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**SIRT4 Regulates ATP homeostasis and mediates a
retrograde signaling via AMPK**

by

LINH HO

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

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1 Chapter 1

GENERAL INTRODUCTION

1.1 Mammalian Sirtuin family

1.1.1 Phylogenic classification of sirtuins

The founding member of the Sirtuins group of proteins is Silent Information Regulator 2 (Sir2) in the budding yeast *Saccharomyces cerevisiae* [Frye, 1999]. The gene encoding the Sir2 enzyme, SIR2, was first discovered by its role in transcriptional silencing of mating-type loci in budding yeast [Smith, 1997][Bryk, 1997]. Following studies proved that SIR2 was involved in silencing at yeast telomeres and in recombinant DNA [Smith, 1997], in partitioning of carbonylated proteins between mother and daughter cells [Aguilaniu, 2003], and in extending the life span of budding yeast by repressing genome instability [Smith, 1997][Sinclair, 1997]. Sir2 was soon later found to be a nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylase (HDAC) [Imai, 2000]. Since then, orthologs of SIR2, known as sirtuins, have been found in most organisms, including plants, bacteria, and animals, and play pivotal roles in promoting the health and survival of an organism [Sinclair, 2006].

Sirtuins were described as NAD⁺-dependent deacetylases [North, 2004]. Sirtuins can be categorized into five classes, I–IV and U, based on the conservation of a 250 amino acid core domain (Figure 1.1). SIRT1, SIRT2, and SIRT3 are class I sirtuins, which show high homology to yeast homologs (Sir2, Hst1, Hst2) and exhibit robust deacetylase activity, whereas weak deacetylases, SIRT4 and SIRT5, belong to classes II and III, respectively [Frye, 2000]. There are 7 mammalian sirtuins, SIRT1–7 (Table 1.1), which are evolutionarily highly conserved in the NAD⁺-binding region and in the 250 amino acid catalytic domain [Frye, 2000]. Despite this

similarity, mounting evidence suggests that their localizations differentially determine their expression patterns, catalytic activities, targets, and, ultimately, biological functions. The mammalian sirtuins have localized in different subcellular compartments, with SIRT1, SIRT6, and SIRT7 predominantly residing in the nucleus, SIRT2 in the cytoplasm, and SIRT3, SIRT4, and SIRT5 in mitochondria [Houtkooper]. Most sirtuins have deacetylase activity on lysine residues of protein substrates. SIRT4 is the only sirtuin whose biochemical role is still being debated as to whether it is an ADP-ribosylase, a deacetylase, or a possible deacylase [Laurent][Haigis, 2006][Du, 2009][Feldman]. SIRT5 displays an effective demalonylase and desuccinylase activity, but very weak deacetylase activity [Du, 2009][Zhang]. A very recent study reported that human SIRT6 could act as a demyristoylase and depalmitoylase from lysine residues on proteins [Jiang]. The discovery of novel deacylase activities of SIRT5 and SIRT6 opens up the possibility that sirtuins in other classes with weak deacetylase activity, such as SIRT4, might possess other kinds of deacylase activity.

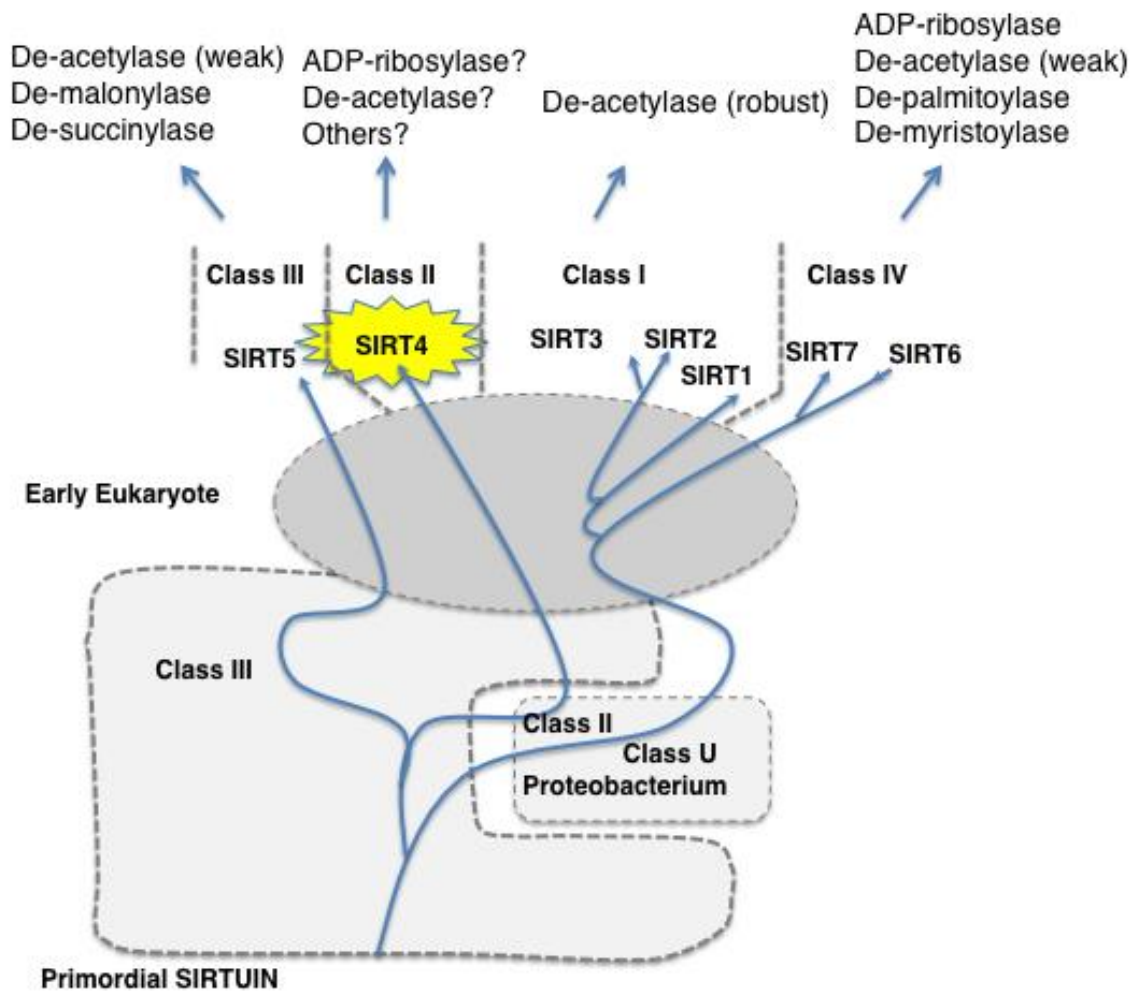


Figure 1.1. Phylogenetic classification of sirtuins

Phylogenetic analysis divides sirtuins into five classes, class I, II, III, IV, and U. SIRT3, SIRT4, and SIRT5 belong to classes I, II, and III, respectively. Sirtuins within a specific class seem to possess unique deacylase activities, although the activity for some classes is currently unknown (adapted from [Frye, 2000]). In addition, it is proposed that α -proteobacterium became engulfed by an archaean cell to form the first eukaryotic cell. The engulfed α -proteobacterium is characterized in a class II sirtuin and a class U sirtuin and the archaean parent contributed the class III sirtuin.

Table 1.1 Summary of the mammalian sirtuins

Sirtuin	Class	Localization	Activity	Targets	Biology	Null phenotype
SIRT1	I	Nucleus, cytoplasm	Deacetylation	PGC-1 α , FOXO1, FOXO3a, FOXO4, AceCS1, p53, P73, Notch, NF- κ B, HIF1 α , HIF2 α , PTEN, Smad3, Smad7, β -catenin, DKK1, Dvls, RelA, Ku70, XPA, XPC, NPS1, LXR, FXR, SREBP1c, and more	Metabolism, stress, DNA damage repair, tumor suppressor, autophagy, cancer, neurodegeneration, cardiovascular diseases	Developmental defects, lethal in some backgrounds
SIRT2	I	Cytoplasm	Deacetylation	Tubulin, PEPCK, FOXO1, PAR3	Cell cycle	Developmentally normal
SIRT3	I	Mitochondria	Deacetylation	LCAD, HMGCS2, GDH, OXPPOS complexes, SOD2, IDH2, OTC, AceCS2, and more	Thermogenesis, ATP production, metabolism, OXPPOS	Developmentally normal
SIRT4	II	Mitochondria	ADP-ribosylation, Deacetylation	GDH, MCD	Insulin secretion, fat metabolism, cancer	Developmentally normal
SIRT5	III	Mitochondria	Deacetylation, demalonylation, desuccinylation	CPS1	Urea cycle	Developmentally normal
SIRT6	IV	Nucleus	Deacetylation, ADP-ribosylation, demyristoylation, depalmitoylation	H3K9, H3K56	Base excision repair, metabolism, cancer, neurodegeneration	Premature aging
SIRT7	IV	Nucleolus	Deacetylation	H3K18, p53, PAF53	rDNA transcription, cardiovascular function, cancer	Smaller size, short lifespan, heart defects

PGC-1 α , Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; ; FOXO-1, -3a, -4, Forkhead box o-1, -3a, -4; AceCS1, Acetyl-CoA synthetase 1; NF- κ B, Nuclear Factor Kappa B; HIF-1 α , -2 α , Hypoxia-inducible factor 1-alpha, 2-alpha; PTEN, Phosphatase and tensin homologue; DKK1, Dickkopf-related protein 1; Dvls, Dishevelled proteins; XPA, Xeroderma pigmentosum group A; XPC, Xeroderma pigmentosum C; PEPCK, Phosphoenolpyruvate *carboxylkinase*; HMGCS2, 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2; GDH, Glutamate dehydrogenase; OXPPOS, oxidative phosphorylation; SOD2, Superoxide dismutase 2; IDH2, Isocitrate dehydrogenase; OTC, Ornithine carbamoyltransferase; AceCS2, acetyl-CoA synthetase 2; MCD, Malonyl-CoA decarboxylase; CPS1, Carbamoyl-phosphate synthase 1.

1.1.1 Sirtuin subcellular localization.

Mammalian sirtuins are found in different subcellular localizations depending on their primary amino acid signal sequences. The subcellular localization of these proteins probably depends upon cell type, stress status, and molecular interactions. For example, SIRT1 and SIRT2 were found to localize in both the nucleus and the cytoplasm and to interact with both nuclear and cytosolic proteins [Cohen, 2004][Tanno, 2007][North, 2007]. SIRT1 is mainly localized in the nucleus but is also present in the cytosol [Tanno, 2007]. In addition to two nuclear localization signal regions, SIRT1 possesses two nuclear export signals [Tanno, 2007]. Therefore, the exposure of nuclear localization signals versus nuclear export signals may explain shuttling localization of SIRT1. Although the physiological relevance of this shuttling is unclear, it is possible that either cytosolic protein targets could be deacetylated or that shuttling is another level of control on nuclear target proteins. SIRT2 is primarily cytosolic but is also present in the nucleus in the G2 to M phase transition of the cell cycle [Vaquero, 2006]. SIRT3, SIRT4 and SIRT5 possess amino (N)-terminal mitochondrial targeting sequences and proved to localize to the mitochondrial matrix [Huang]. SIRT6 is predominantly nuclear [Mostoslavsky, 2006], and SIRT7 was reported to widely express in the nucleolus [Ford, 2006], but further research is necessary to confirm this localization and its physiological relevance.

1.1.2 Sirtuin enzymatic activity

Originally, sirtuins were described as NAD-dependent type III HDACs, as the founding member in yeast, Sir2, silenced specific genomic loci by deacetylating histones H3 and H4 [Braunstein, 1996]. Sirtuin catalyzed deacetylation of proteins was first

reported in 2000 [Imai, 2000][Landry, 2000][Smith, 2000]. The enzymatic reaction is shown in Figure 1.1. Unlike other known protein deacetylases, which simply hydrolyze acetyl-lysine residues, the sirtuin-mediated deacetylation reaction couples lysine deacetylation to NAD hydrolysis. In this process, NAD^+ is reacted with an acetyllysine substrate to produce nicotinamide, the deacetylated substrate, and an unusual compound called 2'-*O*-acetyl-ADP-ribose (2'-AADPR) [Sauve, 2001]. Interestingly, class I sirtuins not only target histones, but also deacetylate a wide range of proteins in different subcellular compartments (Table 1.1). In addition, SIRT4 [Haigis, 2006] and SIRT6 [Liszt, 2005] were reported to function as ADP-ribosyltransferases, even though SIRT6 also can act as a deacetylase [Zhong][Michishita, 2008]. Recently, it was reported that human SIRT6 efficiently removes long-chain fatty acyl groups, such as myristoyl and palmitoyl, from lysine residues on protein substrates [Jiang]. SIRT5 was initially reported to deacetylate the urea cycle enzyme carbamoyl phosphate synthetase 1 (CPS1) [Nakagawa, 2009], but was recently shown to primarily demalonylate and desuccinylate proteins [Peng, 2011], including CPS1 [Du, 2011] [Rardin and He, 2013](Figure 1.2).

The role of NAD^+ in the enzymatic reaction catalysed by sirtuins is as a substrate, where its concentration is determined by the cellular nutritional state [Houtkooper]. Sirtuins convert NAD^+ to nicotinamide and, at higher concentrations, nicotinamide can inhibit sirtuin activity in a non-competitive manner as a feedback mechanism [Anderson, 2003][Bitterman, 2002]. Interestingly, nicotinamide binds to a conserved C-pocket adjacent to NAD^+ to block NAD^+ hydrolysis and therefore inhibits the deacetylation reaction [Avalos, 2004][Bitterman, 2002]. *O*-acetyl-ADP-ribose, the other by-product of the sirtuin deacetylase reaction, was also indicated to be a signaling molecule [Liou,

2005][Kustatscher, 2005], but its exact role in metabolic control needs further studies
[Tong][Houtkooper].

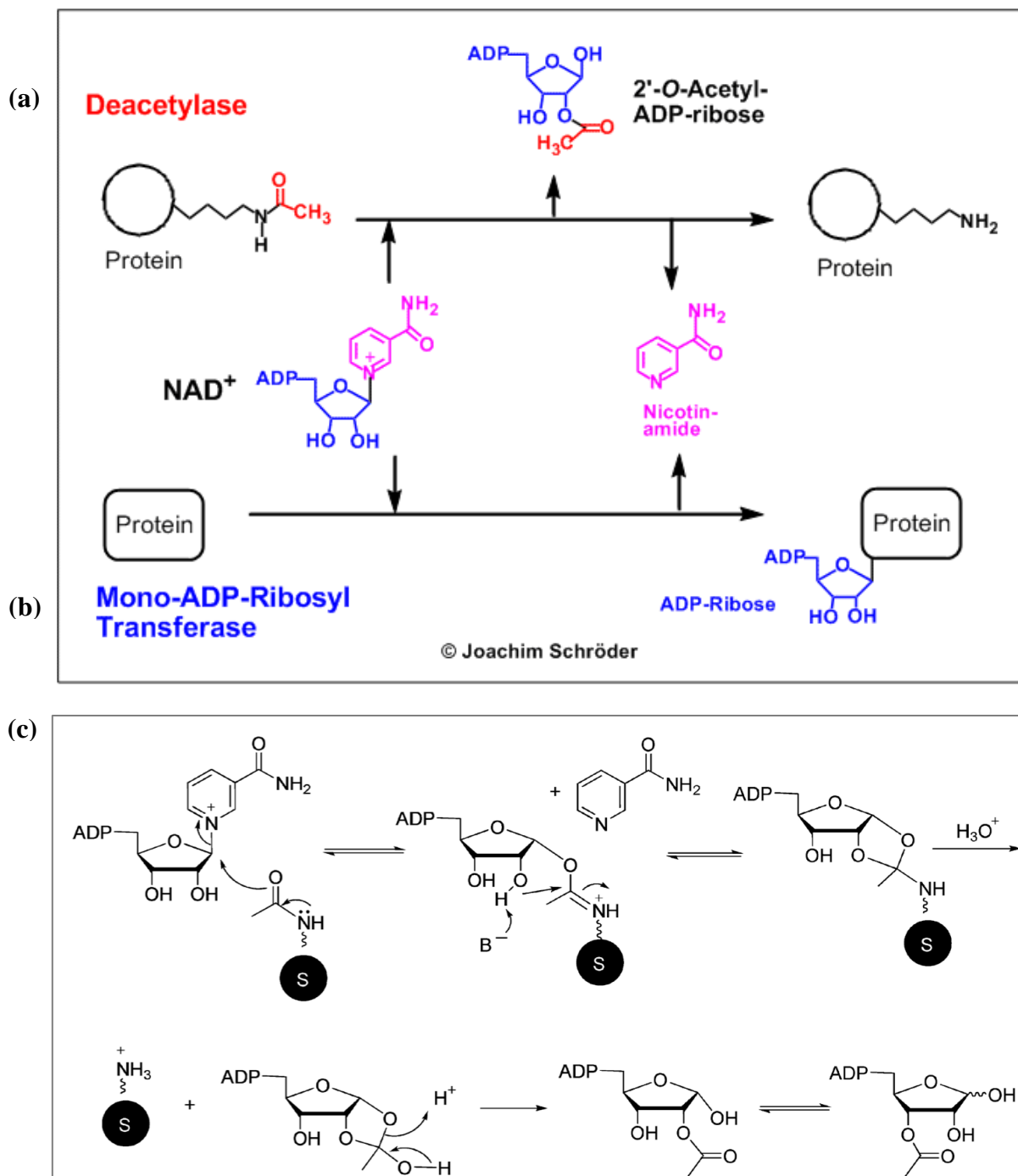


Figure 1.1. Enzymatic mechanisms of sirtuins

(a) Sirtuin-catalyzed deacetylation and (b) ADP-ribosylation reactions. (c) Mechanism of sirtuin-catalyzed NAD^+ -dependent deacetylation of proteins. NAD^+ is consumed as a substrate, along with an acetylated protein substrate, and a water molecule. Upon reaction, the acetyl-group at the N(ϵ)-position is removed and nicotinamide and 2'-AADPR are formed as co-products. (Based on [Du, 2009])

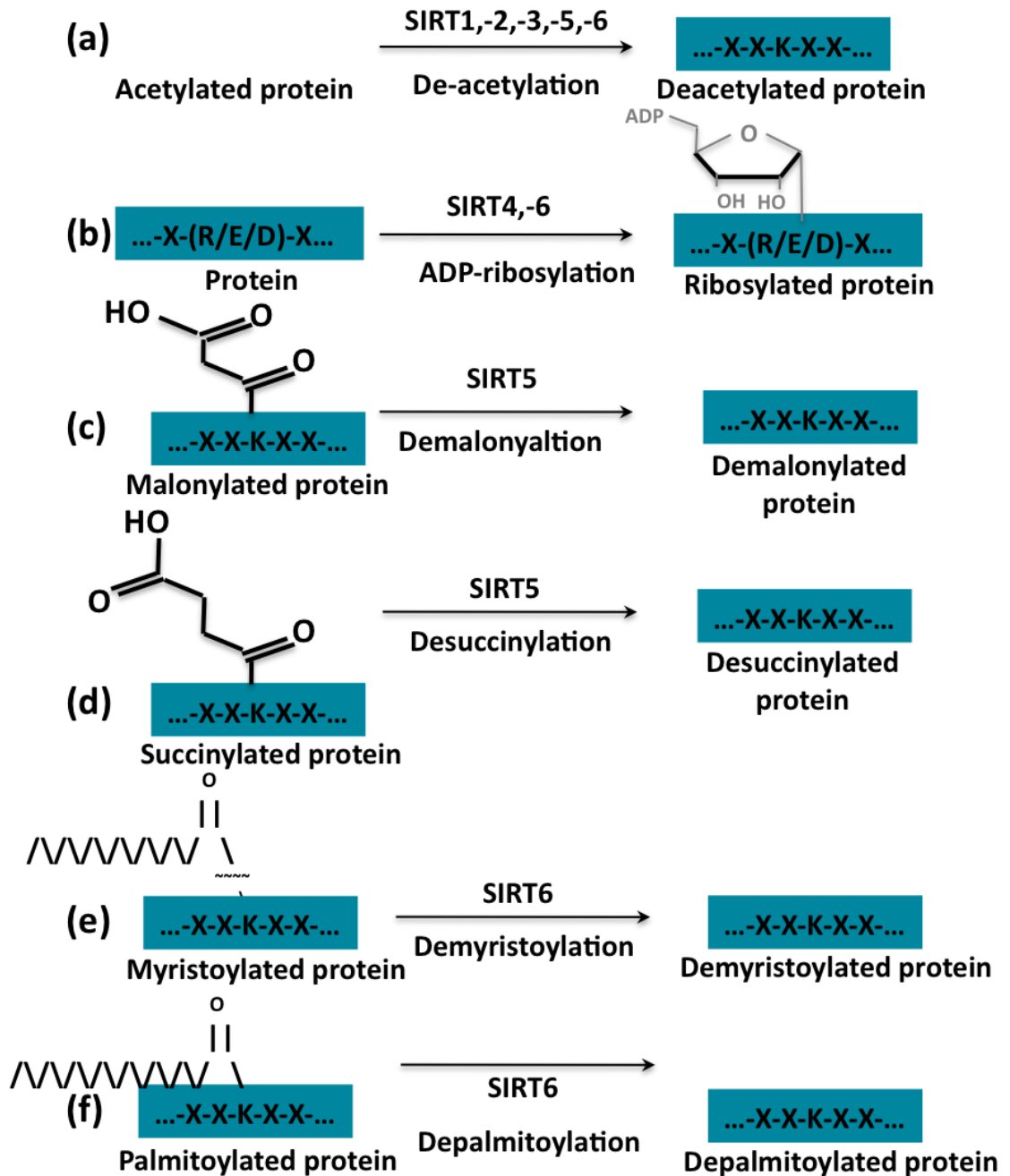


Figure 1.2. Summary reactions of sirtuins

(a) Sirtuins remove an acetyl moiety from a histone or protein, (b) SIRT4 and SIRT6 were described to act as ADP-ribosyl transferases, (c) SIRT5 possesses demalonylase activity and (d) desuccinylase activity, SIRT6 can remove (e) myristoyl and (f) palmitoyl groups from proteins.

1.1.3 SIRT1

1.1.3.1 SIRT1 and metabolism

SIRT1 has been involved in various signaling pathways and cancers (Table 1.1). Growing evidence indicates that SIRT1 is a significant target for alleviating metabolic dysfunction syndromes. SIRT1 expression levels increase in response to caloric restriction (CR), and the enzyme can deacetylate a variety of transcription factors and enzymes in charge of central metabolism and energy expenditure [Houtkooper]. An important factor among these factors is peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α), a transcriptional coactivator that regulates expression of metabolic genes involved in processes such as hepatic gluconeogenesis, fatty acid oxidation (FAO), and mitochondrial biogenesis [Gerhart-Hines][Dominy]. The deacetylation of PGC-1 α by SIRT1 increases PGC-1 α activity, enhancing the expression of transporters and catabolic enzymes for the uptake and utilization of free fatty acids (FAs) [Canto, 2009][Gerhart-Hines, 2007]. Consistent with these data, SIRT1 stimulates browning of adipose tissue by increasing the interaction between PPAR γ and PRDM16 [Qiang], a potent inducer of *PGC-1 α* expression [Kajimura, 2008].

Increased level of SIRT1 can protect against metabolic disorder. Specifically, mild overexpression of SIRT1 in mice on a high-fat diet (HFD) and in diabetic *db/db* mice resulted in improved glucose tolerance, with a significant reduction in blood glucose and plasma insulin levels without significant changes in body weight or fat composition [Pfluger, 2008][Banks, 2008][Herranz]. These effects were partly due to the ability of SIRT1 to maintain β cell integrity and function, as β cell-specific overexpression of SIRT1 remarkably improves insulin secretion and glucose tolerance

[Moynihan, 2005]. In addition, pancreatic expression of SIRT1 reduces the level of β cell death and the extent of glucose intolerance induced by the pro-diabetic drug streptozotocin [Tang]. Moreover, the protective effects of SIRT1 may occur through attenuation of inflammatory responses, as SIRT1 overexpression alleviates HFD-induced hepatic steatosis and adipose tissue-specific inflammation [Pfluger, 2008][Gillum]. In contrast, hepatocyte-specific SIRT1 deletion increased hepatic steatosis and inflammation and endoplasmic reticulum stress with chronic HFD feeding [Purushotham, 2009], as well as an age-dependent onset of hepatic steatosis with normal diet feeding [Wang]. These findings suggest a role for SIRT1 in treating age-dependent non-alcoholic fatty liver disease.

Table 1.1 SIRT1 targets in multiple signaling pathways

Signaling pathways	Targets	Reference
Tumor suppressor	p53, p73, and HIC1	[Chen, 2005][Dai, 2007][Luo, 2001][Vaziri, 2001]
Energy homeostasis	FOXO1, FOXO3a, and FOXO4	[Schug][Yeung, 2004][Kong]
	PGC-1 α , PPAR γ , and AceCS1	[Hallows, 2006][Han][Rodgers, 2005][van der Horst, 2004][Motta, 2004][Brunet, 2004]
Autophagy	Atg5, Atg7, and Atg8	[Powell]
Hypoxia-inducible factors	HIF-1 α and HIF-2 α	[Leiser][Lim][Dioum, 2009]
PI3K/AKT pathway	PTEN	[Ikenoue, 2008]
TGF- β signaling pathway	Smad3 and Smad7	[Li][Kume, 2007]
Wnt signaling pathway	β -catenin, DKK1, and Dvls	[Cho][Holloway][Firestein, 2008]
NF- κ B signaling	RelA/p65	[Chen, 2001][Kiernan, 2003][Chen, 2002]
DNA damage repair pathway	Ku70, XPA, XPC, and NBS1	[Yuan, 2007][Ming][Fan][Jeong, 2007]

PI3K, Phosphoinositide 3-Kinase; AKT, Protein Kinase B; HIC1, Hypermethylated In Cancer 1; NF- κ B, Nuclear Factor Kappa B; FOXO-1, -2, -4, Forkhead box O-1, -2, -4; PPAR γ , Peroxisome Proliferator-Activated Receptor gamma; AceCS1, Acetyl-CoA Synthetase 1; Atg-5, -7, -8, Autophagy-related genes; HIF-1 α , -2 α , Hypoxia-Inducible Factor 1-alpha, 2-alpha; PTEN, Phosphatase and Tensin homologue; Dvls, Dishevelled proteins; DKK1, Dickkopf-related protein 1; XPA, Xeroderma pigmentosum group A; XPC, Xeroderma pigmentosum C; NBS1, Nijmegen breakage syndrome 1.

1.1.3.2 SIRT1 and cardiovascular diseases

SIRT1 suppresses the development of atherosclerosis by modulating the activity of endothelial nitric oxide synthase (eNOS), FoxO1, and p53, and the expression of angiotensin II type 1 receptor (AT1R) [Borradaile, 2009][Brandes, 2008]. SIRT1 also promotes vasodilatory and regenerative functions in endothelial and smooth muscle cells of the vascular wall [Borradaile, 2009][Brandes, 2008]. SIRT1 also deacetylates liver X receptor proteins (LXR) at K432 to promote ubiquitination to alter cholesterol

biosynthesis in the liver and macrophages [Li, 2007] and to reduce serum lipid levels [Feige, 2008]. These results suggested SIRT1 play a major role in the prevention of atherosclerosis and other aging-associated diseases by calorie restriction.

SIRT1 is highly expressed in the vasculature during blood vessel growth, where it controls the angiogenic activity of endothelial cells. Loss of SIRT1 function impairs sprouting angiogenesis and branching morphogenesis of endothelial cells with consequent down-regulation of genes involved in blood vessel development and vascular remodeling [Potente, 2007]. Moreover, activation of SIRT1 in endothelial tissues may be of benefit in the protection of endothelial cell function with age. Mice with endothelial-specific SIRT1 overexpression on an *Apoe*^{-/-} genetic background exhibit attenuated development of atherosclerotic lesions [Zhang, 2008]. Conversely, SIRT1 insufficiency results in greater foam cell formation and atherosclerotic lesioning [Stein]. Taken together, these findings uncover a novel and unexpected role for SIRT1 as a critical modulator of endothelial gene expression governing postnatal vascular growth and its protective effects in atherogenesis.

Sirt1 could have beneficial effects on retard aging and confer stress resistance to the heart in vivo at low to moderate doses (up to 7.5-fold of *Sirt1*) [Alcendor, 2007]. Overexpression of SIRT1 at low to moderate levels in transgenic mouse hearts attenuates age-dependent increases in cardiac hypertrophy, apoptosis/fibrosis, cardiac dysfunction, and expression of senescence markers such as p15^{INK4b} and p19^{ARF} [Alcendor, 2007]. On the other hand, a high level of SIRT1 increased apoptosis and hypertrophy and decreased cardiac function, thereby stimulating the development of cardiomyopathy [Alcendor, 2007]. These data suggested that stimulation of *Sirt1* may be useful for an

antiaging therapy for the heart, more careful evaluations on dosages of Sirt1 need to be investigated.

1.1.3.3 SIRT1 and cancer

SIRT1 expression is heightened in various cancers, including prostate cancers [Huffman, 2007], acute myeloid leukemia [Bradbury, 2005], colon carcinomas [Stunkel, 2007], and non-melanoma skin carcinomas [Hida, 2007]. In these diseases, SIRT1 can be considered a cancer promoter, thus, inhibition of SIRT1 may be a strategy to induce cell cycle arrest and apoptosis in transformed cells [Lain, 2008][Ota, 2006][Heltweg, 2006][Ford, 2005]. Furthermore, the SIRT1 activator SRT1720 was indicated to promote metastasis in a breast cancer mouse model [Suzuki]. In contrast, SIRT1 can be a tumor suppressor. The rescue of SIRT1 levels in some cancer types, such as mutant breast cancer 1 (early onset–mutant (BRCA1) breast cancer cells that have lower levels of SIRT1 compared to BRCA1), can result in inhibition of tumor growth [Wang, 2008]. Furthermore, SIRT1 overexpression reduces the growth rate of fibrosarcoma cells in a tissue allograft model [Lim]. In other cancer mouse models, SIRT1 has been shown to protect against the development and progression of intestinal tumors in a β -catenin–driven colon cancer model [Firestein, 2008], sarcomas, lymphomas, teratomas, and carcinomas arising from deletion of p53 [Wang, 2008], HFD-induced hepatocarcinomas [Herranz], and age-associated spontaneous tumor development [Herranz]. However, what cellular determinants for SIRT1 to be a tumor suppressor or a tumor promoter remain to be uncovered.

1.1.3.4 SIRT1 and neurodegeneration

Progress in understanding the neurobiological benefits of SIRT1 is still in its early stage and has been studied in different human CNS diseases. A hallmark of Alzheimer's disease (AD) is the accumulation of plaques of Abeta 1-40 and 1-42 peptides, which result from the sequential cleavage of APP by the beta and gamma-secretases. In Alzheimer's disease mouse models, brain-specific knockout of SIRT1 showed a dramatic increase in β -amyloid, plaques, and reactive gliosis [Donmez]. These effects were decreased by deacetylation of retinoic acid receptor- β as a result of SIRT1 overexpression [Donmez]. Moreover, SIRT1 has been proposed to have a neuroprotection role in three different mouse models of Huntington's disease [Jiang][Jeong]. Brain-specific deletion of SIRT1 results in exacerbation of neuropathology and mutant Huntingtin neurotoxicity in a mouse model of Huntington's disease. Conversely, overexpression of SIRT1 improves the survival, neuropathology, mutant Huntingtin neurotoxicity, and the expression of brain-derived neurotrophic factor (BDNF) in these same mice [Jeong][Jiang]. The neuroprotective function of SIRT1 in Huntington's disease models is thought to be modulated by the activation of multiple targets, including cAMP response element-binding protein (CREB), CREB regulated transcription coactivator 1, and FOXO3a.

In a mouse model of Parkinson's disease with A53T α -synuclein, overexpression of SIRT1 reduced α -synuclein aggregates, gliosis, and lethality [Donmez]. However, knockout of SIRT1 significantly increased aggregates, worsened gliosis, and hastened mortality [Donmez]. Deacetylation and activation of the heat shock factor HSF1 is proposed to be the crucial mechanism by which SIRT1 protects against Parkinson's disease in this mouse model. Lastly, in a mouse model of injury-induced axonal

degeneration, stimulation of NAD⁺ biosynthesis, and its support of SIRT1 activity, was indicated to be a major factor in delaying axonal degeneration [Araki, 2004][Sasaki, 2006].

1.1.4 SIRT2.

1.1.4.1 The biological properties of the SIRT2

SIRT2 is located primarily in the cytoplasm, where the protein colocalizes with microtubules and deacetylates α -tubulin, the major component of microtubules, at lysine 40 [North, 2003]. SIRT2 also transiently migrates to the nuclei during G2/M transition and deacetylates histone H4 at lysine 16, thereby modulating chromatin condensation during metaphase [Vaquero, 2006]. SIRT1 also deacetylates lysine 16 of histone H4, indicating a possible synergistic relationship or functional redundancy between these sirtuins [Vaquero, 2006][Imai, 2000]. Interestingly, SIRT2 overexpression delays cell cycle progression [Dryden, 2003]. More specifically, SIRT2 expression levels are variable during different cell cycle phases and increase during mitosis. Together, these results suggested SIRT2 might have an important role in regulation cell cycle [Inoue, 2007].

Sirtuin deacetylases and FOXO (Forkhead box, class O) transcription factors have important roles in many biological pathways, including cancer development. SIRT2 deacetylates forkhead transcription factors of class O, FOXO1, and FOXO3 [Zhu, 2012][Wang, 2007][Li, 2007]. Since FOXO transcriptional factors are involved in multiple cellular processes, such as DNA repair, the cell cycle, apoptosis, metabolism, and aging, SIRT2 is therefore proposed to regulate these diverse pathways [Calnan, 2008]. One such mechanism of this regulation is through p300, a histone acetylase that also regulates the function of p53, a protein important for conserving genome stability, known as tumor suppressor. p300 has been shown to inactivate SIRT2 by acetylation, an

effect that may indirectly regulate the activity of p53. More specifically, SIRT2 deacetylates lysine residues in the catalytic domain of p300, which maintains its active form by autoacetylation [Black, 2008]. As a consequence, p300 can inactivate SIRT2 by acetylation, which relieves the inhibitory effect of SIRT2 on the transcriptional activity of p53 [Han, 2008].

Interestingly, post-translational phosphorylation also negatively modulates SIRT2 activity. Phosphorylation at serine 331 inhibits SIRT2 catalytic activity [Pandithage, 2008]. SIRT2 is phosphorylated by various cyclin dependent kinase (CDK) complexes at serine 331 and is dephosphorylated by dual specificity protein phosphatase (CDC14B [Pandithage, 2008][Dryden, 2003]. In addition, several proteins such as 14-3-3 β/γ and homeobox transcription factor 10 are binding partners, but not deacetylation substrates, of SIRT2 [Jin, 2008][Bae, 2004]. The currently known SIRT2 substrates/binding partners suggest a complex and apparently diverse function for this sirtuin in the cell (Table 1.2).

Table 1.2 SIRT2 substrates and interactors and their biological relevance.

Interactor/substrate	Interaction type	Biological relevance	Reference
α -Tubulin	Substrate (Lys-40)	Cytoskeleton regulation Oligodendroglial differentiation	[Zhu][North, 2003]
Histone H3	Substrate (Lys-56)	Cell cycle regulation	[Das, 2009]
Histone H4	Substrate (Lys-16)	Cell cycle regulation	[Vaquero, 2006]
FOXO1	Substrate (Lys-residues surrounding Ser-253)	Adipocyte differentiation	[Li, 2007][Wang, 2009]
FOXO3	Substrate (residues not yet identified)	Regulation of oxidative stress	[Zhu]
		Cell growth arrest apoptosis	
Par-3	Substrate (Lys-831, 848, 881, 1327)	Modulation of peripheral myelination	[Beirowski]
P300	Substrate (several Lys-residues)	Regulation of p300 autoacetylation	[Black, 2008]
PEPCK1	Substrate (residues not yet identified)	Blood glucose homeostasis	[Jiang]
p65	Substrate (Lys-310)	Regulation of NF- κ B dependent gene expression	[Rothgiesser]
HOXA10	Binding partner	Not known	[Bae, 2004]
HDAC6	Binding partner	Cytoskeleton dynamics	[North, 2003]
14-3-3 β/γ	Binding partners	Downregulation of p53 activity	[Jin, 2008]
CDK1, cyclinE/CDK2, cyclinA/CDK2, p35/CDK5	Phosphorylation of SIRT2 at Ser-331,-368	Inhibition of SIRT2 catalytic activity	[Pandithage, 2008][Southwood, 2007]

CDC14A/B, Dual-specificity protein phosphatase 14A/B; CDK1, Cyclin-dependent kinase 1; CyclinA/CDK2, Cyclin A/cyclin-dependent kinase 2 complex; CyclinE/CDK2, Cyclin E/cyclin-dependent kinase 2 complex; p35/CDK5, p35/cyclin-dependent kinase 5 complex; FOXO, Forkhead box protein of class O; HDAC6, Histone deacetylase 6; HOXA10, Homeobox protein A10; Lys, Lysine; NF- κ B, Nuclear factor-kappa B; Par-3, Polarity protein par-3; PEPCK1, phosphoenolpyruvate carboxykinase 1; P300, Histone acetyltransferase ac-P300; p53, tumor suppressor protein 53; p65, transcription factor p65; Ser, Serine; 14-3-3 β/γ , 14-3-3 Protein β/γ isoforms.

1.1.4.2 SIRT2 and metabolic syndromes

Accumulating evidence indicates a role for SIRT2 in regulating adipose tissue development and function. SIRT2 expression is the most abundant sirtuin in adipocytes [Li, 2007]. SIRT2 level decreases during preadipocyte differentiation, whereas reducing SIRT2 expression promotes adipogenesis [Jing, 2007][Wang, 2009]. SIRT2 expression is also elevated in adipose tissue during CR [Wang, 2009] and is significantly reduced in white adipose tissue of obese patients [Krishnan]. As mentioned previously, SIRT2-mediated deacetylation targets include the FOXO family of transcription factors. For example, SIRT2 deacetylates FOXO1, promoting the interaction of FOXO1 with PPAR γ thereby repressing PPAR γ transcriptional activity and inhibiting adipocyte differentiation [Wang, 2009][Jing, 2007]. Additionally, in cell-based assays, SIRT2 increases expression of oxidation and mitochondrial genes in a PGC-1 α -dependent manner [Krishnan]. Thus, discovering the role of SIRT2 activators in metabolic homeostasis may be beneficial in the protection against obesity.

Interestingly, SIRT2 expression is increased in white adipose tissue and kidney of calorie-restricted mice [Zhu]. In another observation, SIRT2 deacetylates and stabilizes phosphoenolpyruvate carboxykinase 1 (PEPCK1) in regulation of glucose homeostasis [Jiang]. In low glucose conditions, SIRT2 is activated, which stabilizes PEPCK1 and shifts the equilibrium toward the generation of glucose from non-carbohydrate carbon sources. This mimics a fast or exercise state in the organisms. In contrast, under high glucose conditions, SIRT2 expression is decreased, resulting in degradation of PEPCK1 by the ubiquitin proteasome system [Jiang]. Further studies need to be conducted to clarify

how nutrient availability affects SIRT2 protein level and cross-talk between sirtuin members during regulation of metabolic homeostasis.

1.1.4.3 SIRT2 and cancer

The known function of SIRT2 at the mitotic checkpoint confers a role for SIRT2 in tumorigenesis [Inoue, 2007]. For example, SIRT2 protein and RNA levels are decreased in gliomas, melanomas, and gastric carcinomas [Peters][Hiratsuka, 2003], and SIRT2 expression inhibits colony formation in glioma cells [Hiratsuka, 2003]. Additionally, overexpression of SIRT2 mediates a delay in cellular proliferation dependent on serine 368 phosphorylation [North, 2007]. Furthermore, overexpression of SIRT2 with a mutation at serine 368 (phosphorylation site) leads to a reduction of hyperploid cells under mitotic stress [North, 2007].

SIRT2 is also known to play a role in the regulation of cell cycle progression [Dryden, 2003][Pandithage, 2008][North, 2007] and chromatin assembly [Vaquero, 2006][Das, 2009]. Knockout of SIRT2 in mice led to tumorigenesis due to failed mitosis complex regulation and disrupted genomic integrity [Kim]. However, studies have reported that pharmacological inhibition of SIRT2 suppresses tumor growth by activating p53 [Jin, 2008][Lain, 2008]. More evidence is needed to discover the therapeutic potential of targeting SIRT2 regulation of the cell cycle.

SIRT2-deficient mice develop gender-specific tumorigenesis. In a recent study, SIRT2 deficiency in mice caused heighten levels of serine-threonine kinases Aurora-A and -B, which direct centrosome amplification, aneuploidy, and mitotic cell death [Kim]. Interestingly, SIRT2-deficient mice develop gender-specific tumorigenesis, with females primarily developing mammary tumors, and males developing more hepatocellular

carcinoma (HCC) [Kim]. These data suggested that SIRT2 is a tumor suppressor by its role in regulation of mitosis and genome integrity.

In accordance with these findings, further studies to explore role of SIRT2 in tumor development has been reported. In fact, another study reported that lysine 56 of histone H3, which is deacetylated by SIRT2 and SIRT1, was hyperacetylated in cancer cells, suggesting that SIRT2 may act as a tumor suppressor gene [Das, 2009]. In addition, SIRT2 downregulation can cause apoptosis in a cancer cell line such as HeLa, but not in normal cells, suggesting SIRT2 could be a possible intervention therapy for cancer [Li]. Moreover, SIRT2 regulates the expression of the pro-apoptotic protein BIM by deacetylating FOXO3 [Wang]. In general, SIRT2 overexpression reduces cell proliferation and regulates cell death in response to stress-induced DNA damage, suggesting that SIRT2 is a tumor suppressor gene [Das, 2009]. Further studies will be important to elucidate the role of SIRT2 activation in specific human cancers and will shed light on feasible approaches for using SIRT2 for cancer interventions and treatments [Bosch-Presegue].

1.1.4.4 SIRT2 and the central nervous system and neurodegenerative disorders

SIRT2 is also the most abundant sirtuin in brain [Zhu,] and is primarily found in oligodendrocytes (OL), the myelin producing cells of the central nervous system (CNS) [Pandithage, 2008][Zhu]. Notably, the SIRT2.2 isoform is expressed significantly high in adult CNS, with maximal accumulation in aged brain, whereas SIRT2.1 is predominately expressed in skeletal muscle and immortalized cells in culture [Maxwell]. SIRT2 expression in OL plays a role in myelinogenesis, although its function is essentially unknown in neurons. In OLs, SIRT2 is localized to the myelin sheath [Werner, 2007]

where the sirtuin has been shown to function at sites of microtubule remodeling by deacetylating α -tubulin to downregulate OL differentiation [Li, 2007] [Harting, 2010][Southwood, 2007]. These results demonstrated a counterbalancing role of SIRT2 against a facilitatory effect of tubulin acetylation on oligodendroglial differentiation. Selective SIRT2 availability to oligodendroglia may have important implications for myelinogenesis, myelin–axon interaction, and brain aging [Li, 2007].

The formation of myelin by Schwann cells (SCs) occurs via a series of orchestrated molecular events. Interestingly, Schwann cell–specific SIRT2 deletion in mice or overexpression of SIRT2 have uncovered a role for the sirtuin in demyelinating neuropathies in the peripheral nervous system [Beirowski]. In neurons, SIRT2 is found in the cytoplasm, neurites, and growth cones [Pandithage, 2008]. In postmitotic hippocampal neurons, neurite outgrowth and growth cone collapse are inhibited by SIRT2 [Pandithage, 2008]. This effect is abolished by CDK-dependent phosphorylation of SIRT2 [Pandithage, 2008]. This provides insights into the role of SIRT2 in control a pivotal polarity pathway in SCs during myelin assembly and the association between intracellular metabolism and SC plasticity [Pandithage, 2008].

SIRT2 is associated with the aggregation of α -synuclein and Huntingtin, proteins involved in Parkinson’s disease (PD) and Huntington’s disease (HD), respectively [Luthi-Carter][Pallos, 2008][Outeiro, 2007]. Inhibition of SIRT2 by pharmacological and genetic means led to a rescue of α -synuclein toxicity in models of PD, namely dopaminergic neurons and an in vivo fly model [Outeiro, 2007]. Several studies reported that reduction of SIRT2 is protective in fly and worm models of HD by increasing photoreceptor neuron survival [Pallos, 2008] and decreasing sterol biosynthesis [Luthi-

Carter]. Partial rescue of toxicity was also observed in a *Drosophila* model of PD [Outeiro, 2007]. However, SIRT2 knockout mice lacked a significant phenotype related to neurodegeneration [Bobrowska], calling into question the effect of SIRT2 on neuronal health in mammals.

1.1.5 Mitochondrial SIRT3

1.1.5.1 SIRT3 and metabolism

Located in mitochondria, SIRT3 is the best-characterized major deacetylase [Lombard]. Basal SIRT3 expression varies widely but is highly expressed in the most metabolically active tissues, including liver, kidney, and heart [Palacios, 2009][Ahn, 2008]. Diet and nutrient status significantly affect SIRT3 levels in different tissues, such that SIRT3 expression in liver and adipose tissue increases in glucose-poor, fasting states including CR [Alhazzazi][Tauriainen][Hallows][Caton][Hirschey]. Expression in skeletal muscle also increases in CR [Tauriainen][Jing], but has been reported to both increase [Caton][Palacios, 2009] and decrease [Jing] with fasting. Exercise increases SIRT3 expression in skeletal muscle in both mice [Gurd][Palacios, 2009] and humans [Lanza, 2008][Koltai]. Interestingly, although a HFD initially induces SIRT3 expression in liver and skeletal muscle, a chronic HFD decreases the expression of the sirtuin [Bao][Tauriainen][Palacios, 2009][Hirschey, 2010][Jing, 2011]. SIRT3 expression is also decreased in mouse models of type 1 or 2 diabetes mellitus [Palacios, 2009][Jing, 2011]. These findings suggest SIRT3 might play an important role in diabetes and metabolism, especially, fat metabolism through its deacetylation activity.

The first identified SIRT3 substrate was human acetyl-CoA synthetase 2 (AceCS2) [Schwer, 2006][Hallows]. AceCS2 is a mitochondrial matrix enzyme that

converts acetate to acetyl-CoA for oxidation through the tricarboxylic acid (TCA) cycle to produce ATP and carbon dioxide (CO₂) [Fujino, 2001]. AceCS2 expression is abundant in the heart and muscle and is upregulated during starvation and ketogenesis [Fujino, 2001]. SIRT3 has been shown to activate AceCS2 by a deacetylation reaction at lysine 642 (human) or lysine 635 (mouse) in the active site of the enzyme [Hallows][Schwer, 2006]. Since these studies were published, more than a dozen additional SIRT3 deacetylation targets have been reported (Table 1.3). Many of these SIRT3 targets play a role in metabolism switch to regulate fasting metabolism, metabolic adaptation toward energy source availability as well as pathologic energy demands of cells.

Table 1.3 Known substrates of mitochondrial SIRT3

Substrate name	Biological relevance	Reference
PPID (Peptidylprolyl Isomerase D or Cyclophilin D)	Glycolysis	[Shulga]
LCAD (Acyl-CoA Dehydrogenase, Long Chain)	Fatty Acid Oxidation	[Hirschey]
HMGCS2 (3-Hydroxy-3-Methylglutaryl-CoA Synthase 2, Mitochondrial)	Ketone Body Synthesis	[Shimazu]
ACSS2 (Acyl-CoA Synthetase Short-Chain Family Member 2)	Acetate Metabolism	[Hallows, 2006][Schwer, 2006]
OTC (Ornithine Transcarbamoyltransferase)	Urea Cycle	[Hallows]
GDH (Glutamate dehydrogenase 1)	Amino Acid Catabolism	[Schlicker, 2008]
NDUFA9 (NADH Dehydrogenase (Ubiquinone) 1 α Subcomplex, 9, 39-kDa)	Oxidative Phosphorylation	[Ahn, 2008]
SDHA (Succinate Dehydrogenase Complex, Subunit A, Flavoprotein)		[Cimen][Finley]
ATP5A (F1F0-ATPase Subunit α)		[Bao]
IDH2 (Isocitrate Dehydrogenase 2, Mitochondrial)	TCA Cycle	[Someya]
SOD2 or MnSOD (Superoxide Dismutase 2, Mitochondrial)	ROS	[Chen][Tao][Qiu]
ALDH2 (Aldehyde Dehydrogenase 2 Family, Mitochondrial)	Ethanol Metabolism	[Xue]
MRPL10 (Mitochondrial Ribosomal Protein L10)	Mitochondrial Protein Synthesis	[Yang]
FOXO3 (Forkhead Box O3)	Transcriptional Activation	[Sundaresan, 2009]
LBK1 or STK11 (Serine/Threonine Kinase 11)	Tumor Suppressor, AMPK Signaling	[Pillai]
HISTH3 (Histone Cluster 3, H3) (Specific to H3K56-Ac)	DNA Damage Response	[Vempati]
XRCC6 or Ku70 (X-ray Repair Complementing Defective Repair in Chinese Hamster Cells 6)	Apoptosis	[Sundaresan, 2008]

SIRT3 functions to deacetylate and activate enzymes important during CR, such as 3-hydroxy-3-methylglutaryl-CoA synthase 2 for generation of ketones [Shimazu] and long-chain acyl-CoA dehydrogenase for oxidation of long-chain FAs [Hirschey]. SIRT3 also deacetylates and activates components of the electron transport chain, thereby increasing oxidative phosphorylation [Cimen][Kim][Ahn, 2008][Kendrick][Bao]. Supporting evidence reported a role for SIRT3 in metabolic disorders. Mice lacking SIRT3 who were fed a HFD showed accelerated obesity, glucose intolerance, insulin resistance, hyperlipidemia, and steatohepatitis [Hirschey]. These effects were partly due to increased stearoyl-CoA desaturase 1 (SCD1) activity in the liver that was caused by increased saturated lipid levels. Deletion of SCD1 rescued both wild-type and SIRT3-knockout mice from HFD-induced metabolic disorders [Hirschey]. Furthermore, SIRT3-knockout mice show impaired insulin signaling in skeletal muscle by increased oxidative stress, which led to activation of the c-Jun N-terminal kinase (JNK) and decreased insulin receptor substrate-1 (IRS-1) signaling following insulin receptor activation [Jing, 2011].

In an independent study, SIRT3 knockout mice under 48 hours fasting showed increased levels of liver palmitoylcarnitine and of various blood acylcarnitines, indicating deficient hepatic β -oxidation [Ahn, 2008]. Furthermore, an analysis of an array of acetylated mitochondrial peptides identified potential targets of deacetylation by SIRT3, including some enzymes involved in FA β -oxidation, such as short-chain L-3-hydroxyacyl-CoA dehydrogenase (SCHAD), the short/branched-chain acyl-CoA dehydrogenase (SBCAD), very-long-chain acyl-CoA dehydrogenase (VLCAD), and long chain acyl-CoA dehydrogenase (LCAD) [Ahn, 2008]. Prominently, patients with a functional SNP in SIRT3, such as the rs11246020 SNP that has a point mutation in the

catalytic domain that decreases its activity, have an increased susceptibility to developing metabolic syndrome [Hirschey, 2010]. However, it is still unknown which tissues are directly responsible for mediating the effects of SIRT3, emphasized by a recent report of both muscle-specific and liver-specific *Sirt3*^{-/-} mice lacking overt metabolic phenotypes [Fernandez-Marcos]. Moreover, acetate produced in the liver from acetyl-CoA is another soluble form of lipid-derived energy that can be distributed to extrahepatic tissues [Shimazu]. SIRT3 also deacetylates and activates AceCS2, an enzyme present in extrahepatic tissues that is used to activate acetate into acetyl-CoA [Hallows, 2006][Schwer, 2006]. Therefore, SIRT3 alleviates the catabolism of FAs in the liver and the peripheral use of lipid-derived acetate and ketone bodies during fasting.

1.1.5.2 SIRT3 and nitrogen metabolism

SIRT3 stimulates amino acid metabolism and nitrogen waste disposal by deacetylating and activating the mitochondrial matrix enzyme glutamate dehydrogenase (GDH) [Schlicker, 2008]. Notably, GDH is ADP-ribosylated by SIRT4, leading to decreased GDH activity and insulin secretion in response to amino acids [Haigis, 2006]. These results suggest a co-ordinate regulation of sirtuins on GDH activity. More specifically, the transfer of the α -amino moiety to α -ketoglutarate by an aminotransferase forms glutamate in catabolism of most amino acids. GDH regenerates α -ketoglutarate from glutamate, and releases nitrogen to the urea cycle as ammonia [Shimazu]. SIRT3 accelerates the urea cycle by deacetylating and activating ornithine transcarbamoylase (OTC), the key mitochondrial enzyme in the urea cycle. Mice lacking SIRT3 exhibit a metabolic profile similar to human disorders of the urea cycle, including increased serum ornithine and reduced citrulline levels – the substrate and product, respectively, of OTC

[Hallows, 2011]. The results suggest that under low energy source, Sirt3 promotes amino acid catabolism and β -oxidation in mitochondria.

1.1.5.3 SIRT3 and mitochondrial respiration

SIRT3 facilitates mitochondrial oxidative phosphorylation (OXPHOS) by deacetylating and activating several components of the respiratory transport chain, including NAD dehydrogenase (ubiquinone 1) alpha subcomplex subunit 9 (NDUFA9) (complex I) [Ahn, 2008] and **Succinate dehydrogenase complex, subunit A (SDHA)** (complex II) [Cimen][Finley]. Accordingly, mice lacking SIRT3 show reduced complex I and II activity in the liver and brown adipose tissue [Finley][Cimen]. SIRT3 also regulates complex IV/V activity, probably by deacetylating ATP synthase [Bao]. Together, these effects on OXPHOS might be explained for the 10% reduced O₂ consumption and up to 50% reduced ATP production observed in mice lacking SIRT3 [Jing, 2011][Ahn, 2008]. These indicate protein acetylation and SIRT3 play important roles in mitochondrial respiration to regulate basal ATP levels.

1.1.5.4 SIRT3 and ROS

SIRT3 also increases the ability of mitochondria to manage with reactive oxygen species (ROS) generated as a by-product of OXPHOS. First, SIRT3 deacetylates and activates isocitrate dehydrogenase 2 (IDH2), an enzyme in the TCA cycle that helps to replenish the mitochondrial pool of nicotinamide adenine dinucleotide phosphate (NADPH) [Someya]. NADPH is used by glutathione reductase to maintain glutathione in its reduced antioxidant form [Berg, 2012]. Normal mice develop age-related hearing loss caused by ROS-induced cochlear cell damage. CR could ameliorate this phenotype via SIRT3-dependent deacetylation of IDH2, leading to reduced ROS levels [Someya].

Second, SIRT3 deacetylates and activates the ROS-scavenging enzyme Mn superoxide dismutase (SOD2), thereby reducing oxidative damage in the liver [Chen, 2011][Tao, 2010][Qiu, 2010]. These results reveal the role of SIRT3 to promote oxidative stress resistance and to reduce cellular ROS, suggesting possible approaches to combat aging and oxidative stress-related diseases.

1.1.5.5 SIRT3 and cardiovascular diseases

SIRT3 has also been proposed to play a role in aging-related heart disease. SIRT3-deficient mice show mild cardiac hypertrophy and interstitial fibrosis at baseline and severe cardiac hypertrophy in response to hypertrophic stimuli, whereas SIRT3-overexpressing mice are protected from similar stimuli [Sundaresan, 2009]. Although the exact mechanism for the anti-hypertrophic effects of SIRT3 remains unclear, some have suggested that the cardiac pathology of SIRT3 knockout mice stems from an increased activation of the mitochondrial permeability transition pore (mPTP), leading to apoptosis of cardiomyocytes [Hafner]. SIRT3 deacetylates and inhibits cyclophilin D, a regulatory component of mPTP, and cardiomyocytes from SIRT3 knockout mice show an age-dependent increase in mitochondrial permeability transition [Hafner]. These data suggest that SIRT3 is necessary to prevent mitochondrial dysfunction and cardiac hypertrophy during aging.

1.1.5.6 SIRT3 and cancer

SIRT3 modulates carbohydrate metabolism in cancer cells [Alhazzazi]. The Warburg effect refers to the preference of cancer cells for glucose utilization as energy source [Bayley]. Downregulation of SIRT3 can enhance the Warburg effect by two mechanisms [Finley][Kim][Shulga]. First, SIRT3 regulates hypoxia-inducible factor 1 α

(HIF1 α), a transcription factor that drives the expression of glycolytic genes, via cellular levels of ROS. In the absence of SIRT3, ROS production is increased, which leads to deactivation of prolyl hydroxylases [Kaelin, 2008] and stabilization of HIF1 α [Finley]. Second, hyperacetylation of the peptidyl-prolyl isomerase cyclophilin D in the absence of SIRT3 helps maintain hexokinase II (HK2), an enzyme plays a pivotal role in highly malignant cancer cells in promoting cell growth and survival, in an active state on the outer mitochondrial membrane, facilitating the rapid production of glucose-6-phosphate [Pedersen, 2002][Shulga, 2010]. These results suggested that SIRT3 might have an important role in metabolism of some cancer cells and their susceptibility to mitochondrial injury and cytotoxicity.

Several studies indicate that SIRT3 is a tumor suppressor [Haigis]. Important among these are these findings is that SIRT3-deficient mice develop mammary tumors, and embryonic fibroblasts from these mice exhibit a malignancy-prone phenotype with increased stress-induced superoxide levels and genomic instability [Kim]. These cellular defects can be rescued by the expression of SOD2, which is a target of SIRT3 deacetylation that is involved in the detoxification of ROS, as discussed previously [Qiu][Tao]. Coinciding with animal data, SIRT3 expression is decreased in many human cancers, with loss of at least one copy of SIRT3 occurring in 40% of human breast and ovarian cancers [Finley][Kim]. Nonetheless, there are also reports that SIRT3 overexpression promotes survival and enhances tumorigenesis, such as with oral cancer, emphasizing the importance of tumor type [Alhazzazi, 2011]. It seems SIRT3 function varies in different normal and tumor tissues and may be cell- and tumor-type specific. It

is necessary to conduct more thorough studies to determine whether SIRT3 as a tumor promoter or suppressor in each cancer type separately.

1.1.6 Mitochondrial SIRT4: still a mysterious enzyme

Among the three mitochondrial sirtuins, SIRT4 is the least known in terms of mechanism, substrates and regulating pathways in metabolism. SIRT4 is a soluble protein in mitochondrial matrix that becomes cleaved at amino acid 28 after importation into mitochondria [Michishita, 2005][Haigis, 2006][Ahuja, 2007]. SIRT4 is present in most tissues, with high levels of expression in liver, heart, kidney, brain and pancreatic β cells [Haigis, 2006][Ahuja, 2007]. The expression of SIRT4 slightly decreases during CR and increases in culture cell models of diabetes [Schwer, 2006][Nasrin][Haigis, 2006]. Interestingly, fasting and/or CR is incorporated with increased expression of hepatic SIRT1 and SIRT3 [Schwer, 2009][Hirschey][Cohen, 2004][Civitarese, 2007][Nasrin] suggesting that sirtuins might compensate their impacts in different states of metabolic demands to roundly regulate the production and utilization of energy in cells.

Although seven mammal sirtuins share a conserved catalytic core domain [Frye, 2000], while SIRT3 plays a major role in deacetylation of mitochondrial proteins, SIRT4 has displayed no detectable NAD-dependent deacetylase activity [North, 2003][Lombard, 2007][Schwer, 2006][Haigis, 2006][Ahuja, 2007]. $Sirt4^{-/-}$ and $Sirt5^{-/-}$ mice show no change in mitochondrial protein acetylation, in contrast, $Sirt3^{-/-}$ mice exhibit a striking hyperacetylation of mitochondrial proteins [Lombard, 2007]. This is not excluded from the possibility that SIRT4 has very strict deacetylation substrate specificity.

Nevertheless, SIRT4 is the only mitochondrial sirtuin that ADP-ribosylates and down-regulates GDH, leading to negative regulation of insulin secretion in islet β cells

[Haigis, 2006][Ahuja, 2007]. SIRT4 inhibits the enzymatic activity of GDH and limits the metabolism of glutamate and glutamine to generate ATP, thereby suppressing the ability of β cells to secrete insulin in response to these amino acids [Haigis, 2006]. SIRT4 knockout mice, which express lower levels of ADP-ribosylated GDH, exhibit higher GDH activity and upregulates amino acid-stimulated insulin secretion [Haigis, 2006]. Intriguingly, SIRT3 has been reported to deacetylate and activate GDH [Schlicker, 2008][Lombard, 2007] implicating a possible cooperated regulation of GDH activity by SIRT3 and SIRT4.

SIRT4 also regulates functions of proteins that are substrates of other sirtuins. In an independent model, knockdown of SIRT4 via siRNA from insulin-producing INS-1E cells led to an increase in insulin secretion in response to glucose, while overexpression of SIRT4 in these cells suppressed insulin secretion [Ahuja, 2007]. In contrast to negative insulin regulation of SIRT4, SIRT1 positively regulates insulin secretion in pancreatic β cells by regulating genes involved in β cell function, including mitochondrial uncoupling protein 2 (Ucp2) [Moynihan, 2005][Bordone, 2006]. Thus it is of interest to address questions about the role of sirtuins in mice lacking either SIRT1 or SIRT4 in insulin secretion. Interestingly, SIRT4 was shown to interact with insulin degrading enzyme (IDE) and the ADP/ATP carrier proteins ANT2 and ANT3, although the biochemical and physiological inter-relationship of these interactions is not yet determined [Ahuja, 2007].

In a recent study, Du et al stated that ADP-ribosylation activity of sirtuins is weak and may not be physiologically relevant [Du, 2009]. The sirtuin dependent-ADP-ribosylation activity is >1000-fold slower than that of a bacterial ADP-ribosyltransferase or their deacetylation activity [Du, 2009]. This claim has cast doubt upon whether sirtuins

serve as physiologically relevant ADP ribosyltransferases. Moreover, two most updated reports by Peng et al and Du et al show that lysine malonylation and succinylation as two new post translational modifications and that SIRT5 is a major enzyme to NAD-dependent demalonylate and desuccinylate multiple cellular proteins [Peng][Du]. Nonetheless, physiological relevance of reversible malonylation and succinylation is still need to be identified. While class I sirtuins, including SIRT1, SIRT2 and SIRT3 [Frye, 2000].exhibit robust deacetylase activity in different cellular compartments [North, 2003], class II (SIRT4), class III (SIRT5), and class IV (SIRT6 and SIRT7) have undetectable or weak deacetylase activity and weak ADP-ribosylase activity [Liszt, 2005][Kawahara, 2009][Michishita, 2008][Mao][Nakagawa, 2009][Haigis, 2006]. Taken together, these findings challenge scientists as to whether SIRT4, SIRT6, or SIRT7 might have an undiscovered role in post-translational modifications or that they might have very strict deacetylation substrate specificity.

In addition to the data supporting SIRT4 as an important metabolic regulator through its effects on insulin secretion in β cells, a recent study has revealed roles for SIRT4 in negatively regulating FAO in muscle and liver [Nasrin]. Knockdown of SIRT4 expression both in vitro and in vivo increased the expression of genes involved in enhancing FAO and oxidative phosphorylation, including SIRT1, medium chain acyl-CoA dehydrogenase (MCAD), carnitine palmitoyltransferase 1 (CPT1), PGC-1 α , cytochrome *c*, ATP synthase, and IDH2 [Nasrin]. The effect of SIRT4 knockdown in enhancing FAO is dependent on SIRT1 [Nasrin]. These results once again show that SIRT4 activities might antagonize SIRT1 and SIRT3 functions. Whether sirtuins share

common substrates in pathways of energy metabolism or whether sirtuins have primary or secondary effects on other sirtuins or their targets remains elusive.

Recent study emphasizes more on the role of SIRT4 in glutamine metabolism via regulating of GDH activity that SIRT4 expression is reduced in human cancer, and its overexpression reduces cell proliferation, transformation, and tumor development [Csibi]. In addition, SIRT4 is an important regulator of lipid homeostasis by deacetylation of malonylcoA decarboxylase (MCD) [Laurent]. These results demonstrated again the role of SIRT4 in regulation of glutamine and fatty acid metabolisms and thus its new role in cancer development.

1.1.7 Mitochondrial SIRT5: the new role of protein deacylation

Enzymatic activity of SIRT5 was unclear until recent finding demonstrated that it acts as potent demalonylase and desuccinylase [Peng][Du]. Malonyl-lysine and succinyl-lysine modifications have been identified in a number of organisms, including yeast, worms, flies, mice, and humans [Zhang][Peng]. For example, mice lacking SIRT5 showed global protein hypermalonylation and hypersuccinylation, suggesting that SIRT5 is the major protein demalonylase and desuccinylase in mammals [Peng]. Accordingly, tyrosine 102 (Y102) and arginine 105 (R105), located within the catalytic pocket of SIRT5, are required for the demalonylase and desuccinylase activities [Du]. The positive charge of this arginine can explain the preference of SIRT5 for negatively charged acyl groups, such as malonyl-lysine and succinyl-lysine. Remarkably, these two amino acids are specifically conserved across class III sirtuins from lower organisms to mammals [Du], but are not conserved between different classes, suggesting a structural basis for

distinct enzymatic activities between sirtuin classes. The biological relevance of these two new postmodifications of SIRT5 is currently undiscovered.

Substrate proteins of demalonylation and desuccinylation of SIRT5 have been also identified using a variety of chemical and biochemical methods. Many of the malonylated or succinylated proteins identified so far are important metabolic enzymes, including IDH2, serine hydroxymethyltransferase, glyceraldehyde 3-phosphate dehydrogenase, GDH, malate dehydrogenase 2, citrate synthase, carbamoyl phosphate synthetase 1 (CPS1), HMGCS2, thiosulfate sulfurtransferase, and aspartate aminotransferase [Zhang, 2011][Peng, 2011][Du, 2011]. Among these novel enzymatic activities, the most known function of SIRT5 as a protein deacetylase is on increasing the activity of the urea cycle enzyme CPS1 [Nakagawa]. SIRT5 has also been reported to regulate CPS1 activity through desuccinylation [Du]. It raises interest in further studies to identify more bona fide substrates of SIRT5 and their physiological significances.

1.1.8 SIRT6: an additional discovery of protein deacylation

1.1.8.1 SIRT6 and metabolism

SIRT6 is found in heterochromatin, where the sirtuin plays a role in histone modification of metabolically relevant genes. SIRT6 was shown to negatively regulate glycolytic gene expression through deacetylation of histone H3 lysine 9 (H3K9) at their promoters [Zhong]. SIRT6 knockout mice show severe hypoglycemia around 4 weeks of age [Mostoslavsky, 2006]. In addition, SIRT6 liver-specific knockout mice display increased glycolysis and disruption of glucose homeostasis [Kim]. Interestingly, mice with neural deletion of SIRT6 have normal glycemia but develop adult-onset obesity [Schwer]. In sharp contrast, SIRT6 overexpression protects from diet-induced obesity,

with increased glucose tolerance and reduced fat accumulation [Kanfi]. SIRT6 overexpression also highly decreases the insulin-like growth factor 1 (IGF-1) signaling pathway, resulting in increased life span in male mice [Kanfi]. Other studies supporting a protective role for SIRT6 activation from the onset of age-related metabolic dysfunction (i.e., diabetes and obesity) have been reported, including regulation of the inflammatory pathway [Kawahara, 2009], DNA damage response [Mostoslavsky, 2006][Kaidi], and genomic integrity [Michishita, 2009][Yang, 2009].

In a most recent study reported that human SIRT6 efficiently removes long-chain fatty acyl groups, such as myristoyl, from lysine residues [Jiang]. SIRT6 promotes the secretion of tumor necrosis factor- α (TNF- α) by removing the fatty acyl modification on lysine 19 (K19) and lysine 20 (K20) residues of TNF- α [Jiang]. The discovery of SIRT6 in regulation of protein lysine fatty acylation shed light on new opportunities to investigate the physiological relevance of a little known protein post-translational modification.

1.1.8.2 SIRT6 and cancer

Current evidence indicates that SIRT6 is important in the preservation of genomic integrity from DNA damage and stress stimuli [Michishita, 2008][Yang, 2009][Mao]. For example, SIRT6 knockout mice have an impaired DNA damage response, including genomic instability by irradiation [Mostoslavsky, 2006]. Notably, although SIRT6 transgenic mice do not show a remarkable decrease in age-associated tumor formation [Kanfi], SIRT6 overexpression may still be considered as protective against tumorigenesis. Further studies are required for the application of SIRT6 activation as a therapeutic strategy in cancer.

1.1.9 SIRT7

1.1.9.1 SIRT7 and cardiovascular/metabolism functions

Although SIRT7 is least characterized among the seven mammalian sirtuins, the outlook for future studies of this protein seem promising. SIRT7-deficient mice develop a lethal heart hypertrophy [Vakhrusheva, 2008]. This phenotype can be explained via the role of SIRT7 to deacetylate and activate p53 leading to increased apoptosis in the myocardium [Vakhrusheva, 2008]. Further studies are needed to identify more SIRT7 targets relating to cardiovascular function.

A recent study has reported that SIRT7 negatively regulates hypoxia-induce factor 1 alpha (HIF-1 α) and HIF-2 α protein levels in a non-enzymatic mechanism that is independent of prolyl hydroxylation and proteasomal or lysosomal degradation [Hubbi, 2013].

Interestingly, SIRT7 deacetylates and activates polymerase-associated factor 53 PAF53, a subunit of RNA polymerase I (Pol I) at lysine 373 [Chen, 2013]. SIRT7 is released from nucleoli in response to different stress conditions, leading to hyperacetylation of PAF53 and decreased Pol I transcription [Chen, 2013]. Remarkably, SIRT7-deficient mice develop chronic hepatosteatosis resembling human fatty liver disease [Shin, 2013]. This phenotype of fatty liver can be relieved by Myc inactivation or pharmacological suppression of endoplasmic reticulum (ER) stress [Shin, 2013].

1.1.9.2 SIRT7 and cancer

An additional deacetylation target of SIRT7, H3K18, was recently identified [Barber]. Surprisingly, hypoacetylation at H3K18 has been linked to aggressive cancer phenotypes [Seligson, 2009]. Furthermore, Myb-binding protein 1a Mybbp1a is capable

of inhibiting the deacetylation activity of H3K18Ac by SIRT7 [Karim]. In fact, SIRT7 knockdown reduces tumor growth of human cancer cell xenografts in mice [Barber][Kim]. Notably, SIRT7 inhibits transcriptional activation of p21 (WAF1/Cip1) by repressing miR-125a-5p and miR-125b-tumor suppressors [Kim]. In another recent study demonstrated that SIRT7 knockdown efficiently suppresses both RNA and protein synthesis via regulating Pol III transcription through the mammalian target of rapamycin mTOR and the transcriptional factor IIIC2 (TFIIIC2) complex, thereby inhibits tumor growth [Tsai]. Taken together, SIRT7 downregulation appears to have an anti-tumor effect, partly through inhibition of protein synthesis.

1.2 Mitochondria and ATP production

During aerobic growth in nonphotosynthetic cells, mitochondria convert nutrient influx into ATP for cellular energy supply by mitochondrial OXPHOS, and also generate ROS due to electron leakage from the respiration chain to molecular oxygen [Schonfeld, 2008]. The respiratory complex performs a sequence of redox reactions in which electrons passing through a series of enzymes, located in the inner mitochondrial membrane, release free energy for the translocation of protons across this membrane [Chaban]. This proton flow from the matrix to the intermembrane space creates a difference in proton electrochemical potential, called the proton-motive force or mitochondrial membrane potential ($\Delta\psi_m$) ([Saraste, 1999]. This force is used by different energy transducers for OXPHOS, such as membrane bound ATP-synthase to synthesize ATP from ADP and phosphate [Saraste, 1999][Mitchell, 1961]. OXPHOS supports two important cell functions, the redox state maintenance (mainly nicotinamide adenine dinucleotide (NADH) reoxidation) and ATP synthesis [Saraste, 1999][Mitchell, 1961].

Interestingly, all living organisms need to maintain both a high phosphate potential and an intracellular redox balance for sustained metabolic function [Saraste, 1999]. The cells need to have mechanisms to adapt to physiological fluctuations by varying the ratio of ATP/O (ATP synthesis over oxygen consumption - NADH reoxidation) in response to different demands in energy. There are several mechanisms that affect to ATP/O ratio to allow for modulation between ATP synthesis and NADH reoxydation. First, the passive proton leak decreases the coupling efficiency to reduce the rate of ATP synthesis [Nobes, 1990]. This mechanism is dependent on the proton-motive force and cannot be widely modulated [Nobes, 1990]. Second, uncoupling proteins (UCPs), discussed in more detail below, allow modulation of membrane proton permeability by dissipating the proton gradient before it can be used to provide energy for OXPHOS. Third is H⁺ transport, which is well established and highly regulated by guanosine triphosphate (GTP) as an inhibitor, and fatty acids as a stimulator [Nicholls, 1984]. One example of this is facilitated H⁺ transport. This function has been reported in yeast with a decrease in the proton-motive force by the activity of external NADH or glyceraldehyde 3-phosphate (G3P) dehydrogenases, leading to a decrease in redox pressure with little effect on ATP synthesis [Mourier]. In *Saccharomyces cerevisiae*, for example, complex I is not expressed, and the NADH and other dehydrogenases are not coupled to proton flux. These findings result in an increase in proton permeability associated with a high activity of these dehydrogenases is independent of the rest of the respiratory chain and the ATP synthase proton pump [Mourier]. Lastly, slip or intrinsic uncoupling is a mechanism tht decreases the efficiency of a proton pump because of a partial and variable decoupling of the chemical reaction from the proton transport

[Luvisetto, 1991]. In summary, cells use different mechanisms including passive proton leak, uncoupling protein generated heat, proton transport and proton permeability, and intrinsic uncoupling to modulate the balance between redox state and ATP synthesis.

1.3 Adenine Nucleotide Translocators (ANTs)

Adenine nucleotide translocator (ANT), also known as the ADP/ATP translocator, is the most abundant protein in the inner mitochondrial membrane [Kaukonen, 2000]. There are four ANT isoforms (ANT1, ANT2, ANT3, and ANT4) with their distinct tissue-specific expression patterns. ANT1 is majorly expressed in the heart, skeletal muscle, and brain. ANT2 predominates in the liver and in proliferating cells. ANT3 is ubiquitously expressed, and ANT4 is found in the liver, testis, and undifferentiated embryonic stem cells [Dolce, 2005].

ANT plays an important role in the translocation of ATP and ADP across the mitochondrial matrix [Kaukonen, 2000]. ANT exchanges ATP synthesized in the mitochondria for cytosolic ADP in order to supply continuous ADP for maintaining the OXPHOS process in mitochondria. ATP/ADP exchange by ANT is crucial for the maintenance of ATP synthase activity and normal levels of mitochondrial membrane potential ($\Delta\psi_m$) [Vander Heiden, 1999]. Conversely, in the cellular context of impaired function of ATP/ADP exchange, ANT displays an essential role in inducing cell apoptosis and generating ROS [Bakker, 2000][Won].

1.3.1 ANT and apoptosis

Mitochondrial membrane permeabilization (MMP) is a rate-limiting step of apoptosis and is modulated by the mitochondrial permeability transition pore (mtPTP) [Halestrap, 2009]. mtPTP is a nonspecific pore, permeable to all molecules of less than

1.5 kDa, and is formed by the voltage-dependent anion channel (VDAC), the proapoptotic and antiapoptotic Bax/Bcl2 protein family members, cyclophilin D, and the ANTs [Zoratti, 1995]. mtPTP opening causes swelling of the mitochondrial matrix and rupturing of the outer membrane to allow the release of cytochrome c and other proapoptotic proteins into the cytosol [Halestrap, 2009]. Interestingly, ANT is thought to be part of the mtPTP complex [Halestrap, 1990], however, ANT has been reported to interact with VDAC located in the outer mitochondrial membrane to form a large protein-permeable channel [Leung, 2008]. Moreover, liver mitochondria from ANT knockout mice can still undergo normal permeability transition resulting in release of cytochrome c but need more Ca^{2+} than usual to activate the mtPTP. Interestingly, once the mtPTP was activated, it could no longer be regulated by ANT ligands [Kokoszka, 2004]. In addition, hepatocytes without ANT remained competent to respond to various initiators of cell death [Kokoszka, 2004]. Therefore, ANTs are non-essential structural components of the mtPTP, although they do contribute to regulation of mtPTP. Despite these arguments, ANT is still considered to have an important role in the genesis of mtPTP [Leung].

1.3.2 ANT and mitochondrial uncoupler

The flow of electrons through the electron transport chain (ETC) of cellular respiration is tightly coupled to ATP synthesis under physiological conditions [Boudina, 2006]. In other words, mitochondrial oxygen consumption is tightly coupled to ATP synthesis, such that the two sets of reactions cannot occur without the other. More specifically, the proton flux re-enters the matrix via the F_0F_1 ATPase to regenerate ATP from ADP through coupling OXPHOS. A small proportion of proton can bypass the F_0F_1 ATPase, leading to mitochondrial oxygen consumption that is not coupled to ATP

synthesis (mitochondrial uncoupling) [Boudina, 2006]. UCP1, a protein found in the inner mitochondrial membrane of brown adipose tissue, as described previously, regulates this process [Nicholls, 1984]. Following, four more UCP homologues have been discovered (UCP2, UCP3, UCP4 and UCP5/BCMP1 [brain mitochondrial carrier protein 1]) [Ledesma, 2002]. These proteins mediate proton leak across the mitochondrial membrane and decrease $\Delta\psi_m$ [Teshima, 2003], thereby decreasing ATP production. Since ROS production parallels an increase in $\Delta\psi_m$, UCP-mediated uncoupling has been proposed to play a role in decreasing mitochondrial ROS production [Teshima, 2003][Lee, 2005]. This implicates a possible mechanism in which mitochondria protect themselves from oxidative damage through uncoupling [Brand, 2005], which may delay mitochondrial ROS production, protect against cellular damage, and reduce insulin secretion [Brand, 2005]. Therefore, uncouplers are important potential targets for treatment of aging, degenerative diseases, diabetes, and, perhaps, obesity [Brand, 2005].

Several reports suggested that ANT is also a mitochondrial uncoupler and is responsible for basal uncoupling or proton leak [Boudina, 2006]. In rodents, ANT1 and ANT2 were shown to mediate uncoupling by fatty acids and to lower mitochondrial membrane potential in heart and skeletal muscle [Roussel, 2000]. Additionally, ANT1-deficient mice were demonstrated to have a 50% decrease in proton conductance in skeletal muscle [Brand, 2005]. Furthermore, a 4-hydroxy-2-nonenal-induced proton leak could be inhibited by the ANT inhibitor carboxyatractyloside [Echtay, 2003]. These observations suggest that ANT might decrease mitochondrial ROS production by functioning as an uncoupler.

1.3.3 ANT and metabolic syndrome (impairs ATP/ADP translocase activity)

Obesity is known to be associated with increased cytosolic triglyceride stores in non-adipose tissue such as muscles, liver and pancreatic β cells [Oakes, 1997][Lee, 1997][Bakker, 2000]. Cytosolic triglyceride is a source of long chain acyl-CoA esters (LCACs), the metabolically active form of fatty acids. LCACs might be accumulated under pathological conditions with excess lipid supply, such as obesity, and mitochondrial fatty acid β -oxidation defect [Franch, 2002]. LCACs were shown to inhibit the ATP/ADP translocase activity of the ANT by competitive displacement of the nucleotide from its binding site on the protein. Accordingly, increased concentrations of LCACs in the cell contribute to mitochondrial dysfunction in metabolic defects through inhibition of the ANT [Bakker, 2000][Ciapaite, 2006]. Inhibition of ATP/ADP translocase activity lowers cytosolic ATP and matrix ADP availability, and increases mitochondrial membrane potential ($\Delta\psi_m$) [Bakker, 2000]. These reactions promote the formation of ROS, resulting in impaired cellular functions and cell death [Bakker, 2000][Ciapaite, 2006].

1.3.4 ANT and PGC-1 α (increase ATP/ADP translocate activity preventing endothelial apoptosis)

PGC-1 α is a transcriptional coactivator of nuclear receptors and plays an important role in energy metabolism [Lin, 2005], mitochondrial biogenesis [Lin, 2005] and intracellular ROS [St-Pierre, 2006]. In a recent study, PGC-1 α has been reported to regulate ROS generation and endothelial cell apoptosis by increasing FAO and enhancing ATP/ADP translocase activity by ANT [Won]. It is well known that fatty acids, such as linoleic acid (LA), increase ROS generation and cell apoptosis in endothelial cells [Lee, 2005]. In addition, LA increased the mRNA and protein expression of antioxidant

enzymes, including manganese superoxide dismutase, copper-zinc superoxide dismutase, catalase, glutathione peroxidase, and uncoupling protein2 (UCP2), consistent with the notion that oxidative stress can induce cellular antioxidant responses [Bianchi, 2002]. Interestingly, LA significantly increased ANT1 expression and decreased ATP/ADP translocase activity, with a significant increase in $\Delta\psi_m$ (hyperpolarization) and ROS generation [Won]. Surprisingly, inhibitors of fatty acyl CoA synthase and ceramide synthase decreased LA-induced effects on ATP/ADP translocase activity [Won]. This suggests that lipid metabolites, such as LCAC, diacylglycerol, and ceramide caused an LA-induced impairment of ATP/ADP translocase activity [Won]. These results also imply that increased ROS generation with LA may be responsible for the increase in ANT1 expression. More importantly, changes in ANT1 expression may not account for the decrease in ATP/ADP translocase activity with LA.

1.4 AMPK activation and metabolism

In mammalian cells, AMPK was activated by an increase in cellular 5' adenosine monophosphate-activated protein (AMP), ADP or Ca^{2+} under different metabolic conditions. This mechanism of AMPK activation may explain reduced production of ATP during stress, such as starvation for glucose [Salt, 1998] or oxygen supply [Marsin, 2000], a chemically-induced cellular stress response [Corton, 1994], or by stresses that increase ATP consumption, such as muscle contraction [Winder, 1996]. AMPK is also turned on by several drugs and xenobiotics, including antidiabetic drugs (such as metformin, phenformin and thiazolidinediones [Fryer, 2002][Zhou, 2001]), resveratrol from grapes and red wine [Baur, 2006], epigallocatechin gallate from green tea, capsaicin

from peppers [Hwang, 2005], curcumin from turmeric [Lim, 2009], and even garlic [Lee]) and plant products (berberine [Lee, 2006] and hispidulin [Lin]).

Increasing evidence has shown that some cellular stresses activate AMPK by non-classical mechanisms that may not involve increases in AMP, ADP or Ca²⁺ levels. AMPK is activated by ROS, such as hydrogen peroxide (H₂O₂), in a cell culture model [Alexander] or by DNA damaging agents such as etoposide [Fu, 2008], doxorubicin [Ji] and ionizing radiation [Sanli]. Interestingly, at high ROS concentrations, AMPK activation might be secondary to the inhibition of mitochondrial ATP synthesis, with consequent rises in AMP and ADP levels [Alexander]. This non-canonical AMPK activation of pathways may be more direct through oxidation or glutathionylation of two conserved Cys residues in the AMPK α -subunit [Zmijewski] or cytoplasmic form of the phosphoinositide 3-kinase-like kinase (PIKK) ataxia telangiectasia mutated (ATM) [Alexander]. Nuclear ATM has a role in the DNA damage-sensing pathway and in the response to oxidative stress [Ditch, 2009]. In another word, some interesting pathways including ROS and ATM dependent that can activate AMPK, leading to secondary alterations in AMP and ADP concentrations to redirect cellular metabolism from anabolic and energy-consuming processes.

Cells turn on metabolic switch, specifically fatty acid metabolism to adapt changes in AMP and ADP levels upon AMPK activation. The activation of the AMP-activated protein kinase leads to phosphorylation and inactivation of the enzyme acetyl-CoA carboxylase (ACC) under the control of malonyl-CoA levels, and thus FAO, during exercise [Winder, 1996]. Cytosolic malonyl-CoA is synthesized by carboxylation of acetyl-CoA by ACC. In contrast, the decarboxylation of malonyl-CoA by MCD

regenerates acetyl-CoA [Saggerson, 2008]. On the other hand, the mitochondrial malonyl-CoA, is generated by activity of propionyl-CoA carboxylase (PCC) on acetyl-CoA, with the reverse reaction again catalyzed by MCD [Saggerson, 2008]. The activities of both ACC and MCD are regulated by many physiological factors including glucose, insulin, or AMPK, through allosteric or phosphorylation mechanisms [Saggerson, 2008]. Malonyl-CoA is not only the precursor for de novo FA synthesis but is also an inhibitor of FAO. Specifically, Malonyl-CoA binds to and inhibits CPT1 on the mitochondrial outer membrane, leading to the inhibition of the transport of FAs into mitochondria for β -oxidation [Saggerson, 2008]. In mammals, there are two ACC isoforms, ACC1 and ACC2, with distinct tissue expression and function. ACC1 is dominant in lipogenic tissues where it produces malonyl-CoA in the cytoplasm for lipogenesis. Meanwhile, ACC2 is highly expressed in oxidative tissues where it negatively regulates FA oxidation [Tong, 2005]. ACC2 is proposed to inhibit CPT1 by creating a local high concentration of malonyl-CoA at the mitochondrial outer membrane [Abu-Elheiga, 2000]. Activation of AMPK leads to decreased Malonyl-coA by down-regulating ACC activity, resulting in inhibiting fatty acid synthesis, and increasing fatty acid oxidation.

2 Chapter 2

SIRT4 REGULATES ATP HOMEOSTASIS AND MEDIATES A RETROGRADE SIGNALING VIA AMPK

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2.1 Abstract

Efficient coupling of cellular energy production to metabolic demand is crucial to maintain organismal homeostasis. Here, we report that the mitochondrial sirtuin, Sirt4, regulates mitochondrial ATP homeostasis. We found that Sirt4 affects mitochondrial uncoupling via the adenine nucleotide translocator 2 (ANT2). Loss of SIRT4 expression led to decreased cellular ATP levels in vitro and in vivo, while SIRT4 overexpression was associated with increased ATP levels. Further, we provide evidence that lack of SIRT4 activated a retrograde signaling response from the mitochondria to the nucleus that includes AMPK, PGC1- α , key regulators of β -oxidation, such as Acetyl-CoA carboxylase, and components of the mitochondrial respiratory machinery. This study highlights the ability of Sirt4 to regulated ATP levels via ANT2 and a feedback loop involving AMPK.

Keywords: Sirt4, ANT2, AMPK, ATP, mitochondrial signaling

2.2 Introduction

Maintenance of energy balance is vital for cellular and organismal physiology. In addition to affecting cellular functions, deregulated energy homeostasis is associated with metabolic diseases, such as obesity and diabetes. Rates of utilization and metabolic fates of carbohydrates and lipids are coupled to the energy demands of a cell (and an organism). Organismal energy balance is regulated by multiple mechanisms and involves mitochondria, which contain the main intracellular machinery for ATP production. As such, mitochondria are key components that couple metabolic inputs to energy homeostasis. Integration of factors intrinsic and extrinsic to mitochondria affects mitochondrial functions to maintain the balance between metabolic inputs and energy homeostasis [Nunnari].

Sirtuins are NAD⁺-dependent enzymes that mediate cellular physiology in response to metabolic inputs and energy demands [Schwer, 2008][Haigis]. While all sirtuins rely on NAD⁺ as a cofactor [Imai, 2000][Ghosh], only SIRT1, 2, and 3 exhibit robust deacetylase activity, and SIRT5 and 6 exhibit novel deacylase catalytic activities [Jiang][Kawahara, 2009][Du][Mostoslavsky, 2006][Laurent]. Three mitochondrial sirtuins, SIRT3, SIRT4 and SIRT5 in mammals, affect cellular and organismal physiology by regulating mitochondrial functions [Huang][Verdin]. SIRT4, the evolutionarily conserved mitochondrial sirtuin in metazoans, was initially identified as an ADP-ribosylase [Haigis, 2006] and shown to regulate insulin secretion [Haigis, 2006][Ahuja, 2007]. In addition, the ability of SIRT4 to regulate glutamine metabolism via GDH has been shown to be important for cancer development [Jeong][Csibi]. SIRT4 is a critical regulator of fat metabolism [Nasrin][Laurent]. SIRT4 deficiency leads to

increased β -oxidation, which involves an increased transcription of FAO genes in the nucleus [Nasrin]. However, the mechanism by which SIRT4 in mitochondria regulates nuclear transcriptional response is unknown. Recently, SIRT4 was shown to possess deacetylase activity [Laurent], and its ability to deacetylate malonyl-CoA-decarboxylase (MCD) has been implicated in regulating fat metabolism. In agreement with this finding, SIRT4 knockout mice burn more fat and hence are protected from high fat diet-induced obesity [Laurent].

The metabolic switch between carbohydrate and fat is coupled with ATP homeostasis [Hock, 2009][Locasale]. Specifically, at the mitochondrial level, the transition between fed and starved conditions is regulated by mechanisms that alter carbon chain utilization to match cellular energy demand [Hock, 2009][Locasale]. Despite this knowledge, it is still unclear if SIRT4 has a role in controlling cellular ATP levels as an underlying cause of the described phenotypes of SIRT4 deficiency in cells and animals. Thus, understanding the function of SIRT4 in coupling metabolic flux and energy homeostasis is of major importance, and could potentially be exploited therapeutically.

AMPK is a central sensor of cellular energy status [Hardie, 1997][Steinberg, 2009]. During energy-deprived conditions, such as starvation, fat oxidation is increased and mitochondrial functions are elevated [Hardie, 1997][Nunnari]. AMPK phosphorylates and inhibits ACC, leading to a decrease in malonyl-CoA levels, which ultimately results in increased CPT1-dependent transport and oxidation of fatty acids in mitochondria [Munday, 1988][Winder, 1997][Saggerson, 2008]. AMPK also regulates the expression of nuclear-encoded mitochondrial genes via the peroxisome proliferator-

activated receptor gamma coactivator-1 α (PGC-1 α) [Steinberg, 2009][Hock, 2009][Hardie]. AMPK-dependent phosphorylation of PGC-1 α results in increased transcription of genes involved in FAO and mitochondrial biogenesis [Steinberg, 2009][Hock, 2009][Hardie][Canto, 2009]. Although AMPK senses AMP/ATP ratios, its role in mediating retrograde signaling from mitochondria to nucleus (and particularly, in a sirtuin-dependent manner) is poorly appreciated.

In this study, we have investigated the role of SIRT4 in energy homeostasis. Moreover, we establish a functional relationship between SIRT4 and the ANT2, which is also known to act as an uncoupler [Klingenberg, 2008][Halestrap, 2003][Fiore, 1998]. Our results describe a SIRT4-dependent molecular mechanism that mediates a retrograde signaling to the nucleus.

2.3 Materials and methods

2.3.1 Antibodies and Reagents

Antibodies used were specific for monoclonal and polyclonal AMPK α , Phospho-AMPK α (Thr172), Acetyl-CoA Carboxylase (ACC), Phospho-ACC (Ser79) (Cell Signaling Technology), PGC-1 α (Millipore and Sant Cruz), and SIRT4 (generous gift from Marcia Haigis (as described [Haigis, 2006])), FLAG-M2, MYC, Tubulin and β -Actin (Sigma Aldrich). Medium 199 (for culturing primary hepatocytes), Oligomycin, Trifluorocarbonylcyanide phenylhydrazine (FCCP), Rotenone and XF Assay medium (Seahorse Bioscience or Sigma Aldrich), AICAR, pyruvate and 2-deoxy glucose (Sigma Aldrich). Fetal bovine serum (FBS) and Horse serum (Invitrogen), and DMEM (Sigma Aldrich).

2.3.2 Animal Studies

All animal studies were performed using IACUC-approved protocols. Studies used male WT littermate and SIRT4KO 129Sv mice [Haigis, 2006], at 10-14 weeks of age, maintained on a standard chow diet (5053 PicoLab diet), unless otherwise indicated. Mice were sacrificed at 7 hours for fed mouse studies, or transferred to a new cage without food for 18 hours (overnight), and then sacrificed for fasted mouse studies.

2.3.3 Cell Culture and treatments

HEK293T, HepG2, C2C12 and HHL-17 [Clayton, 2005] cells were grown in DMEM supplemented with 10% FBS containing 5 mM (low) or 25 mM (high) glucose. Confluent C2C12 cells differentiated into myotubes using 1% horse serum. Cells were grown in high glucose medium unless otherwise mentioned. Transfected cells were treated with 20 mM 2-deoxy-glucose (2-DG) for 2 hours to transiently inhibit glycolysis and 500 μ M AICAR for 16 hours for AMPK activation. Cells treated with PBS or DMSO were used as controls.

2.3.4 Transfection

For SIRT4 and ANT2 overexpression, cells were either stably transfected and selected on puromycin or transiently transfected. Empty pBABE-puro or pcDNA3.1 were used for control transfection. 20 nM (unless mentioned otherwise) of scrambled and SIRT4 siRNAs were transfected in HEK293T and 50 nM in C2C12. Lipofectamine 2000 (Invitrogen) was used for transfections per manufacturer instructions.

2.3.5 ATP and ADP/ATP ratio measurement

ATP was measured spectrophotometrically as described previously [Saha] using the method of Oliver H and Lowry JVP (A flexible system of enzymatic analysis, Academic Press, 1972, p 151-153). Total ATP and ADP/ATP from cells were determined using ATP bioluminescence assay mix (Sigma Aldrich) and ADP/ATP kits (Mitosciences), respectively.

2.3.6 Immunoblot analysis

Organs were harvested and homogenized using a tissue homogenizer. Cell and tissue lysates were prepared at 4°C in ice-cold RIPA buffer (50 mM Tris [pH 7.4], 0.1% SDS, 0.5% sodium deoxycholate, 150 mM NaCl, 1% NP40) containing protease inhibitors (EDTA-free Complete; Roche Molecular Biochemicals)), halt or PHOSTOP phosphatase inhibitor cocktail (Thermo scientific and Roche). The lysates were clarified by centrifugation for 15 minutes at 4°C at 14,000 rpm and protein concentrations were estimated by BCA assay. The lysates were resolved on SDS-PAGE gel, electrophoretically transferred, immunoblotted and signal was detected using ECL (Amersham Biosciences, Thermo Scientific or Roche).

2.3.7 Plasmids, siRNA and Primers

Human SIRT4 and ANT2 cDNAs were cloned into pBABE-puro and pCDNA3.1 vectors, respectively. Scrambled and SIRT4 siRNAs were obtained from Imagenes and Sigma Aldrich. ANT1 and ANT2 siRNAs were obtained from Sigma Aldrich. List of primers used for cloning is provided in Supplementary Table 1.

2.3.8 RNA isolation and quantitation

Total RNA was isolated using Trizol reagent (Invitrogen) and reverse transcribed using random hexamers and SuperScript-III kit (Invitrogen). Real-time PCR was carried out using primer pair listed in Supplementary Table 1 using Eppendorf or Roche real-time machines, and SYBR kit (Kapa Biosystems).

2.3.9 Oxygen consumption

For primary hepatocytes from SIRT4KO and WT littermates: Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured in XF assay media (nonbuffered DMEM containing 25 mM glucose, 2 mM L glutamine, and 2 mM sodium pyruvate) under basal conditions and in response to 1 μ M oligomycin, 0.5 μ M fluoro-carbonyl cyanide phenylhydrazone (FCCP), and 2 μ M rotenone with the Seahorse XF-96 Extracellular Flux Analyzer (Seahorse Bioscience). The XF-96 measures the concentration of oxygen and free protons in the medium above a monolayer of cells in real-time. Protein concentration was determined for each well using a standard BCA protein assay. OCR and ECAR values are normalized to mg/protein and plotted as the mean \pm standard deviation.

For cultured cells: Cells were trypsinized and oxygen consumption flux was determined using OROBOROS high resolution respirometer in DMEM (with 25 mM glucose and 2 mM L glutamine with or without 2 mM pyruvate) under basal and in response to 2.5 mM rotenone, 10 mM succinate, 1 mM ADP, 1 mM orthophosphate (Pi), 0.5 μ M FCCP and 1 mg/mL oligomycin. Two million cells in a total volume of 2.1 ml were used for the assays.

2.3.10 ATP synthase activity assay

Complex-V enzyme activity was measured with ATP synthase enzyme activity microplate assay kit (Mitosciences) according to manufacturer instruction. Briefly, the ATP synthase enzyme, from equal amounts of mitochondria, isolated from cells (as described above), was immunocaptured within the wells of the microplate and the enzyme activity was measured.

2.3.11 Estimation of mtDNA

Total genomic DNA was isolated using the genomic DNA isolation kit (Bangalore Genei/Merck). The relative mitochondrial DNA (mtDNA) content was quantified by qRT-PCR, using primers for cytochrome b (mitochondrial genomic DNA) and nuclear β -Actin (for nuclear genomic DNA).

2.3.12 Data processing and Statistical Analyses

Student's T-test and analysis of variance (ANOVA) were used for statistical analysis (p value: * < 0.05, ** < 0.01, *** < 0.001, or as indicated). Microsoft Excel was used for data processing, and statistical significance was calculated using Excel or Sigmaplot. Results are given as the mean \pm standard error. All experiments were performed at least three times.

2.4 Results

2.4.1 SIRT4 regulates cellular ATP levels

To test whether SIRT4 regulates cellular bioenergetics, we assayed for total ATP in cells after upregulating or downregulating SIRT4 expression in several cell types (Figure 2.1 A-F and Figure 2.9 [Supplementary Figure S1A]). Overexpression of SIRT4

led to an increase in cellular ATP in both HEK293T and HHL-17 cells (Figure 2.1 B and C). Conversely, knocking down endogenous SIRT4 led to a marked decrease in total ATP in HepG2, HEK293T and C2C12 myotubes (Figure 2.1 D-F and Figure 2.9 [Supplementary Figure 1A]).

Next, we tested whether SIRT4 is involved in energy homeostasis *in vivo*. Measurement of total ATP levels in muscles and liver from wild-type and SIRT4 knockout (SIRT4KO) mice showed a significant decrease in ATP content in SIRT4KO tissues (Figure 2.1G and H; Figure 2.10 [Supplementary Figure S1B]). Interestingly, lack of SIRT4 led to reductions in ATP under both fed and starved conditions (Figure 2.1G and H).

The total cellular ATP pool is affected by ATP production from glycolysis and mitochondrial OXPHOS [Locasale]. To determine if the SIRT4-dependent increase in ATP was due to mitochondrial ATP synthesis, ATP levels were measured in cells treated for 2 hours with 2-deoxy-glucose (2-DG), an inhibitor of glycolysis. Interestingly, the difference in total ATP levels was maintained in 2-DG treated cells (Figure 2.9 [Supplementary Figure S1C]). To determine whether this change was secondary to an elevated total nucleotide pool or a change in ATP homeostasis, we measured ADP/ATP ratios. We found that cells overexpressing SIRT4 have a lower ADP/ATP ratio than controls (Figure 2.9 [Supplementary Figure S1D]). Conversely, ADP/ATP ratios increased in SIRT4KO tissues (Figure 2.9 [Supplementary Figures S1E and S1F]). Therefore, these data provide evidence that SIRT4 regulates ATP synthesis within mitochondria.

2.4.2 Sirt4 regulates mitochondrial respiration via ANT2-mediated coupling efficiency

Net changes of intracellular ATP levels are a function of its production and consumption. Mitochondrial respiration and the coupled flux through the ETC drive ATP synthesis. To determine if components of cellular ATP production are regulated by SIRT4, we examined mitochondrial respiration in loss- and gain-of-function experiments. Since endogenous SIRT4 expression was drastically reduced during fasting (Figure 2.2A), we wanted to assess the effect of SIRT4 perturbations on respiration under normal and conditions that mimic starvation by adding 2 mM pyruvate to the culture medium [Rodgers, 2005]. Transient knockdown of SIRT4 in HEK293T cells led to increased oxygen consumption under basal and FCCP treated conditions (Figure 2.2B). In contrast, SIRT4 overexpression (SIRT4OE) led to decreased oxygen consumption (Figure 2.2C). Interestingly, knocking down SIRT4 in cells under starvation-like conditions abolished the difference in basal oxygen flux (Figure 2.2D). In agreement with these findings, basal oxygen consumption was unaffected in primary hepatocytes (media supplemented with pyruvate) isolated from SIRT4KO mice. However, oxygen consumption during FCCP-mediated uncoupling was much greater in SIRT4KO mice than in control animals (Figure 2.2E). The rate of extracellular acidification, which indicates the amount of glycolysis within a cell, remained unchanged (Figure 2.2F).

To test whether SIRT4 directly affects ATP synthase activity, we measured the activity of immunocaptured ATP-synthase and detected a comparable enzymatic activity in control and SIRT4OE cells (Figure 2.9G [Supplementary Figure 1G]). Failure to maintain ATP levels despite increased oxygen consumption in SIRT4-deficient cells and

the lack of change in ATP-synthase activity indicate that SIRT4 affects the coupling of ETC flux to ATP production and OXPHOS efficiency.

UCPs and ANTs regulate the coupling efficiency of the electrochemical gradient across the inner mitochondrial membrane. Interestingly, ANTs have a dual role in regulating energy homeostasis: they transport ADP-ATP across the inner mitochondrial membrane, and they act as uncoupling proteins. Acylated ANTs uncouple mitochondria and reduce the efficiency of OXPHOS [Klingenberg, 2008][Forman, 1983][Brustovetsky, 1994]. Moreover, uncoupling in the liver predominantly depends on ANT2 [Shabalina, 2006]. Our results show that SIRT4-dependent changes in ATP and mitochondrial respiration correlate negatively.

Since we previously reported that SIRT4 interacts with endogenous ANT2 [Ahuja, 2007], we tested whether SIRT4 affects the coupling efficiency of OXPHOS in an ANT2-dependent manner and determined whether SIRT4-dependent changes in oxygen consumption are due to coupling efficiency. The reduced oxygen flux in SIRT4OE cells was lost when cells were treated with Rotenone (inhibitor of complex-I) (Figure 2.3A). Further, the difference between control and SIRT4OE cells was retained when permeabilized cells were provided with succinate and ADP/Pi (Figure 2.3B). This difference in oxygen consumption (when succinate was provided as a substrate) also ruled out possible effects from alterations in SIRT4-mediated and GDH-dependent changes in TCA flux via α -ketoglutarate (α -KG). Importantly, the decrease and increase in oxygen consumption observed in SIRT4KD and in SIRT4OE cells was insensitive to Oligomycin treatment (ATP synthase inhibitor) (Figure 2.3C, D; Figure 2.11

[Supplementary Figure 3]). Based on these results, we conclude that SIRT4 regulates coupling efficiency.

Next, we tested whether the increased uncoupling in SIRT4KD cells was mediated by ANT2. As shown earlier, knocking down SIRT4 led to increased oxygen consumption (Figure 2.3C, D). Interestingly, downregulation of ANT2 in these cells suppressed the basal rate of respiration (Figure 2.3C, D). Importantly, the oxygen flux between SIRT4KD and SIRT4KD/ANT2KD was significantly different post-Oligomycin treatment (Figure 2.3). Knocking down ANT2 along with SIRT4 restored oxygen consumption to control levels (Figure 2.3D). These results indicate that SIRT4 regulates coupling efficiency in an ANT2-dependent manner.

2.4.3 SIRT4 regulates ATP in an ANT2-dependent manner

The results presented above prompted us to investigate whether the SIRT4-ANT2 interaction was required for maintaining cellular ATP levels. As shown earlier, we detected elevated ATP levels in SIRT4OE cells. Importantly, this increase was reduced to control levels when ANT2 was knocked down simultaneously (Figure 2.4A and Figure 2.10 [Supplementary Figure S2A]).

ANT protein family members are expressed in a tissue-specific manner and may have different functions [Klingenberg, 2008][Doerner, 1997]. While mice have two proteins (ANT1 and ANT2), humans have four (ANT1–4) [Doerner, 1997]. Of these four, the functions of ANT1 and ANT2 are well characterized. To check if the ability of SIRT4 to affect ATP homeostasis was restricted to its interaction with ANT2, we knocked down ANT1 in SIRT4OE HEK293T cells (Figure 2.10 [Supplementary Figure S2B]). In contrast to what we had seen with ANT2 knockdown, downregulation of ANT1 did not

reduce the SIRT4-mediated increase in total ATP (Figure 2.4B). Importantly, only ANT2 is expressed in liver (Figure 2.10 [Supplementary Figure S2D]) [Shabalina, 2006][Doerner, 1997], and these results demonstrate the specificity of the SIRT4-ANT2 interaction in energy metabolism.

2.4.4 Reduction of ATP levels by Sirt4 deficiency initiates a homeostatic feedback loop via ANT2/AMPK

Sensing and signaling of cellular energetic status depends on the activity of AMPK, which is allosterically activated by AMP/ADP [Steinberg, 2009][Hardie][Canto, 2009]. Since SIRT4 activity regulates ATP levels, we addressed whether AMPK activity was affected. Importantly, overexpression of SIRT4 led to a marked decrease in pAMPK levels (Figure 2.5A). Conversely, knocking down SIRT4 resulted in an increase in AMPK activity (Figure 2.5B). In agreement with these observations, measurement of pAMPK *in vivo* showed a strong increase in its levels in livers of mice lacking SIRT4 in comparison to littermate controls (Figure 2.5C).

Notably, the decrease of pAMPK in SIRT4OE cells was rescued when ANT2 was simultaneously knocked down, demonstrating that the cross-talk between SIRT4 and AMPK requires ANT2 (Figure 2.5D). These results establish a novel functional interaction between SIRT4 and AMPK and its dependency on ANT2.

Increased FAO during fasting depends on AMPK activity. AMPK-mediated phosphorylation of ACC leads to decreased malony-CoA levels, derepression of CPT1 [Winder, 1997][Saggerson, 2008][Munday, 1988] and increased mitochondrial FAs uptake [Alam, 1998]. Since SIRT4 has been implicated in the regulation of FA metabolism [Nasrin][Laurent], we tested the effect of SIRT4 on ACC phosphorylation. In

agreement with the observed increase in AMPK activity, we found that mice lacking SIRT4 showed increased phosphorylation of ACC (Figure 2.5E).

In addition to modifying metabolic enzymes, AMPK also regulates the transcriptional coactivator PGC-1 α . We found that PGC-1 α protein levels were inversely correlated with SIRT4 in cells (Figure 2.5F). Induction of PGC-1 α was also observed in liver from SIRT4KO mice (Figure 2.5G). Treating SIRT4OE cells with AICAR (an AMPK activator) rescued the decrease of both pAMPK and PGC-1 α (Figure 2.12 [Supplementary Figure S4]). These results support the model that SIRT4 and AMPK interact via ANT2 to mediate a retrograde signaling to ACC and PGC-1 α .

2.4.5 Sirt4-AMPK mediated retrograde signaling regulates expression of fatty acid oxidation genes

PGC-1 α plays an important role in the fasting response by activating transcription of FAO genes [Vega, 2000][Leone, 2005]. Having established a retrograde signaling from SIRT4 to PGC-1 α via ANT2 and AMPK, we measured the transcription of FAO genes. As predicted, PGC-1 α and its downstream FAO genes (such as *ERR α* , *CPT1* and *MCAD*) were significantly downregulated in SIRT4OE cells than in controls (Figure 2.6A-D). Importantly, knocking down ANT2 in these cells rescued the downregulation and restored their expression.

2.4.6 Sirt4 regulates mitochondrial biogenesis in a feedback loop via ANT2/AMPK/PGC-1 α signaling

The cellular response to restore energy homeostasis includes mitochondrial biogenesis, which is often seen during exercise, fasting or mild uncoupling [Hock, 2009]. Increased expression of nuclear-encoded mitochondrial genes by AMPK-PGC-1 α

regulates mitochondrial mass under these conditions [Hardie][Hock, 2009]. Transcription of genes, such as *PGC-1 α* , *ERR α* , *TFAM*, *NRF1* and *Cyt.C*, is a hallmark of mitochondrial biogenesis [Hock, 2009].

Measurement of expression of these genes showed lower mRNA levels in *Sirt4*OE cells than in controls (Figure 2.7A-C). Knocking down ANT2 in SIRT4OE cells rescued the decreased mRNA levels of *PGC-1 α* , *ERR α* , *TFAM*, *NRF1*, and *Cyt.C* (Figure 2.7A-C and Figure 2.6B). This finding further strengthens an important role of the SIRT4-ANT2 interaction in mediating a compensatory change in the expression of nuclear-encoded mitochondrial genes.

In agreement with these changes, we also found a significant decrease in the protein levels of transcription factor A (TFAM) and cytochrome C in both HEK293T (Figure 2.7D) and HEPG2 (Figure 2.13 [Supplementary Figure 5]) upon SIRT4 overexpression. We also observed a small but notable effect on Porin levels (Figure 2.7D). Importantly, mtDNA, a marker of mitochondrial biogenesis, was decreased in SIRT4OE cells and this was suppressed in cells lacking ANT2 (Figure 2.7E). Our data indicate that mitochondrial SIRT4 controls key regulators of mitochondrial mass, as well as the transcription of OXPHOS components via a feedback regulatory loop involving ANT2-AMPK-PGC-1 α .

2.5 Discussion and conclusion

The results presented above support an important role for SIRT4 in energy homeostasis. Specifically, the ability of SIRT4 to alter cellular ATP requires the ANT2, a transmembrane protein located on the inner mitochondrial membrane. Furthermore, we show that ANT2-mediated OXPHOS efficiency is regulated by SIRT4, and loss of SIRT4

leads to increased oxygen consumption by uncoupling. SIRT4 deficiency that mimics energy-deprived condition initiates a homeostatic response involving AMPK and PGC-1 α . We propose that SIRT4 controls the efficiency of ATP production. Further, integration of mitochondrial signaling with nuclear transcription couples fluctuations in cellular energy needs with metabolic inputs. In this regard, we have uncovered a previously unknown function of SIRT4 and show that the sirtuin establishes a retrograde signaling to the nucleus to maintain energy homeostasis.

By overexpressing and knocking down SIRT4 as well as using SIRT4 knockout animals to study this gene *in vivo*, we define its role in energy homeostasis. Importantly, lack of SIRT4 led to a decrease in ATP levels, both in cells and in tissues from SIRT4KO mice. SIRT3 has been reported to increase ATP production by deacetylating ETC components and increasing cellular respiration [Shi, 2005][Huang][He][Ahn, 2008]. Our results clearly indicate that, in contrast to SIRT3, the SIRT4-dependent increase in cellular ATP is not due to increased respiration. To dissect out the mechanism of SIRT4-dependent ATP changes, we investigated the role of ANT2, as ANT2 was previously reported to interact with SIRT4 [Ahuja, 2007]. ANTs are ADP/ATP translocators with various expression and functions in different tissues [Klingenberg, 2008][Doerner, 1997]. Although, ANT1 and ANT2 are evolutionarily conserved, ANT2 is more widely expressed [Shabalina, 2006][Doerner, 1997]. By knocking down ANT2 in cells that overexpressed SIRT4, we established that SIRT4 requires ANT2 to affect cellular ATP. We also found that, while the SIRT4-ANT2 interaction was required for ATP homeostasis, ANT1 did not seem to play a role in SIRT4-dependent changes.

While SIRT4 positively influenced ATP levels, the sirtuin was negatively correlated with respiration. Dissipation of the electrochemical gradient by proton leak (or uncoupling) leads to increased respiration and also results in decreased ATP production. Interestingly, ANT2s have dual functions in mitochondria with regards to energy homeostasis [Ko, 2003][Klingenberg, 2008][Brustovetsky, 1994]. While they play a critical role as antiporters for ADP and ATP, they uncouple the mitochondrial membrane upon acylation [Klingenberg, 2008][Forman, 1983][Brustovetsky, 1994]. Thus, we hypothesized that SIRT4 deficiency leads to an ANT2-dependent increase in respiration due to uncoupling. By providing excess substrates (succinate and ADP/Pi), we have shown that the SIRT4 dependent changes in oxygen consumption are not due to alterations in TCA flux. Importantly, we found that the increased respiration in SIRT4 knockdown cells was lost when ANT2 expression was downregulated. These results suggest that ANT2 could be a potential substrate for SIRT4.

SIRT4 exhibits both ADP-ribosylase and deacetylase activities [Laurent][Kawahara, 2009]. Although ANT2 might be ADP-ribosylated [Mowbray, 1996], its activity as an uncoupler has been largely attributed to acylation [Klingenberg, 2008][Forman, 1983][Brustovetsky, 1994]. It will be interesting to investigate whether SIRT4 possesses a deacetylase activity similar to what has been reported for SIRT5 [He]. However, by identifying the nexus of ANT2 and SIRT4, we added a new component to the understanding of the complex biology of SIRT4.

We have shown that SIRT4 modulates AMPK activity and is part of a retrograde signaling pathway from the mitochondria to the nucleus. There was a significant increase in pAMPK levels in the absence of SIRT4 (SIRT4KD cells and SIRT4KO tissues), which

was consistent with energy deficit. Importantly, the decrease in AMPK activity in SIRT4 overexpressing cells was rescued when ANT2 was knocked down, demonstrating the crucial role of ANT2 in mediating a SIRT4-dependent signal to AMPK. As, energy homeostasis is intricately linked to alterations in metabolic flux and mitochondrial functions, our results support the hypothesis that the inputs from mitochondria via AMPK activity restore energy balance.

The increased AMPK-dependent phosphorylation of ACC that we observed in SIRT4KO mice highlights the physiological significance of SIRT4-AMPK signaling in FAO. Specifically, AMPK-dependent pACC-mediated changes in cytosolic malonyl-CoA are critical for regulating CPT1 (on the outer mitochondrial membrane) mediated FA transport [Saggerson, 2008][Alam, 1998]. Interestingly, the lean phenotype displayed by SIRT4 deficient mice was shown to be due to increased activity of MCD in the mitochondria [Laurent]. Our observations demonstrate that the other side of the reaction, mediated via acetyl-CoA carboxylase, is also regulated by SIRT4 via its activation of AMPK.

Finally, we tested the effect of SIRT4-AMPK-mediated retrograde signaling on PGC-1 α dependent transcription of FAO genes [Vega, 2000][Leone, 2005]. Notably, in the absence of SIRT4, AMPK-dependent signaling increased the expression of PGC-1 α and its downstream FAO genes. In agreement with the effects on AMPK activity, reduced expression of *PGC-1 α* , *MCAD*, *CPT1* and *ERR α* in SIRT4-overexpressing cells were rescued by ANT2 knockdown. These results demonstrate that SIRT4 in the mitochondria not only regulates mitochondrial uptake of FAs via AMPK-ACC, but it also controls FAO through AMPK-PGC-1 α signaling to the nucleus.

Our findings show that SIRT4-AMPK-PGC-1 α signaling affects mitochondrial biogenesis (transcription of OXPHOS components) in an ANT2-dependent manner. We provide conclusive evidence to suggest that SIRT4 in the mitochondria creates a feedback loop to regulate mitochondrial homeostasis. In response to calorie restriction, starvation and mild uncoupling, AMPK-PGC-1 α signaling regulates mitochondrial biogenesis [Hock, 2009]. Although, transcriptional regulation of nuclear encoded genes has been extensively studied [Hock, 2009], the ability of mitochondria to provide an instructive cue to control mitochondrial mass and function is poorly understood [Nunnari][Hock, 2009].

We found that mitochondrial SIRT4 mediates retrograde signaling. Crosstalk between the NAD⁺-dependent mitochondrial factor, SIRT4, and an AMP/ADP sensor in the cytoplasm/nucleus (AMPK) orchestrates cellular physiology (Figure 2.8). Retrograde signaling from mitochondria has been well studied in yeast and in plants [Liu, 2006][Butow, 2004][Biswas, 2005]. Although, ATP and calcium dependent signaling are thought to be important in mitochondrial signaling, it is poorly addressed in mammals [Butow, 2004][Biswas, 2005][Liu, 2006]. Therefore, it is interesting to note that the NAD⁺-dependent SIRT4 in the mitochondria provides instructive cues to alter cellular physiology, in addition to regulating a feedback for mitochondrial functions.

To conclude, an inability to utilize FAs has been implicated in the onset and progression of metabolic diseases, such as obesity and diabetes. Efforts to increase FAO, for example by activating AMPK, have been only partially successful because of a lack of concomitant alteration in the energy status of a cell [Steinberg, 2009][Hoehn]. In fact, a simultaneous increase in FAO and energy dissipation (either through exercise or

mitochondrial uncoupling) has been speculated to be critical in providing a therapeutic intervention [Thrush][Klaus][Hoehn]. Thus, our report highlights that the central role of SIRT4 in regulating OXPHOS efficiency, ATP homeostasis and FAO may also have important clinical relevance.

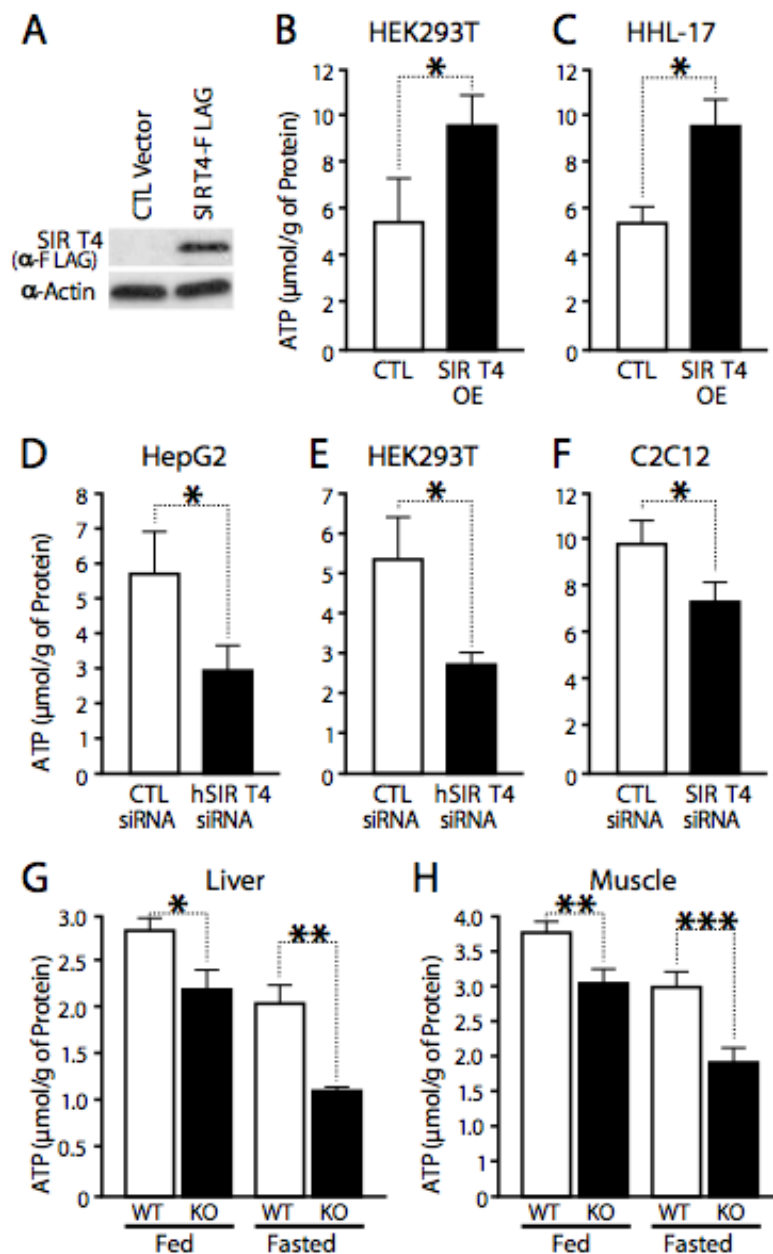


Figure 2.1. SIRT4 regulates cellular ATP levels.

(A) Western blot showing overexpression of SIRT4. (B–F) ATP levels in SIRT4 overexpressing or knockdown cells (HEK293T, HHL-human hepatocytes, HEPG2 and C2C12 myotubes), as indicated. (G, H) ATP levels in wild-type and SIRT4KO (G) liver and (H) muscles under fed and fasted conditions. Statistical significance was calculated using student's t-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, or as indicated. CTL, control.

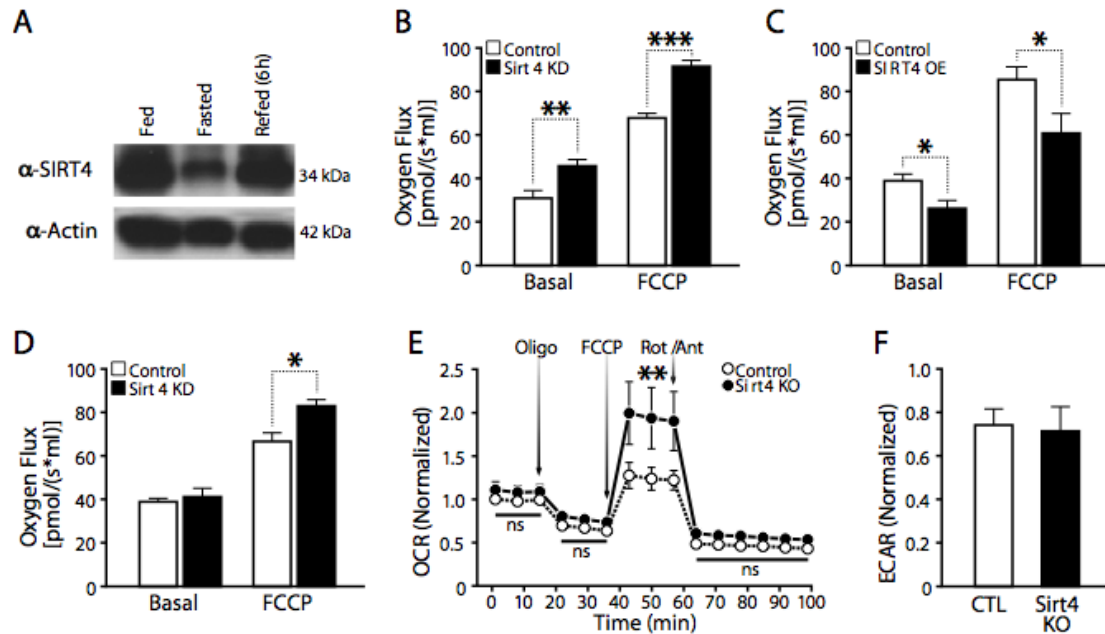


Figure 2.2. Cellular respiration is negatively associated with SIRT4 expression.

(A) Western blot showing liver SIRT4 expression during fed, fasted and refed conditions. (B) and (C) Oxygen flux (respiration) in Control, SIRT4KD (B) and SIRT4OE (C) HEK293T cells. (D) Oxygen flux (respiration) in control and SIRT4KD HEK293T cells in media supplemented with 2mM pyruvate. (E) Oxygen consumption rate (OCR, respiration) in primary hepatocytes isolated from wild-type and SIRT4KO mice. OCR was measured under basal and Oligomycin (Oligo), FCCP and Rotenone/Antimycin-A treated conditions (media supplemented with 2 mM pyruvate). (F) ECAR (glycolysis flux) in primary hepatocytes isolated from wild-type and SIRT4KO mice. Oxygen flux was measured under basal and FCCP treated conditions. Statistical significance was calculated using student's t-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, or as indicated.

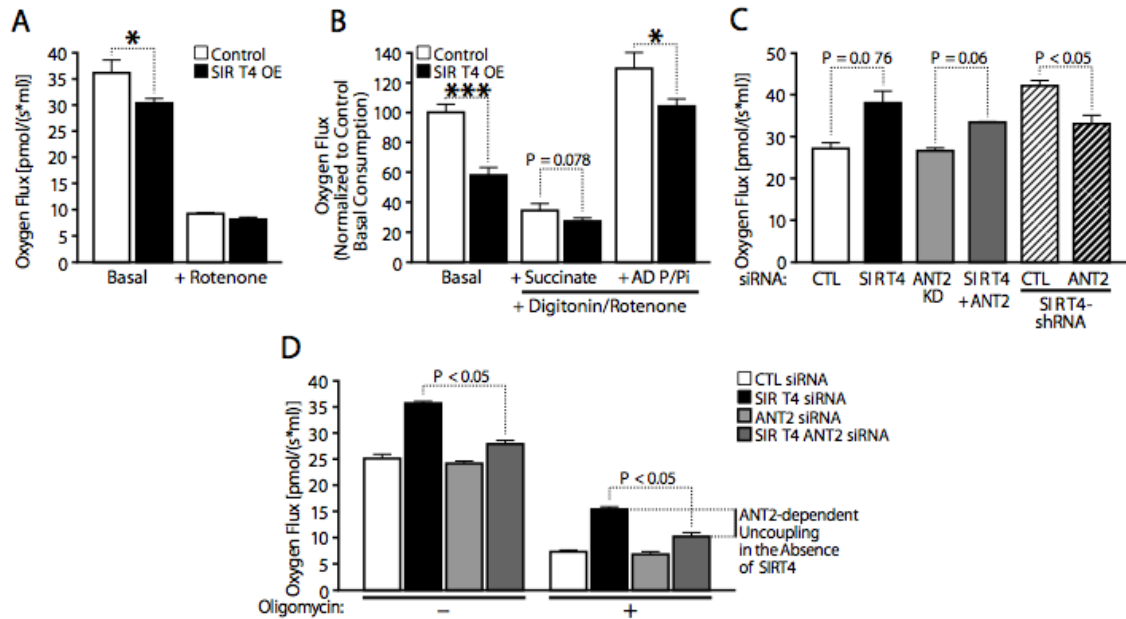


Figure 2.3. SIRT4 regulates mitochondrial respiration via ANT2-mediated coupling efficiency.

(A) Oxygen consumption in Control and SIRT4OE HEK293T cells under basal and Rotenone treated conditions. (B) Oxygen consumption in permeabilized Control and SIRT4OE HEK293T cells under basal conditions and in response to sequential addition of substrates, succinate and ADP/Pi. Rotenone was added to inhibited complex-I when measuring respiration in response to succinate and ADP/Pi addition, as indicated. (C, D) Oxygen consumption in control and SIRT4KD HEK293T cells under basal (C, D) and Oligomycin treated (D) conditions. SIRT4 alone or SIRT4 and ANT2 were simultaneously knocked down to measure ANT2-dependent uncoupled respiration as indicated. Statistical significance was calculated using student's t-test and ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, or as indicated.

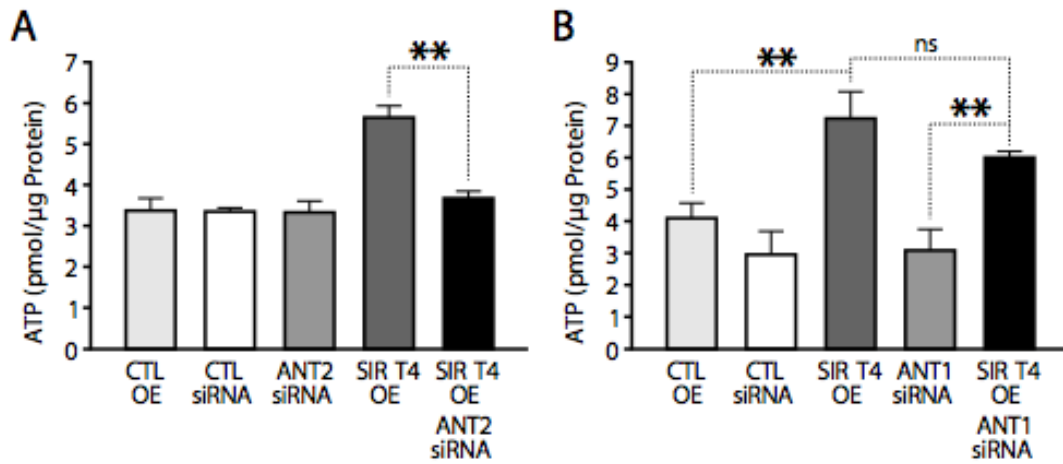


Figure 2.4. SIRT4 regulates ATP in an ANT2-dependent manner.

(A) ATP levels in HEK293T cells that were transfected with Control, ANT2-siRNA, SIRT4-FLAG and SIRT4-FLAG/ANT2-siRNA constructs, as indicated. # indicates $p < 0.05$ for cells transfected with SIRT4-FLAG in the presence or absence of ANT2 (+/- ANT2-siRNA). (B) ATP levels in HEK293T cells that were transfected with Control, ANT1-siRNA, SIRT4-FLAG and SIRT4-FLAG/ANT1-siRNA constructs, as indicated. Statistical significance was calculated using student's t-test and ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, or as indicated.

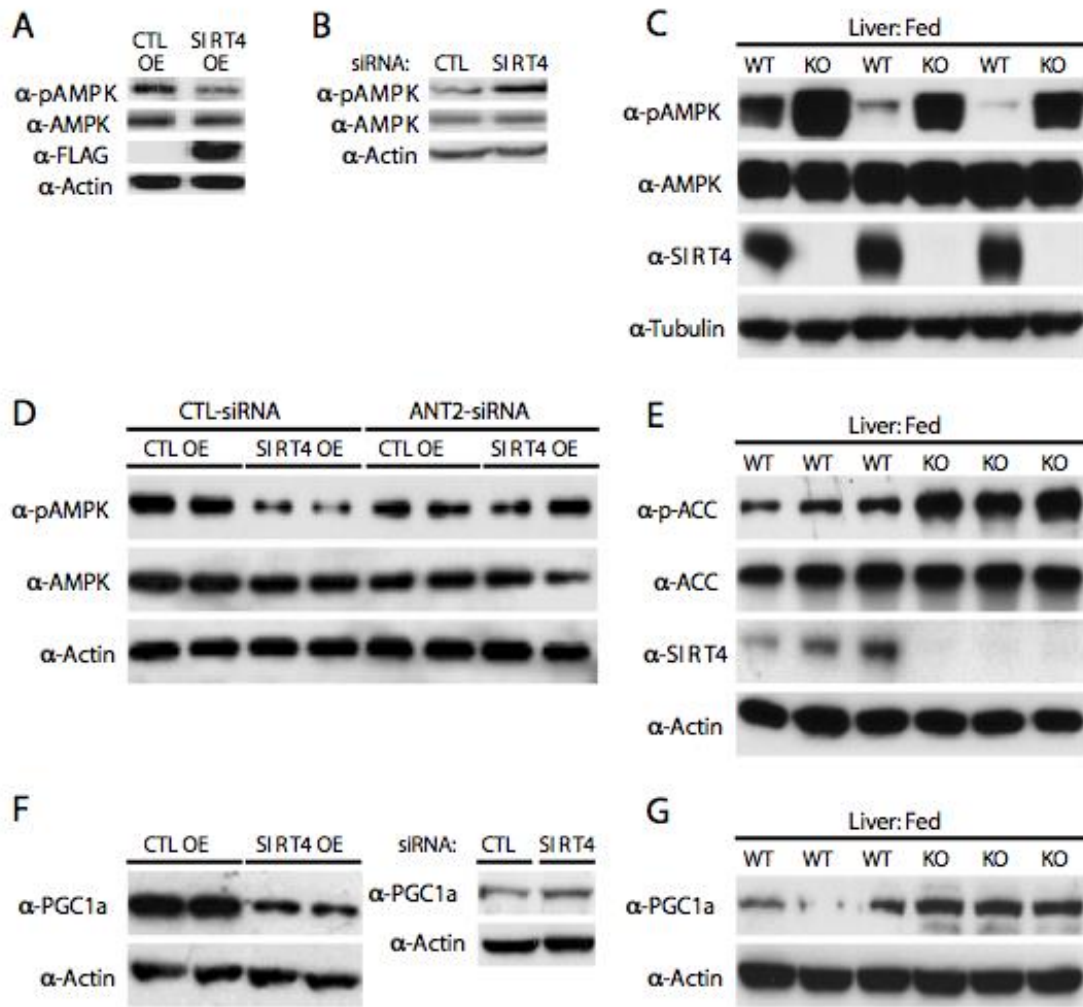


Figure 2.5. SIRT4 deficiency initiates a homeostatic feedback loop via ANT2/AMPK.

(A) Western blots for p-AMPK, AMPK, SIRT4-FLAG and actin in Control and SIRT4OE HEK293T cells. (B) Western blots for p-AMPK, AMPK and actin in Control and SIRT4KD HEK293T cells. (C) Western blots for p-AMPK, AMPK, Sirt4 and tubulin in liver lysates from wild-type and SIRT4KO mice under fed conditions. (D) Western blots for p-AMPK, AMPK and actin in Control and SIRT4OE HEK293T cells in the presence or absence of ANT2 (+/- ANT2-siRNA), as indicated. (E) Western blots for p-ACC, ACC, SIRT4 and actin in liver lysates from wild-type and SIRT4KO mice under fed conditions. (F) Western blots for PGC-1 α and actin in Control and SIRT4OE or SIRT4KD HEK293T cells, as indicated. (G) Western blots for PGC-1 α and actin in liver lysates from wild-type and SIRT4KO mice under fed conditions.

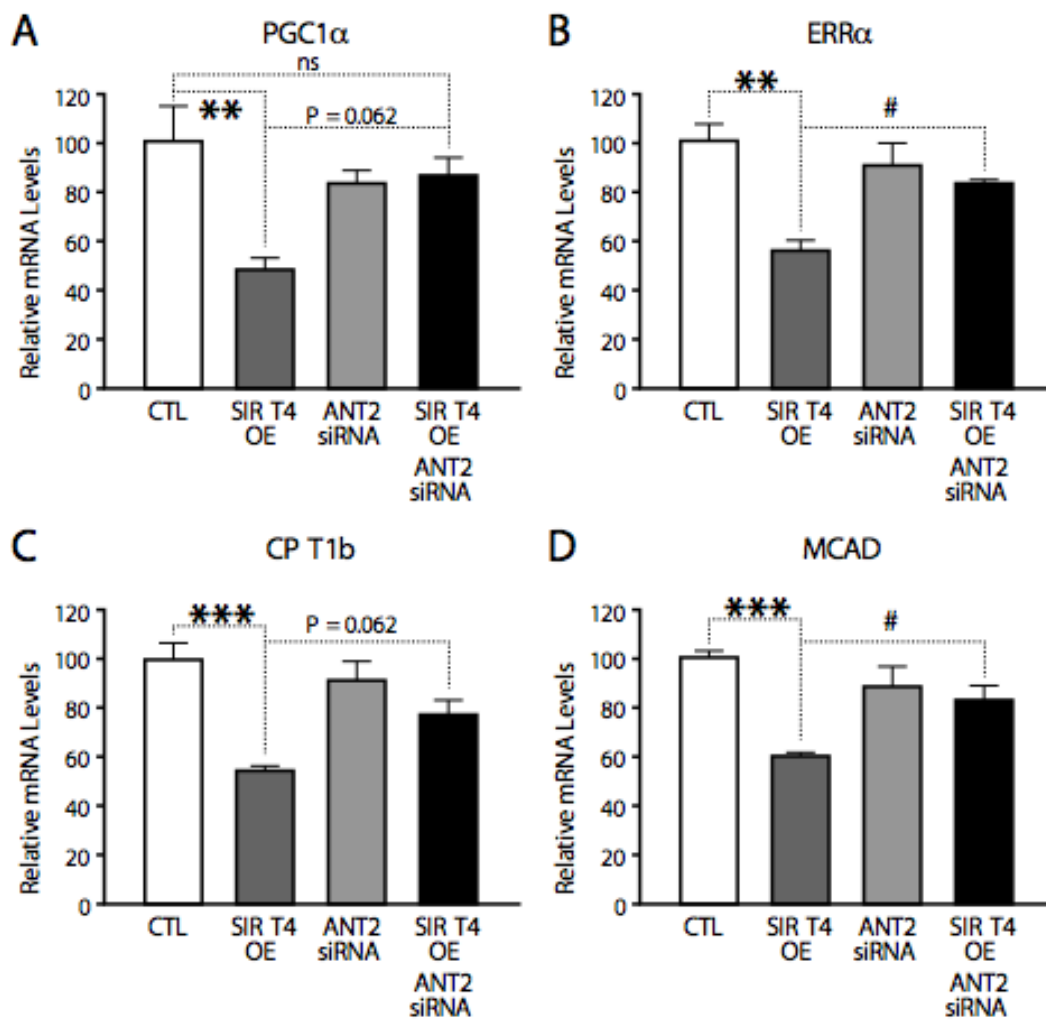


Figure 2.6. SIRT4-AMPK mediated retrograde signaling regulates FAO gene expression.

(A–D) Quantitative RT-PCR of genes involved in fatty acid oxidation (FAO) (A) *PGC-1α*, (B) *ERRα*, (C) *CPT1b* and (D) *MCAD* from RNA isolated from HEK293T cells transfected with Control, SIRT4-FLAG, ANT2-siRNA and SIRT4-FLAG/ANT2-siRNA constructs. Statistical significance was calculated using ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, or as indicated.

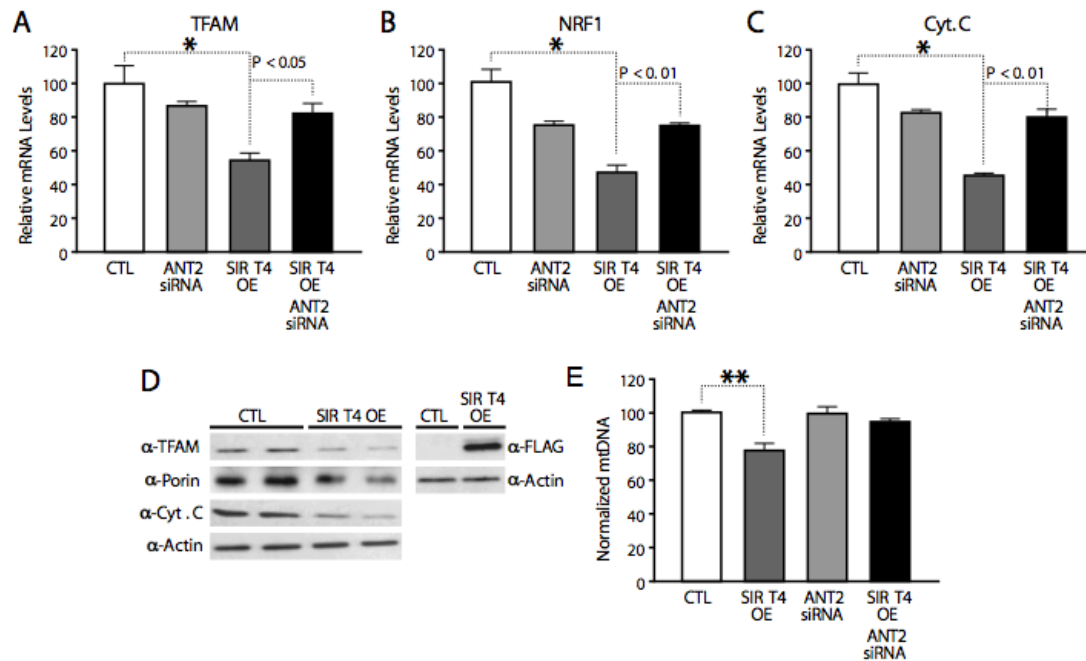


Figure 2.7. SIRT4 regulates expression of nuclear encoded mitochondrial genes in a feedback loop via ANT2/AMPK/PGC-1 α signaling.

(A–C) Quantitative RT-PCR of nuclear encoded mitochondrial genes (A) *TFAM*, (B) *NRF1* and (C) *Cytochrome C* from RNA isolated from HEK293T cells transfected with Control, SIRT4-FLAG, ANT2-siRNA and SIRT4-FLAG/ANT2-siRNA constructs. (D) Western blots for TFAM, Porin, cytochrome c, SIRT4-FLAG and actin in Control and SIRT4OE HEK293T cells. (E) Quantitative RT-PCR of mitochondrial DNA (normalized to nuclear DNA) in DNA isolated from HEK293T cells transfected with Control, SIRT4-FLAG, ANT2-siRNA and SIRT4-FLAG/ANT2-siRNA constructs. Statistical significance was calculated using ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, or as indicated.

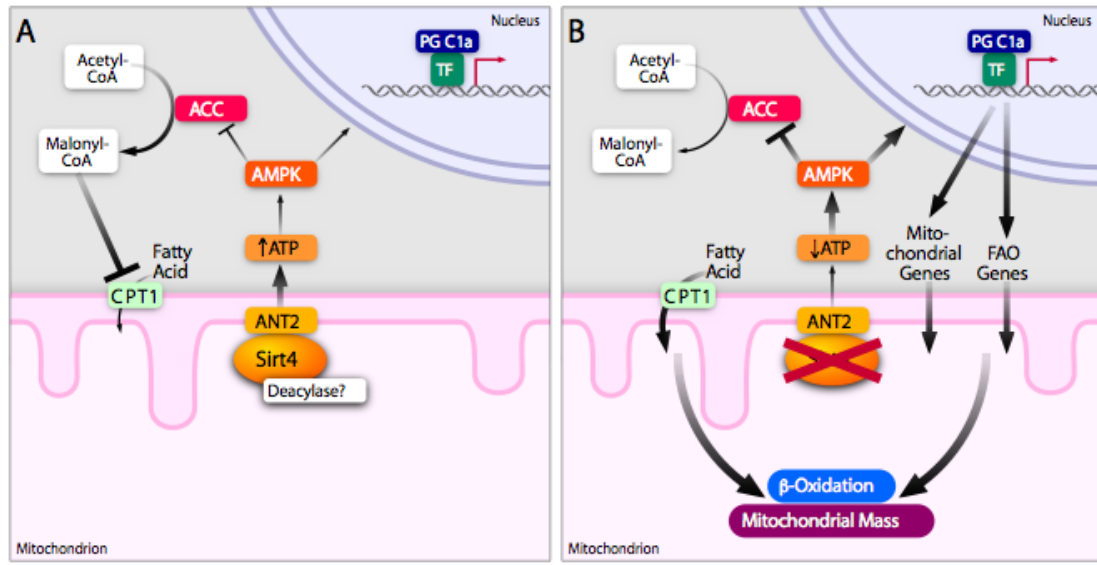


Figure 2.8. SIRT4-ANT2 interplay regulates energy homeostasis and mediates a retrograde signaling from mitochondria.

SIRT4-ANT2 interaction in the mitochondria is required for cellular ATP homeostasis. SIRT4-dependent increase in ATP reduces AMPK activity to mediate a retrograde signaling to affect ACC and PGC-1 α functions. In SIRT4 deficient conditions, ANT2-dependent uncoupling results in a reduction in cellular ATP levels and activates AMPK. Since acylation of ANT2 is known to uncouple mitochondria, we speculate that SIRT4 possesses a deacylase activity. AMPK activation in the absence of SIRT4 consequently leads to phosphorylation of ACC (inhibitory effect), which is known to reduce malonyl CoA levels in the cytosol and thus increase mitochondrial fatty acid (FA) uptake (via depression of CPT1). In addition, AMPK activation in the absence of SIRT4 increases the expression of PGC-1 α and its downstream targets involved in β -oxidation and mitochondrial biogenesis. Together, we propose that SIRT4 in the mitochondria mediates a feedback control to regulate FA metabolism and oxidative phosphorylation (OXPHOS) components.

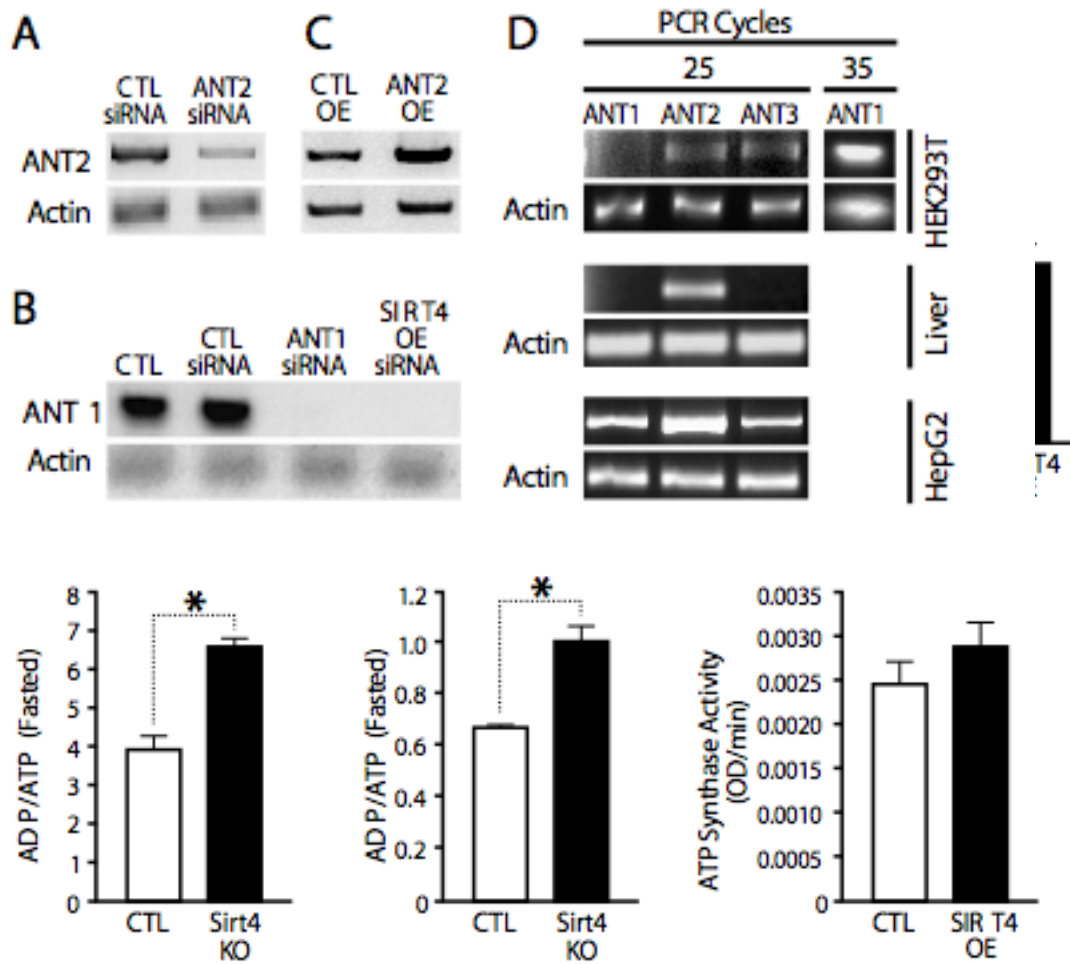


Figure 2.9. Supplementary Figure.

A) RT-PCR to show the efficiency of SIRT4 knockdown. B) Western Blot using anti-SIRT4 antibodies in Control and SIRT4KO mice. C) Total ATP levels in Control and SIRT4-overexpressing HEK293T cells, and in response to 2-deoxyglucose treatment. D-F) ADP/ATP ratios in (D) Control and SIRT4-overexpressing HEK293T cells, (E) Control and SIRT4-knockout liver under fasting conditions and (F) Control and SIRT4KO muscles under fasting conditions G) ATP synthase activity in Control and SIRT4-overexpressing HEK293T cells.

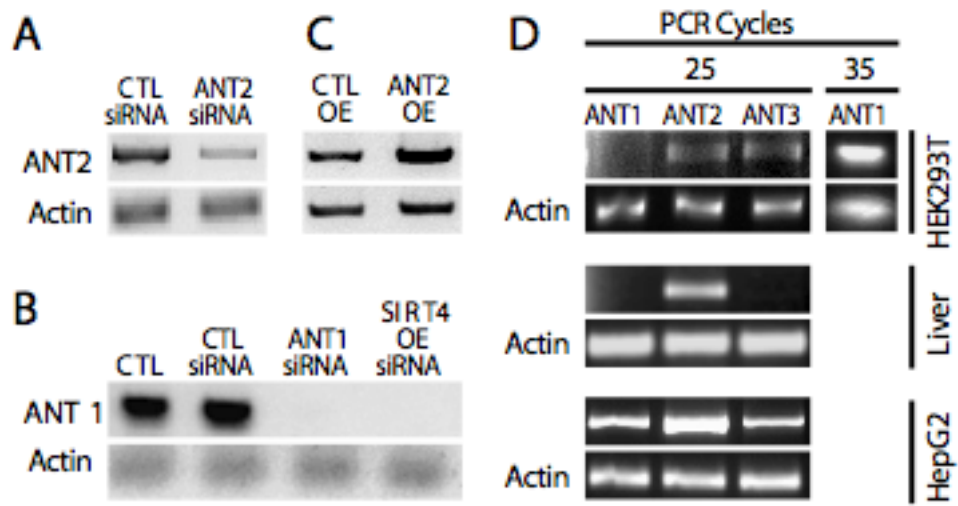


Figure 2.10. Supplementary Figure 2.

A-B) RT-PCR to show knockdown of (A) ANT2 and (B) ANT1 in HEK293T cells. C) RT-PCR to show ANT2 overexpression in HEK293T cells. D) RT-PCR to show differential expression of ANT1, ANT2 and ANT3 in HEK293T cells, HepG2 cells and liver samples.

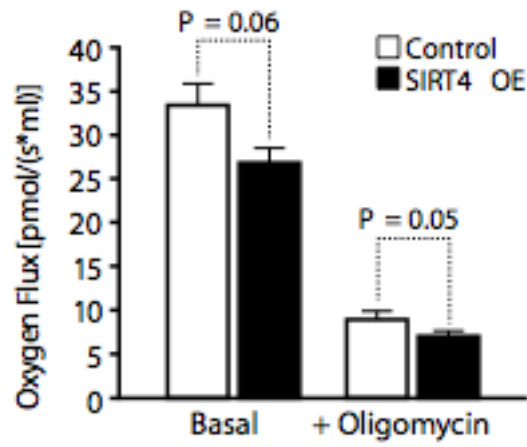


Figure 2.11. Supplementary Figure 3.

Oxygen flux in Control and SIRT4-overexpressing HEK293T cells in response to Oligomycin treatment.

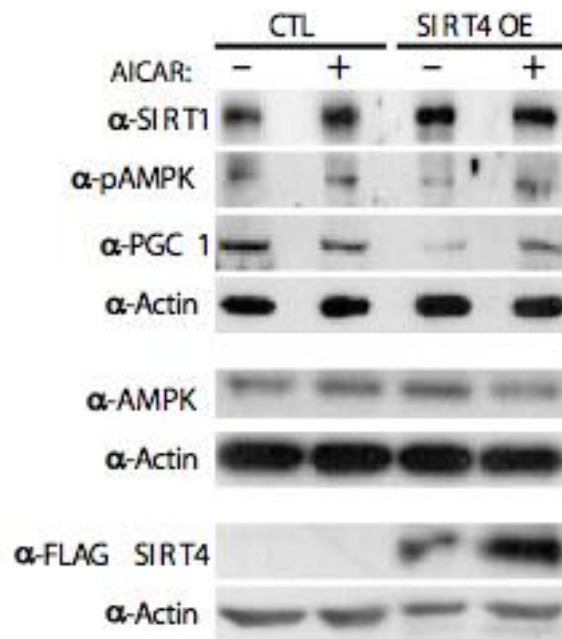


Figure 2.12. Supplementary Figure 4.

Western blot to show the protein levels of SIRT1, phospho-AMPK, SIRT4-FLAG, PGC-1 α and AMPK in Control and SIRT4-overexpressing HEK293T cells, and in response to AICAR treatment.

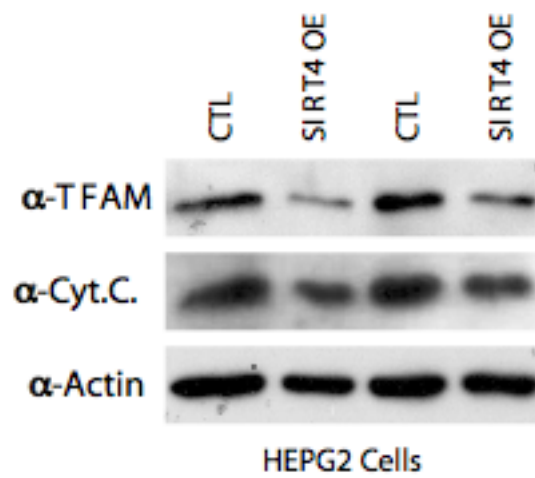


Figure 2.13. Supplementary Figure 5.

Western blot to show the protein levels of TFAM and cytochrome c in Control and SIRT4-overexpressing HepG2 cells.

Table 2.1 Supplementary Table 1: List of primers use for RT-qPCR

ACTIN	F: GCATGGGTCAGAAGGATTCC R: ACGCAGCTCATTGTAGAAGG
CPT1b	F: TCGTCACCTCTTCTGCCTTT R: ACACACCATAGCCGTCATCA
PGC1 α	F: GTGGATGAAGACGGATTGCC R: GCTGAGTGTTGGCTGGTGCC
MCAD	F: TTGAGTTCACCGAACAGCAG R: TCCAAGTCCAAGACCTCCAC
ERR α	F: TGCCAATTCAGACTCTGTGC R: CCAGCTTCACCCCATAGAAA
SIRT4	F: GACAAGGTTGATTTTGTGCAC R: TCAAAGGCAGCAACTCTCCAC
TFAM	F: CTTATAGGGCGGAGTGGCAG R: GCTGAACGAGGTCTTTTTGGT
NRF1	F: CACAGAAAAGGTGCTCAAAGGA R: TTTGGGTCACTCCGTGTTCC
CYT.C	F: GAGATGTTTATGCGGCCAG R: ACGTAGTCCTCACCACCAT
COX-IV	F: GAGTGTTGTGAAGAGTGAA R:TCATCTCGGCGAAGCTCTC
CYT-B for mtDNA	F: CCCATCCAACATCTCCGCAT R: GATGAAAAGGCGGTTGAGGC

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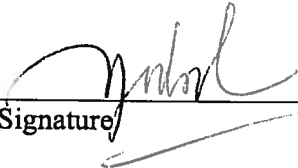
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