochondrial homeostasis (chapter 3.1). Its beneficial effects are associated with an induction of genes involved in oxidative phosphorylation and mitochondrial biogenesis, thereby increasing mitochondrial ability to oxidize substrates. Since berberine has been shown to activate AMPK, which functions are closely related to SIRT1 and mitochondrial biogenesis (Canto et al., 2009), we tested if SIRT1 is involved in the protective mechanisms of berberine in mitochondrial function. Initially, C2C12 myotubes were exposed to high glucose and high fatty acids (FFAs) for 96 h. These exposures induced mitochondrial dysfunction, as demonstrated by a decrease in

mitochondrial membrane potential and COX activity (Figure 3.3.6A-D). Interestingly, treatment with berberine for 96h prevented this loss in mitochondrial function (Figure 3.3.6A-D).

Similarly to berberine, DCHC, a SIRT1 activator (Figure 3.3.6A-D), also prevented the loss of mitochondrial function caused by high glucose or FFAs. Moreover, incubation of C2C12 myotubes with both berberine and EX-527 (a known inhibitor of SIRT1), completely blocked berberine-mediated prevention of mitochondrial dysfunction induced by both high glucose and FFAs (Figure 3.3.6A-D). This indicates that the beneficial effects of berberine on mitochondrial function are, at least partly, mediated through SIRT1.

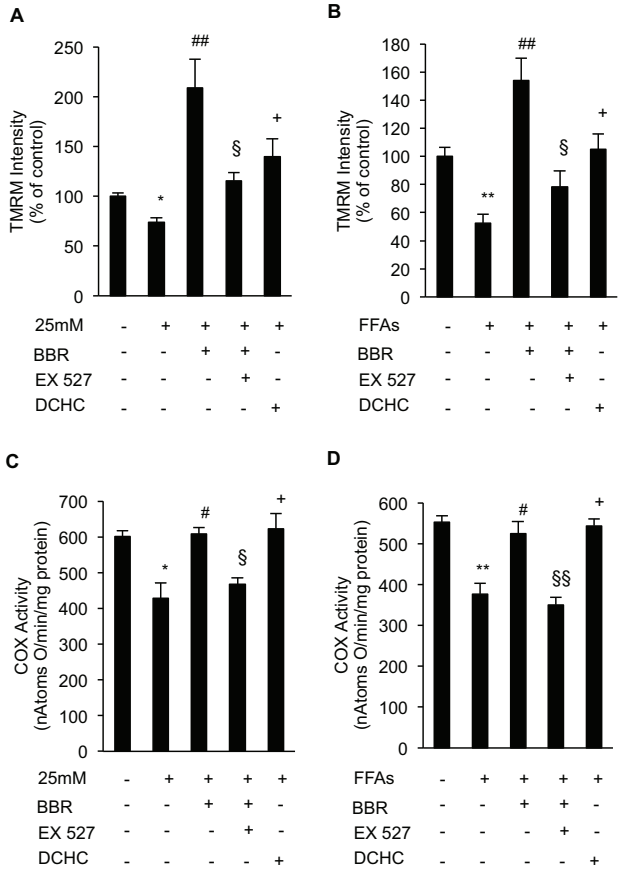


Figure 3.3.6 - SIRT1 is involved in the rescue of mitochondrial dysfunction induced by hyperglycemia and fatty acids in C2C12 myotubes by Berberine. (A and B) Mitochondrial membrane potential measured with TMRM fluorescent probe and normalized to mg of protein in cells after 96 h of

treatment with hyperglycemia (25 mM) (A) or fatty acids (FFAs) (B) and exposed to 5 μM berberine, 10 μM DCHC and 5 μM berberine plus 1 μM EX- 527. Data was analyzed as percentage of untreated cells taken as 100% (n=5 experiments *p<0.05 **p<0.01 versus Ctl, ## p<0.01 versus 25 mM or FFAs, § p<0.05 versus 25 mM+BBR or FFAs+BBR, + p<0.05 versus 25 mM or FFAs). (C and D) Cytochrome c Oxidase activity measured polarographically in cells after 96h of treatment with hyperglycemia (25 mM) (C) or fatty acids (FFAs) (D) and exposed to 5 μM berberine, 10 μM DCHC and 5 μM berberine plus 1 μM EX- 527. Data is expressed in nAtomsO/min/mg protein. (n=4 experiments *p<0.05 **p<0.01 versus Ctl, # p<0.05 versus 25 mM or FFAs, § p<0.05 §§ p<0.01 versus 25 mM+BBR or FFAs+BBR, + p<0.05 versus 25 mM or FFAs). Values are expressed as mean ± SEM.

3.4.2.4 Mitochondrial biogenesis is increased by berberine in a SIRT1-dependent-manner

Hyperglycemia has been reported to induce mitochondrial dysfunction by decreasing mitochondrial biogenesis (Palmeira et al., 2007; Rolo and Palmeira, 2006). Moreover, recent studies report that lipid overload in skeletal muscle may promote mitochondrial dysfunction by supplying an excessive amount of reducing equivalents to the ETC complexes, resulting in their inhibition (Koves et al., 2008; Muoio and Koves, 2007). Therefore, increasing mitochondrial biogenesis may constitute an important approach in preventing FFA-induced mitochondrial dysfunction. Indeed, the increase in citrate synthase activity observed in the *in vivo* study suggested that berberine increased mitochondrial biogenesis (Figure 3.3.5C). Furthermore, citrate synthase activity in C2C12 myotubes was decreased by both hyperglycemia and FFAs while both berberine and DCHC prevented this decrease (Figure 3.3.7). Interestingly, the beneficial effects of berberine were again blocked by treatment with EX-527 (Figure 3.3.7).

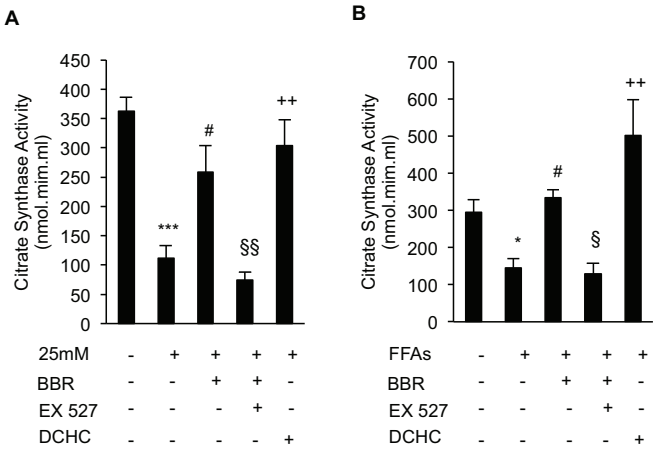


Figure 3.3.7 - SIRT1 is involved in the ability of berberine to protect from hyperglycemia and fatty acids-induced decline in citrate synthase activity in C2C12 myotubes. (A and B) Citrate Synthase activity measured spectrophotometrically in cells after 96h of treatment with hyperglycemia (25 mM) (A) or fatty acids (FFAs) (B) and exposed to 5 μM berberine, 10 μM DCHC and 5 μM berberine plus 1 μM EX- 527. Data is expressed in nAtomsO/min/mg protein. (n=4 experiments ***p<0.001 *p<0.05 versus Ctl, # p<0.05 versus 25 mM or FFAs, §§ p<0.01 § p<0.05 versus 25 mM+BBR or FFAs+BBR, + p<0.05 versus 25 mM or FFAs). Values are expressed as mean ± SEM.

To address if berberine indeed increases mitochondrial biogenesis in a SIRT1-dependent manner, we examined several parameters of mitochondrial biogenesis in SIRT1 knockdown and control cells (Figure 3.3.8A). Berberine prevented the decrease in mitochondrial DNA content (Figure 3.3.8B) and mitochondrial mass (Figure 3.3.8C) induced by hyperglycemia in control cells. However, berberine did not affect mitochondrial DNA content (Figure 3.3.8B) or mitochondrial mass (Figure 3.3.8C) in cells lacking SIRT1.

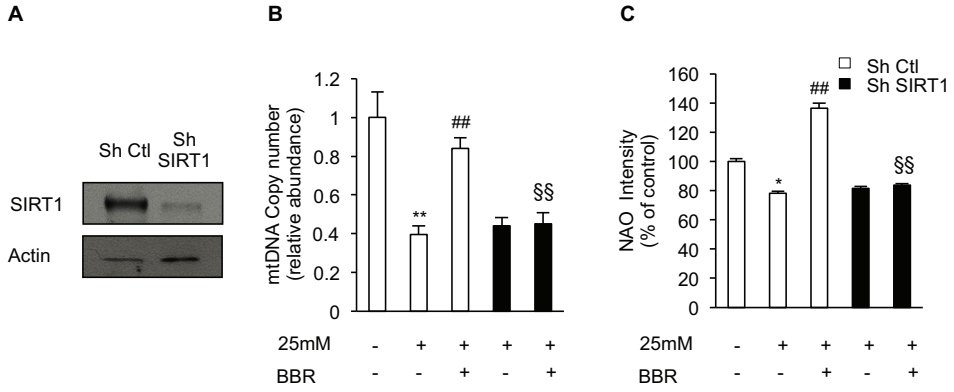


Figure 3.3.8 - Berberine rescues decline in mitochondrial biogenesis induced by hyperglycemia in a SIRT1-dependent manner in C2C12 myoblasts. (A) Representative Imunnoblot for SIRT1 and Actin in C2C12 cells infected with SIRT1 Sh RNA (Sh SIRT1) or non targeting Sh RNA (Sh Ctl). (B) Mitochondrial DNA amount analyzed by means of quantitative PCR in C2C12 cells infected with SIRT1 shRNA (Sh SIRT1) or nontargeting shRNA (sh Ctl) after 96 h of treatment with hyperglycemia (25 mM) and 5 μM berberine (BBR). Relative units are expressed in comparison to untreated cells taken as 1.0. (n=5 experiments **p<0.01 versus Ctl, ## p<0.01 versus 25 mM, §§ p<0.01 versus 25 mM+BBR). (C) Mitochondrial mass measured by quantification of NAO intensity in C2C12 cells infected with SIRT1 shRNA (Sh SIRT1) or nontargeting shRNA (sh Ctl) after 96 h of treatment with hyperglycemia (25 mM) and 5 μM berberine (BBR). Data is expressed as percentage of untreated cells taken as 100% (n=5 experiments *p<0.05 versus Ctl, ## p<0.01 versus 25 mM, §§ p<0.01 versus 25 mM+BBR). Values are expressed as mean ± SEM.

In addition, in control cells, berberine prevented hyperglycemia-induced decrease in the expression of several genes that regulate mitochondrial biogenesis, such as PGC-1alpha, NRF-1, NRF-2 and TFAM (Figure 3.3.9A), as well as nuclear-encoded (NDUFS8 and COX5b) and mitochondrial-encoded mitochondrial genes (ND1 and COX1) (Figure 3.3.9B). However, in the cells lacking SIRT1, berberine did not show any effect on the aforementioned genes. These results were also confirmed at the protein level (Figure 3.3.9C). To assess the effects of berberine on mitochondrial biogenesis *in vivo*, the expression of mitochondrial biogenesis regulators such as PGC-1alpha and TFAM and mitochondrial genes COX1, COX2, and COX IV were analyzed. HFD feeding decreased the expression of these genes, and in all cases, this was rescued by berberine (Figure 3.3.9.D).

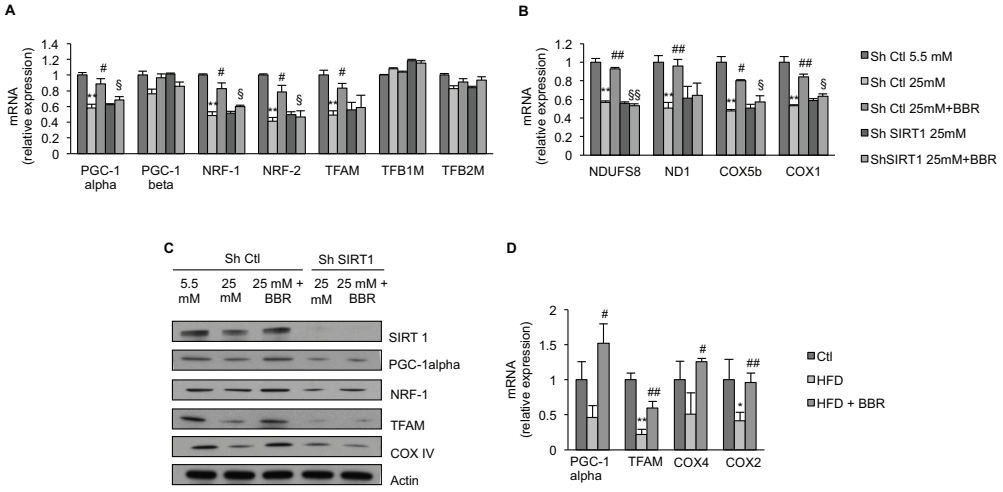


Figure 3.3.9 - Berberine regulates mitochondrial biogenesis in a SIRT1-dependent manner in C2C12 myoblasts and also in skeletal muscle of Sprague-Dawley Rats. (A) PGC-1alpha, PGC-1beta, NRF-1, NRF-2, TFAM, TFB1M and TFB2M mRNA analyzed by means of quantitative RT-PCR in C2C12 cells infected with SIRT1 shRNA (Sh SIRT1) or nontargeting shRNA (sh Ctl) after 96 h of treatment with hyperglycemia (25 mM) and 5 μM berberine (BBR). Relative expression values of the untreated cells were taken as 1.0. (n=4 experiments **p<0.01 versus Ctl, # p<0.05 versus 25 mM, § p<0.05 versus 25 mM+BBR). (B) NDUFS8, ND1, COX5b, COX1 mRNA analyzed by means of quantitative RT-PCR in C2C12 cells infected with SIRT1 shRNA (Sh SIRT1) or nontargeting shRNA (sh Ctl) after 96 h of treatment with hyperglycemia (25 mM) and 5 μM berberine (BBR). Relative expression values of the untreated cells were taken as 1.0. (n=4 experiments **p<0.01 versus Ctl, # p<0.05 versus 25 mM, § p<0.05 versus 25 mM+BBR). (C) Representative Immunoblot for SIRT1, PGC-1alpha, NRF-1, TFAM, COXIV and Actin in C2C12 cells infected with SIRT1 shRNA (Sh SIRT1) or nontargeting shRNA (sh Ctl) after 96 h of treatment with hyperglycemia (25 mM) and 5 μM berberine (BBR). (E) PGC-1alpha, TFAM, COX4, COX2 mRNA analyzed by means of RT-PCR in skeletal muscle of rats fed with Chow diet (Ctl), High Fat Diet (HFD) and High Fat Diet supplemented with berberine (HFD + BRR). (n=4 experiments *p<0.05 **p<0.01 versus Ctl, # p<0.05 ## p<0.01 versus HFD). Values are expressed as mean ± SEM.

3.3.3.5. Berberine increases the NAD^+/NADH ratio

After observing that the effects of berberine on mitochondrial function and biogenesis were dependent on SIRT1, we sought to elucidate the mechanism by which BBR activates SIRT1. Given that SIRT1 is known to be regulated by NAD^+ and the NAD^+/NADH ratio, we measured their content in cells treated with berberine. Interestingly, berberine prevented the decrease in the NAD^+/NADH ratio caused by hyperglycemia (Figure 3.3.10A) in C2C12 myotubes. To explain the increase in

NAD^+/NADH induced by berberine, we evaluated the expression of NAMPT, the rate-limiting factor in NAD^+ biosynthesis and has been shown to be the link between linking high glucose and FFAs with SIRT1 downregulation (de Kreutzenberg et al., 2010). Therefore, we tested whether berberine modulates NAMPT expression in C2C12 cells. Gene expression of NAMPT markedly increased after 6 h of berberine exposure (Figure 3.3.10C), which was paralleled by an increase in the NAD^+/NADH ratio (Figure 3.3.10B). Moreover, FK866, an inhibitor of NAMPT, blocked the induction of genes that regulate mitochondrial biogenesis and also in nuclear-encoded and mitochondrial-encoded mitochondrial genes by berberine (Figure 3.3.10D).

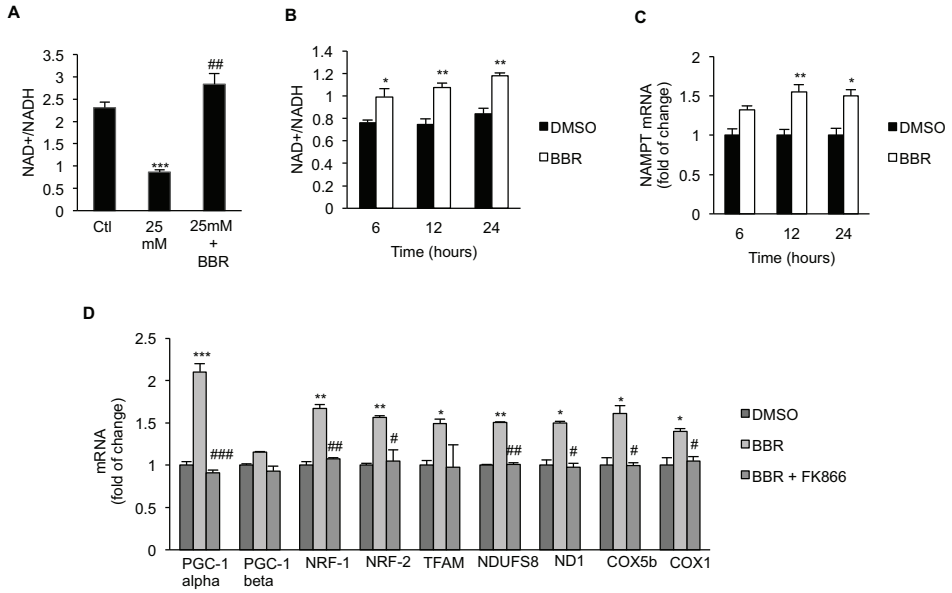


Figure 3.3.10 - Berberine increases NAD^+/NADH ratio through induction of NAMPT expression in C2C12 cells. (A) NAD^+/NADH ratio measured spectophotometricaly in C2C12 myotubes treated for 96 h with hyperglycemia and exposed to 5 μM berberine (BBR) (n=4 experiments ***p<0.001 Ctl, ## p<0.01 versus 25 mM). (B) NAD^+/NADH ratio measured spectophotometricaly in C2C12 myoblasts treated for 6 h, 12 h and 24h with 5 μM berberine (BBR) (n=4 experiments *p<0.05 **p<0.01 versus Ctl). (C) NAMPT mRNA analyzed by means of quantitative RT-PCR in C2C12 cells after 6 h, 12 h and 24 h of treatment with 5 μM berberine (BBR). Relative expression values of the untreated cells were taken as 1.0. (n=4 experiments *p<0.05 **p<0.01 versus Ctl). (D) PGC-1alpha, PGC1-beta, NRF-1, NRF-2, TFAM, NDUFS8, ND1, COX5b, COX1 mRNA analyzed by means of quantitative RT-PCR in C2C12 cells after 12 h of treatment with 5 μM berberine (BBR) or 5 μM berberine (BBR) + 10 nM FK866. Relative expression values of the untreated cells were taken as 1.0. (n=4 experiments *p<0.05 **p<0.01 ***p<0.001 versus Ctl, # p<0.05 ## p<0.01 ### p<0.001 versus BBR). Values are expressed as mean ± SEM.

3.3.3.7. SIRT1 is involved in AMPK phosphorylation induced by Berberine

Berberine is known to activate AMPK (Cheng et al., 2006; Lee et al., 2006) but the precise mechanism by which berberine activates AMPK however is not well established. SIRT1 is known to regulate AMPK through deacetylation and activation of LKB1 (Lan et al., 2008), and was found to be required for resveratrol's ability to activate AMPK (Chapter 3.2). Thus, we aimed to address in our models if berberine-induced AMPK activation is mediated by SIRT1. As described previously, berberine rescued the decrease in AMPK phosphorylation (Thr172) induced by hyperglycemia in C2C12 control cells, (Figure 3.3.11), as well as the decrease in ACC phosphorylation (Ser79) (a direct target of AMPK activity) (Figure 3.3.11). However, these effects were not observed in cells lacking SIRT1 (Figure 3.4.7B and 3.4.7C).

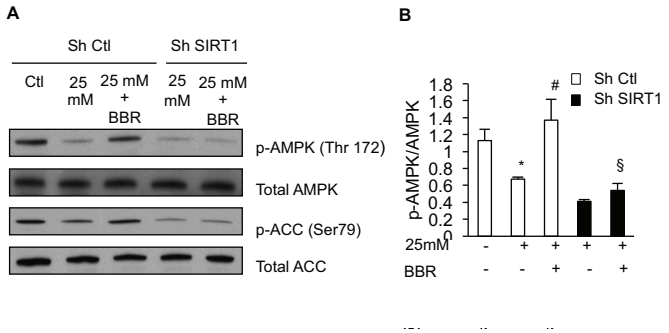


Figure 3.3.11 - Berberine activates AMPK in C2C12 myoblasts in a SIRT1-dependent way. (A) Representative Immunoblot for total AMPK, p-AMPK (Thr172), total ACC and p-ACC (Ser79) in C2C12 cells infected with SIRT1 shRNA (Sh SIRT1) or nontargeting shRNA (sh Ctl) after 96 h of treatment with hyperglycemia (25 mM) and to 5 μM berberine (BBR). (B) Quantification of AMPK activation by Berberine in C2C12 cells infected with SIRT1 shRNA (Sh SIRT1) or nontargeting shRNA (sh Ctl) after 96h of treatment with hyperglycemia (25 mM) and to 5 μM berberine (BBR). (n=4 experiments *p<0.05 versus Ctl, # p<0.05 versus 25mM, § p<0.05 versus 25 mM+BBR). Values are expressed as mean ± SEM.

Since a dose and time-dependent mechanism for AMPK activation by berberine, dependent on superoxide-generation has been shown (Xia et al., 2011b), we compared the effects of 5 and 20 μM berberine on C2C12 cells, at 6, 12 and 24 h. While at 5 μM, berberine increased mitochondrial membrane potential, 20 μM berberine decreased this parameter at all time points (Figure 3.3.12A). This was associated with a decrease in ATP content elicited by 20 μM berberine, that was not affected by knockdown of SIRT1 (Figure 3.3.12C). This effect is consistent with published data

showing inhibition of mitochondrial oxygen consumption by this range of berberine concentrations. Opposingly, 5 μM berberine increased NAD^+/NADH ratio as early as 6h incubation (Figure 3.3.12B), which led to an increase in ATP content only after 12h of incubation with berberine (Figure 3.3.12C). This data further supports a dose-dependent, indirect but positive action of berberine on mitochondria, that is primarily mediated by SIRT1 activation.

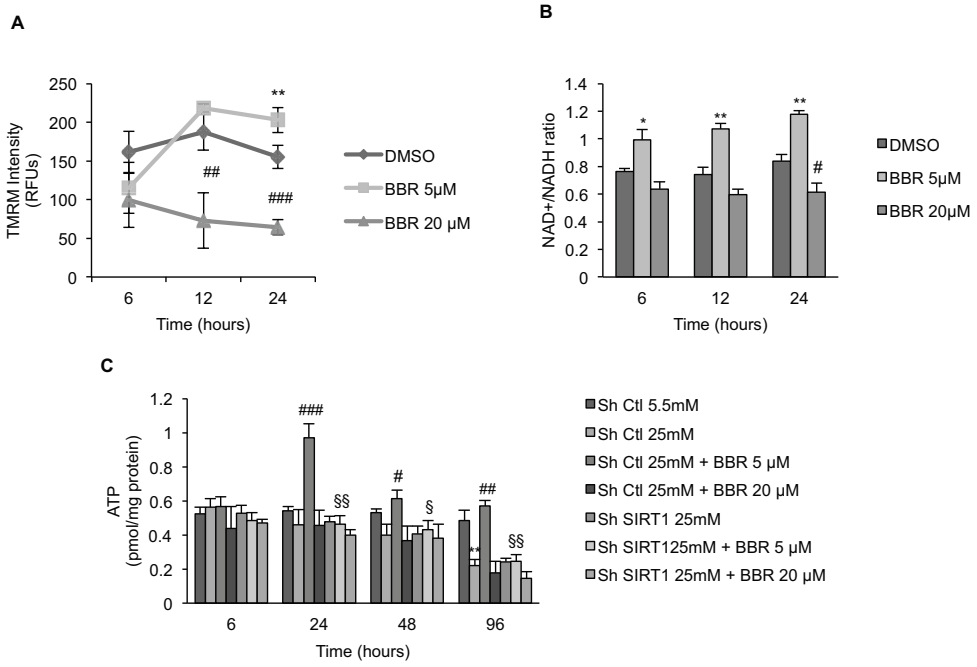


Figure 3.3.12 - Different concentrations of berberine have different effects in mitochondrial function in C2C12. (A) Mitochondrial membrane potential measured with TMRM fluorescent probe (B) and NAD^+/NADH ratio measured spectophotometricaly in C2C12 cells after 6 h, 12 h and 24 h of treatment with 5 μM and 20 μM berberine (BBR). (n=4 experiments *p<0.05 **p<0.01 versus DMSO, # p<0.05 ## p<0.01 ### p<0.001 versus DMSO). (C) ATP content measured in C2C12 cells infected with SIRT1 shRNA (Sh SIRT1) or nontargeting shRNA (sh Ctl) after 6 h, 24 h, 48 h and 96 h of treatment with hyperglycemia (25 mM), 5 μM and 20 μM berberine (BBR). (n=4 experiments **p<0.01 versus Ctl, # p<0.05 ## p<0.01 ### p<0.001 versus 25 mM, § p<0.05 §§ p<0.01 versus 25 mM+BBR). Values are expressed as mean ± SEM.

3.4.3. Discussion

In the present study we have demonstrated that oral treatment with berberine (100 mg/kg/day) rescues key features of the metabolic syndrome, including insulin resistance and hyperleptinemia. This is in agreement with several other studies that have demonstrated a role for berberine in the protection against HFD-induced insulin

resistance and diabetes (Chen et al., 2010; Lee et al., 2006; Xia et al., 2011b). Notably, berberine not only acts in the insulin resistant tissues, but has also been shown to protect pancreatic beta cells function in high caloric and streptozotocin-induce diabetic models (Lu et al., 2009; Wang et al., 2008; Zhou et al., 2009). Several studies have shown that berberine needs to be administrated in high doses (380-560 mg/kg/day) in order to have a beneficial effect (Lee et al., 2006; Turner et al., 2008). However, our data and a recent study (Chen et al., 2010) show that berberine can protect from HFD feeding at a much lower dose than previously described (100 mg/kg/day).

Here, we show for the first time, that the beneficial effects of berberine are accompanied by an increase in mitochondrial function and biogenesis in skeletal muscle. Our results also confirm these findings in a cell-based model. It is important to note that short term treatments with berberine were previously reported to inhibit ETC complex I (Turner et al., 2008), decrease ATP content in hepatocytes (Xia et al., 2011b) and cause mitochondrial fragmentation, depolarization, and oxidative stress in K1735-M2 cells when used at concentrations 2-5 times higher than the concentrations used in this study (Pereira et al., 2007). Moreover, when berberine is incubated with isolated liver mitochondria, mitochondrial respiration is inhibited and mitochondrial permeability transition is induced (Pereira et al., 2008). Therefore, while our findings demonstrate improved mitochondrial function by berberine, they do not rule out the possibility of toxic effects of berberine on mitochondria under different conditions or at higher concentrations.

PGC-1α and some of its targets, such as NRF-1, NRF-2 and TFAM, are known to be the main regulators of the mitochondrial biogenesis pathway (Scarpulla, 2008) and ultimately regulate mitochondrial function and fiber-type switch in skeletal muscle (Rasbach et al., 2010). Our results show that berberine rescues from mitochondrial dysfunction caused by HFD feeding in skeletal muscle, as well as from hyperglycemia and fatty acid exposure. Our data strongly indicate that this effect is due to enhanced mitochondrial biogenesis through the PGC-1α signaling pathway. Furthermore, our results show that the beneficial effects of berberine on mitochondrial biogenesis and function are dependent on the presence of SIRT1, pointing to an essential role for SIRT1 in the molecular pathway mediating the effects of berberine.

A recent study by Xia and colleagues showed that in hepatocytes, short term treatment with berberine results in ATP depletion and that results in AMPK activation (Xia et al., 2011b). Despite the apparent contradiction, we hypothesize that short term

treatments with higher doses of berberine induce mitochondrial toxicity and ATP depletion, while a long term, lower dose results in a SIRT1-dependent AMPK activation and increased mitochondrial biogenesis. This culminates in the prevention and reversion of mitochondrial dysfunction induced by HFD, hyperglycemia and fatty acid exposure. The ability of berberine to activate AMPK has been extensively studied (Cheng et al., 2006; Lee et al., 2006; Lu et al., 2010). Nevertheless, the exact mechanism by which this occurs remains elusive. It was previously reported that berberine inhibits ETC complex I, thereby increasing the AMP/ATP ratio and thus activating AMPK (Turner et al., 2008). Furthermore it was recently showed that mitochondrial-derived superoxide and peroxynitrite are required for berberine-induced AMPK activation in endothelial cells (Han et al., 2010b).

In this work, we demonstrate another mechanism by which berberine is able to counteract the negative effects of excess nutrients on metabolic homeostasis. In this case, AMPK activation is not a primary target but probably a consequence of SIRT1 activation by berberine. This model explains why berberine is able to improve mitochondrial and insulin sensitivity in animals previously fed a high fat diet, and therefore exhibiting mitochondrial damage with impaired ATP formation, prior to berberine treatment. Therefore, under the conditions of our study, inhibition of ATP synthesis by berberine probably is not the main trigger for AMPK activation and improvement of mitochondrial function. This is supported by assays with C2C12 cells exposed to 5 or 20 μM berberine for 6, 12 or 24 h. Indeed, while 20 μM berberine decreases mitochondrial membrane potential and ATP content, 5 μM berberine has the opposite effect being dependent on the formation of NAD^+ associated with increased NAMPT expression and SIRT1 activation. These observations provide evidence that SIRT1 plays a pivotal role in the cellular effects of berberine but does not rule out the involvement of AMPK. Since SIRT1 regulates LKB1 through deacetylation (Lan et al., 2008), a necessary step for AMPK activation, SIRT1 activation by berberine may lead to a secondary activation of AMPK. Indeed, SIRT1 silencing blocks berberine-induced AMPK (Thr172) and ACC (Ser67) phosphorylation. Further investigation will be needed to sort out the interplay between LKB1, AMPK, and SIRT1 in mediating the effects of berberine. However, recent work has shown that the hypoglycemic action of berberine can be attributed to its acute activation of the transport activity of GLUT1, an effect independent of AMPK activation (Cok et al., 2011). Thus, the beneficial effects of

berberine on metabolic abnormalities may involve mechanisms that are yet to be elucidated.

All together, our results demonstrate for the first time that low concentrations of berberine activate SIRT1, through inducing NAMPT expression and thereby raising NAD^+. Moreover, we also show that SIRT1 activity is necessary for the ability of berberine to activate AMPK and protect mitochondrial homeostasis in high caloric conditions (Figure 3.3.13).

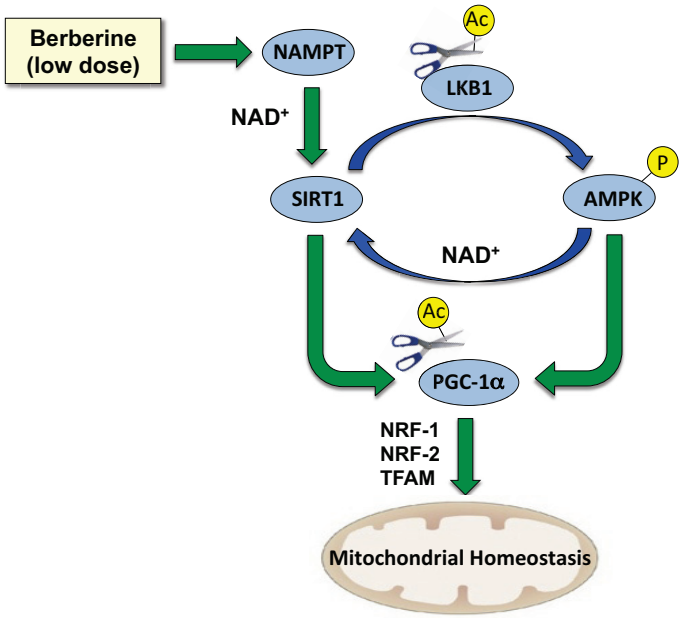


Figure 3.3.12 - Mechanisms of action of low doses of berberine in the regulation of mitochondrial homeostasis.

Chapter 4 – General Discussion

Growing evidence has shown that declining mitochondrial function and content may underlie many metabolic and age-related diseases (Figueiredo et al., 2008; Rolo et al., 2011; Rolo and Palmeira, 2006; Wallace, 2005). Giving further support to this idea, treatments that maintain appropriate mitochondrial mass and function (mitochondrial homeostasis) can delay the progression of some of these disorders (Baur et al., 2006; Horvath et al., 2011; Hwang et al., 2009; Lagouge et al., 2006; Minor et al., 2011). Therefore, upregulation of mitochondrial biogenesis through stimulation of its regulatory pathways is highly likely to be effective in the treatment of diseases where mitochondrial metabolism plays a role. The balance between mitochondrial biogenesis and degradation is essential for the maintenance of appropriate mitochondrial activity (Michel et al., 2012). Indeed, mitochondrial biogenesis stimulation can be beneficial to adapt the organelle and prevent its dysfunction by enhancing mitochondrial activity and promoting the replacement of inefficient mitochondrial components. In support of this, overexpression of PGC-1α, the 'master regulator' of mitochondrial biogenesis, in skeletal muscle was demonstrated to delay many age-related phenotypes and increase lifespan (Wenz, 2009). Consistent with this idea, treatment of animal models with compounds that induce mitochondrial biogenesis through induction of PGC-1α, like metformin, SRT1720, resveratrol and berberine were reported to protect from disorders like diabetes and metabolic syndrome (Baur et al., 2006; Feige et al., 2008; Lagouge et al., 2006; Lee et al., 2006; Minor et al., 2011; Suwa et al., 2006; Turner et al., 2008). Further supporting the potential role of mitochondrial biogenesis as a therapeutically strategy, under conditions of high supply of reduced substrates, like in obesity, increasing mitochondrial biogenesis increases mitochondrial activity and therefore prevent the damage to mitochondrial metabolism induced by the overload of substrates (Rolo et al., 2011) .

Interestingly, SIRT1 is known to regulate mitochondrial biogenesis through deacetylation of PGC-1α (Gerhart-Hines et al., 2007; Rodgers et al., 2005). As many of the compounds mentioned before were reported to induce mitochondrial biogenesis, we hypothesized that SIRT1 might be involved in the regulation of mitochondrial homeostasis induced by them. Further supporting this hypothesis, compounds like resveratrol and SRT1720 were found to directly activate SIRT1 *in vitro* (Dai et al., 2010; Howitz et al., 2003). Moreover, the effects of these compounds in metabolism and inflammation in cell-based systems were found to be, at least in part, mediated by SIRT1 (Feige et al., 2008; Lagouge et al., 2006; Minor et al., 2011).

However, due to the fact that germline whole body SIRT1 knockout mice have several developmental abnormalities (Cheng et al., 2003; McBurney et al., 2003; Sequeira et al., 2008), the role of SIRT1 in mitochondrial biology and the dependence upon SIRT1 of some of these compounds *in vivo* remains to be explored.

In this thesis is described, for the first time, the generation of an adult-inducible whole body SIRT1 KO mouse (chapter 3.1), which has allowed us to directly assess the role of SIRT1 in mitochondrial biology (chapter 3.1) as well as the effects of resveratrol treatment (chapter 3.2) in otherwise healthy adult animals lacking functional SIRT1.

Importantly, using this mouse model we clearly show that the ability of resveratrol to maintain mitochondrial homeostasis in HFD-treated mice in skeletal muscle is entirety dependent upon SIRT1 activity (chapter 3.2). This is evidenced by the lack of resveratrol's ability to upregulate mitochondrial biogenesis, and protect mitochondrial function as well as from the shift towards glycolytic fibers in SIRT1 KO mice. As addressed before, SIRT1 and AMPK perform many similar functions in the cell, including regulation of PGC-1α and mitochondrial biogenesis, and are also able to regulate each other (Canto et al., 2009; Canto et al., 2010; Lan et al., 2008). Interestingly, resveratrol treatment also leads to AMPK activation (Baur et al., 2006; Dasgupta and Milbrandt, 2007) and AMPK was previously found to be necessary for resveratrol's beneficial effects *in vivo*, suggesting that the role of SIRT1 in resveratrol's beneficial effects may be secondary to AMPK activation (Um et al., 2010). Importantly, the work presented in this thesis (chapter 3.2), showed that both, *in vivo* and *in vitro*, the effects of resveratrol are highly dependent on the doses used. Indeed, here is shown that different doses of resveratrol have different mechanisms of action: lower to moderate doses primarily target SIRT1 and consequently lead to AMPK activation through LKB1 deacetylation (Figure 3.2.17); on the other hand, higher doses of resveratrol *in vitro* seem to follow the model of action proposed in the literature (Canto and Auwerx, 2012), where activation of AMPK occurs in a SIRT1 independent fashion probably due to inhibition of the mitochondrial ETC and consequent decline in ATP production, which leads to SIRT1 activation by increasing NAD^+ levels (Figure 3.2.17). Nevertheless, regardless of whether SIRT1 is primarily or secondarily activated by resveratrol, SIRT1 is absolutely required for resveratrol-mediated effects in mitochondrial homeostasis, as shown in chapter 3.2.

In this thesis, it is also shown that berberine, another compound that protects from several diseases including diabetes, reverses some of the key features of metabolic syndrome like insulin resistance, and suggests that it might be mediated, at least in part, by inducing proper fuel utilization due to restoration of mitochondrial homeostasis (chapter 3.3). Indeed, we clearly show that berberine reverses HFD-induced and protects from hyperglycemia-induced mitochondrial dysfunction, declines in mitochondrial content and a shift towards more glycolytic fibers. Interestingly, berberine is also known to activate AMPK, and it has been suggested to do so by inducing a decline in the AMP/ATP ratio through inhibition of the mitochondrial ETC (Turner et al., 2008), similar to the mechanism by which resveratrol was proposed to act. Therefore, we sought to evaluate if the effects of berberine were also mediated by SIRT1. Indeed, in chapter 3.3 is shown for the first time that the effects of berberine in mitochondrial homeostasis are mediated by SIRT1. In the absence of SIRT1 berberine failed to induce AMPK activation and to protect from hyperglycemia-induced mitochondrial dysfunction, and declines in mitochondrial biogenesis. Importantly, and similar to what was observed with resveratrol, dose-dependent mechanisms were also observed for berberine's action in the regulation of mitochondrial homeostasis. Similarly to previous reports (Turner et al., 2008), we observed that higher doses of berberine cause mitochondrial toxicity leading to AMPK activation. However, lower doses of berberine primarily target SIRT1 without inducing mitochondrial toxicity. Further, the data shown in chapter 3.3 clearly show that the ability of berberine to induce SIRT1 activity is, at least in part, mediated through regulation of NAD^+ levels. Berberine was found to raise NAD^+ levels by inducing the upregulation of NAMPT expression, and this increase in NAD^+ levels was necessary for the increase in mitochondrial biogenesis induced by berberine (Figure 3.3.12).

Giving further strength to the fundamental role of SIRT1 in mitochondrial homeostasis, we observed that overexpression of SIRT1 *in vivo* mimics the effects of resveratrol and berberine on mitochondrial metabolism in skeletal muscle (chapter 3.2). Moreover, and in line with the findings from resveratrol and berberine treated animals, SIRT1 overexpression was found to be sufficient to induce AMPK activation in skeletal muscle, which clearly demonstrates that SIRT1 and AMPK do not function independently or linearly in the cell in response to resveratrol or berberine. Together, these results clearly demonstrate that SIRT1 plays a pivotal role in the regulation of mitochondrial homeostasis through induction of mitochondrial function as well as regulation of PGC-1α

induced mitochondrial biogenesis and also that SIRT1 is a key player in the beneficial effects of both resveratrol and berberine.

Importantly, using the adult-inducible whole body SIRT1 KO mouse, we show that loss of SIRT1 induces mitochondrial dysfunction in conditions of normal nutrient supply. However, and surprisingly, there was no change in the classical mitochondrial biogenesis pathway or changes in mitochondrial mass under these experimental conditions (chapter 3.1). These data together with the known redundant nature of the cell, lead to the hypothesis that SIRT1 might regulate mitochondrial homeostasis through PGC-1α independent pathways. Indeed, in chapter 3.1 we demonstrate for the first time that loss of SIRT1 under fed conditions does not change mitochondrial mass but rather has a specific effect on mtDNA maintenance and expression of the 13 mitochondrial-encoded ETC genes in a PGC-1α independent manner, leading to an imbalance between nuclear and mitochondrial encoded ETC subunits and thereby causing mitochondrial dysfunction. Moreover, it is also shown in this chapter that this new PGC-1α independent pathway is mediated by SIRT1's regulation of HIF1α protein stability. SIRT1 was previously shown to regulate HIF1α transcriptional activity under hypoxic conditions (Lim et al., 2010), however in this thesis we describe for the first time a different level of regulation of HIF1α by SIRT1. Indeed, loss of SIRT1 induces HIF1α stabilization under normoxic conditions, which not only promotes a shift towards increased glycolytic genes and a more glycolytic muscle fibers, but also causes an imbalance between nuclear and mitochondrial-encoded ETC subunits and mitochondrial dysfunction through inhibition of c-Myc-induced TFAM expression, adding an extra layer in the metabolic reprogramming induced by HIF1α and suggesting that the imbalance between nuclear and mitochondrial-encoded genes might be involved in the redirection of the carbon flux from oxidative phosphorylation to glycolysis and biosynthetic pathways induced by HIF1α.

As discussed both in the general introduction as well as in chapter 3.1, a tight coordination of the expression of genes encoded in both genomes is essential to promote functional and efficient mitochondria and, therefore, deregulation of this process like that observed with age or in SIRT1 KO mice may underlie the disruption in mitochondrial homeostasis observed during aging and age-related disorders. Indeed, the data presented in chapter 3.1 suggest that the imbalance between the expression of nuclear and mitochondrial-encoded genes is involved in the development of mitochondrial dysfunction during aging in the skeletal muscle and is accompanied by a

decline in NAD^+, an essential factor for SIRT1 activity, and stabilization of HIF1α. Moreover, and giving further strength to this idea, boosting NAD^+ levels in old mice was found to destabilize HIF1α and to reverse mitochondrial dysfunction as well as restoring mtDNA content and the expression of mitochondrial-encoded genes in a SIRT1 and HIF1α dependent manner. Together, this demonstrates that the ability to synchronize the induction of mitochondrial biogenesis in the nucleus and mitochondria is lost with age and strongly suggests that this might be due to a decline in NAD^+ levels and consequent loss of SIRT1 activity leading to HIF1α stabilization.

In conclusion, the work presented in this thesis describes, for the first time, the role of SIRT1 in mitochondrial biology *in vivo*. Indeed, and taking advantage of the new mouse model we have generated (chapter 3.1), we were able to describe for the first time that, in addition of regulation of PGC-1α and mitochondrial biogenesis, SIRT1 also regulates mitochondrial homeostasis through regulation of a HIF1α-c-Myc-TFAM axis (chapter 3.1) and shows the relevance of this pathway for the development of age-related mitochondrial dysfunction. Moreover, the data reported in this thesis also shows that the effects on mitochondrial homeostasis of two widely used natural compounds, resveratrol (chapter 3.2) and berberine (chapter 3.3), are largely mediated by SIRT1 when used at doses that do not induce mitochondrial toxicity. All together, the data presented in this thesis clearly show that SIRT1 is a metabolic sensor that integrates a variety of pathways to maintain mitochondrial homeostasis and therefore supports the idea of SIRT1 as an important target for the development of new drugs to treat diseases where mitochondrial homeostasis is disrupted (Figure 4.1).

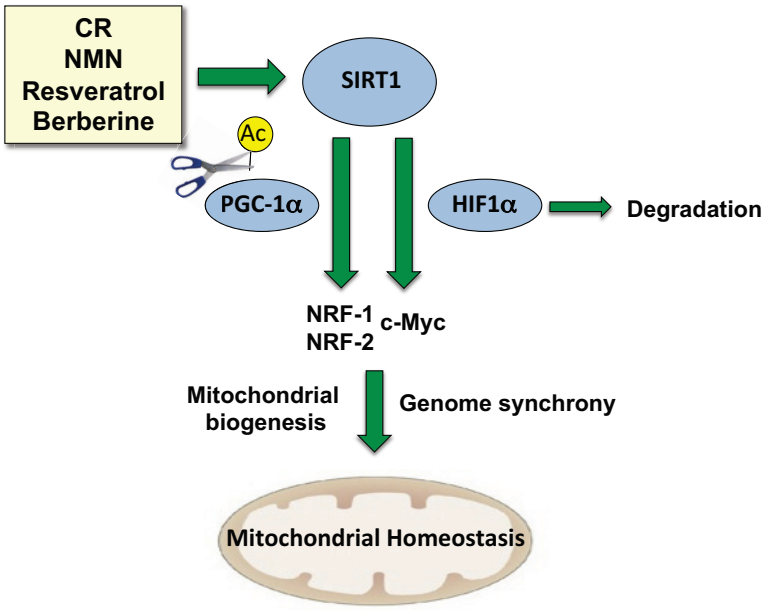


Figure 4.1 – SIRT1 as a central mediator of mitochondrial homeostasis maintenance.

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