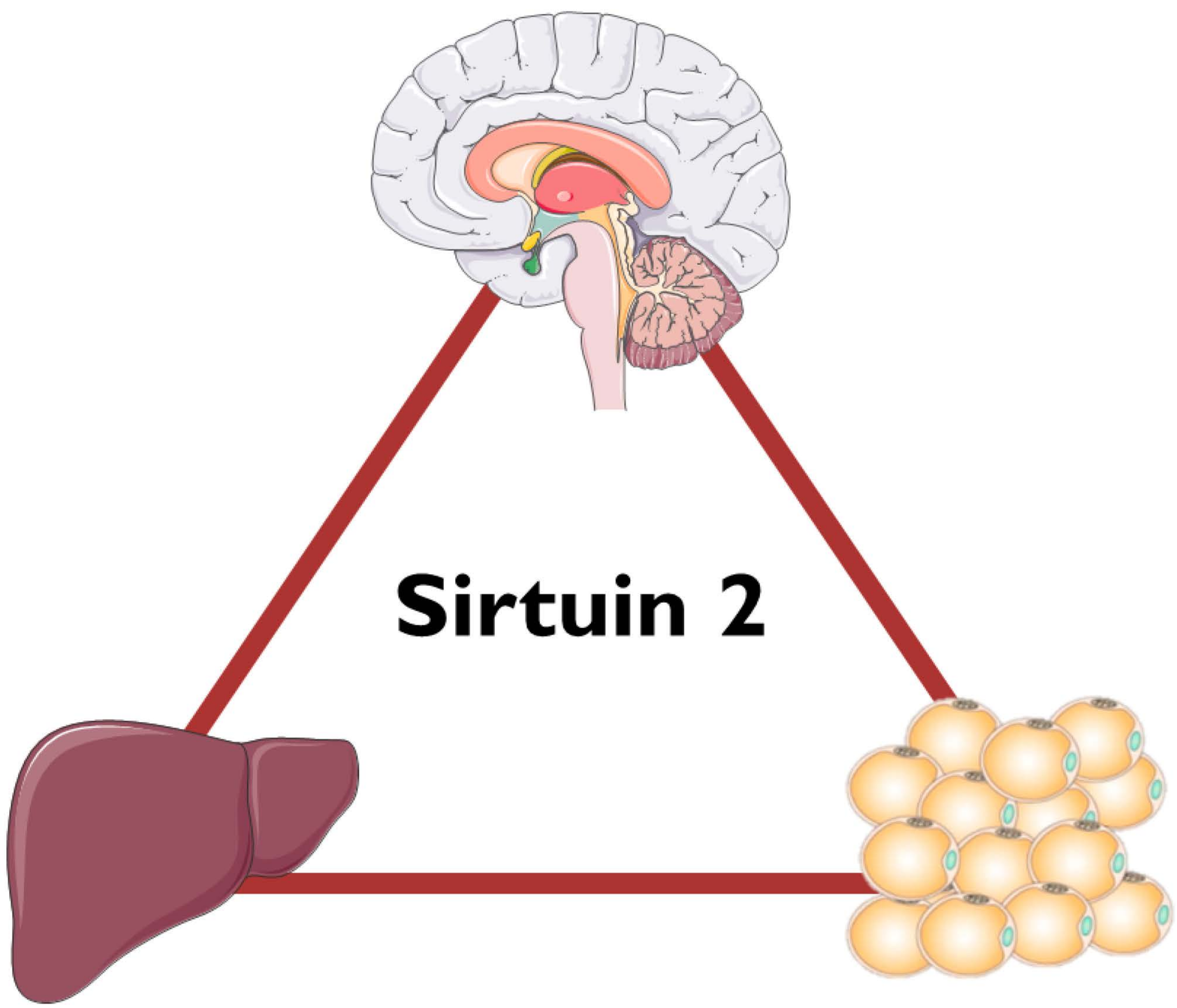

Marta Quatorze Correia

Unraveling the Role of Sirtuin 2 in Metabolic Homeostasis

Dissertação de Mestrado em Biologia Celular e Molecular,
orientada pelo Doutor Pedro Gomes e pela Professora Doutora Emília Duarte,
apresentada ao Departamento de Ciências da Vida da Universidade de Coimbra.

Junho 2016

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UNIVERSIDADE DE COIMBRA

Cover note: Sirtuin 2 (SIRT2) has emerged as an important regulator of the mammalian metabolism. SIRT2 is particularly expressed in metabolic relevant tissues, such as brain, white adipose tissue and liver, where it regulates key processes of the glucose and lipid metabolism.

O trabalho aqui apresentado foi realizado no grupo de Neuroendocrinologia e Envelhecimento do Centro de Neurociências e Biologia Celular (Universidade de Coimbra, Portugal), liderado pela Professora Doutora Cláudia Cavadas, e orientado pelo Doutor Pedro Gomes.

O trabalho foi co-financiado pela FEDER (QREN) através do Programa Mais Centro, no âmbito do projecto "Aging, Stress And Chronic Diseases: From Mechanisms To Therapeutics" (CENTRO-07-ST24-FEDER-002006), bem como pelo Programa Operacional Factores de Competitividade – COMPETE e pela Fundação para a Ciência e Tecnologia (FCT) através do projecto UID/NEU/04539/2013.

The present work was performed in the Neuroendocrinology and Aging group of the Center for Neuroscience and Cell Biology (University of Coimbra, Portugal), headed by Professor Cláudia Cavadas, and under scientific guidance of Dr. Pedro Gomes.

The present work was co-funded by FEDER (QREN) through Programa Mais Centro, under the project "Aging, Stress And Chronic Diseases: From Mechanisms To Therapeutics" (CENTRO-07-ST24-FEDER-002006), as well as by Programa Operacional Factores de Competitividade – COMPETE and FCT – Fundação para a Ciência e a Tecnologia under strategic project UID/NEU/04539/2013.

Agradecimentos

Agradeço antes de mais à Professora Doutora Cláudia Cavadas pela oportunidade de integrar o seu grupo e pela confiança que sempre depositou no meu trabalho.

Ao meu orientador, Doutor Pedro Gomes, pela orientação e pelo apoio que demonstrou durante todo o ano.

A todas as colegas do grupo que me receberam e me apoiaram ao longo do meu percurso. Um muito obrigada por tudo o que me ensinaram. Os meus primeiros passos como cientista foram dados ao vosso lado, tudo o que aprendi até agora foi graças a vocês, e isso vou levar sempre comigo, para onde quer que eu vá. Um especial obrigada à Sara, à Marisa, à Mariana, à Janete e à Joana, que de uma maneira mais activa participaram na realização do trabalho aqui apresentado. Por fim, um grande obrigada à Marta pela ajuda indispensável nesta fase final.

À Dona Isabel e à Sara por toda a ajuda e disponibilidade que demonstraram.

A toda a gente da "sala dos mestrados" pela boa disposição e por todo o apoio, por marcarem este ano de uma maneira tão especial. Vão deixar saudades!

A todos os meus colegas do mestrado que, estando ou não presentes durante este ano, fizeram destes dois anos os melhores anos da minha vida. Obrigada por todos os momentos que passámos, por toda a intensidade e cumplicidade com que foram vividos. Para onde quer que eu vá, levo um bocadinho de cada um de vocês!

Aos meus pais e à minha irmã tenho a agradecer não este trabalho, não este ano, mas tudo o que sou e o que tenho até agora. Sem vocês nada disto era possível.

Muito obrigada a todos!

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Abbreviation

ACLY - ATP-citrate lyase

AgRP - Agouti-related peptide

AMPK - AMP-activated protein kinase

ARC - Arcuate nucleus

BAT - Brown adipose tissue

BBB - Blood-brain barrier

BCA - Bicinchoninic acid

BSA - Bovine serum albumin

CART - Cocaine - and amphetamine-regulated transcript

Cer - Ceramide

ChREBP - Carbohydrate response element binding protein

CPS1 - Carbamoyl phosphate synthase 1

CR - Caloric restriction

DAG - Diacylglycerol

EGF - Epidermal growth factor

ERK - Extracellular-signal-regulated kinase

FAS - Fatty acid synthase FBPase - Fructose-1,6-biphosphatase

FFA - Free fatty acids

FOXO - Forkhead box class O

GDH - Glutamate dehydrogenase

G6Pase - Glucose-6-phosphatase

G6PD - Glucose-6-phosphatase dehydrogenase

Glut4 - Glucose transporter type 4

GSK-3 - Glycogen synthase kinase 3

HD - Huntington’s disease

HFD - High-fat diet

HGP - Hepatic glucose production

HIF-1α - Hypoxia-inducible factor 1 α

ICV - Intracerebroventricular

IpGTT - Intraperitoneal glucose tolerance test

IpITT - Intraperitoneal insulin tolerance test

IRS - Insulin receptor substrates

K_{ATP} channels – ATP-sensitive potassium channels

LCAD - Long-chain acyl CoA dehydrogenase

LCFA - Long-chain fatty acid

LCFA-CoA - Long-chain fatty acyl – CoA

LH - Lateral hypothalamus

LXR - Liver X receptor

MAPK - Mitogen-activated protein kinase

MC - Melanocortin

MCH - Melanin-concentrating hormone

MC3/4R - Melanocortin-3 and -4 receptor

ME - Median eminence

MnSOD - Manganese superoxide dismutase

mTOR - Mammalian target of rapamycin

NAD - Nicotinamide adenine dinucleotide

NAM - Nicotinamide

NF-κB - Nuclear factor-κB

NPY - Neuropeptide Y

NTS - Nucleus tractus solitarius

PEPCK1 - Phosphoenolpyruvate carboxykinase 1

PGAM2 - Phosphoglycerate mutase 2

PGC-1α - Peroxisome proliferator-activated receptor α coactivator-1 α

PI3K - Phosphoinositide 3-kinase

PKB/Akt - Protein kinase B

PKC - Protein kinase C

POMC - Proopiomelanocortin

PPAR - Peroxisome proliferator-activated receptor

PPAR-ϒ - Peroxisome proliferator-activated receptor ϒ

PVN - Paraventricular nucleus

ROS -Reactive oxygen species

SDS - Sodium dodecyl sulphate

SEM - Standard error of the mean

SFA - Saturated fatty acids

SOD2 - Superoxide dismutase 2

Sir 2 - Silent information regulator 2

SIRT - Sirtuin

SNS - Sympathetic nervous system

SREBP-1c - Sterol regulatory element-binding protein-1c

TGF-α - transforming growth factor-α

VMH - Ventromedial hypothalamus

WAT - White adipose tissue

WT - Wild type

UCP1 - Uncoupling protein 1

Abstract

Sirtuin 2 (SIRT2) is one of the seven members (SIRT1-7) of the sirtuin family. Sirtuins are NAD^+-dependent deacetylases with homology to the silent information regulator 2 (Sir2), found to extend longevity in yeast. Although it is still controversial whether sirtuins play a role in longevity, increasing evidence supports that they modulate homeostasis by sensing energy needs and restoring homeostasis during stress conditions. Metabolic diseases, such as obesity and type 2 diabetes, are one of the main causes of mortality and morbidity worldwide and their prevalence is increasing at an alarming rate. SIRT2 is particularly expressed in metabolic relevant tissues, such as adipose tissue, liver and brain, and has emerged as an important regulator of mammalian metabolism. SIRT2 regulates key processes of the lipid and glucose metabolism, such as adipogenesis, gluconeogenesis, fatty acid oxidation, lipogenesis and insulin sensitivity. Moreover, SIRT2 expression is modulated by energy availability, being downregulated during states of energy excess and overexpressed under low-energy conditions. Taking into account these previous findings, the present study aimed to evaluate the role of SIRT2 in the regulation of metabolic homeostasis *in vivo*. This study provides the first comprehensive metabolic characterization of SIRT2 knockout (SIRT2-KO) mice. SIRT2-KO mice developed no obvious phenotype when fed a normal chow diet (CD). Interestingly, when subjected to metabolic stress induced by short-term high fat diet (HFD) feeding, SIRT2-KO mice developed severe metabolic abnormalities, including increased body weight gain and energy intake, hyperglycemia, hypertriglyceridemia, glucose intolerance and insulin resistance. Thus, our data suggest that SIRT2 plays a key role during metabolic stress conditions. Taking into account the present findings, SIRT2 modulation may be a potential therapeutic strategy to counteract the metabolic dysfunction associated with the early onset of metabolic disorders.

Keywords: Sirtuin 2, Obesity, Insulin Resistance, High Fat Diet

Resumo

A Sirtuína 2 (SIRT2) é uma das sete proteínas que constituem a família das sirtuínas (SIRT1-7). As sirtuínas são desacetilases dependentes de NAD^+, homólogas do gene regulador de informação silenciosa 2 (Sir2), conhecido por prolongar a longevidade em leveduras. É cada vez mais evidente que as sirtuínas modulam a homeostasia detectando necessidades energéticas e restaurando a homeostasia em condições de stress. A obesidade e a diabetes tipo 2 são duas das maiores causas mundiais de mortalidade e a sua prevalência tem vindo a aumentar a um ritmo acelerado. A SIRT2 encontra-se particularmente expressa em órgãos com relevância metabólica, tais como o tecido adiposo, o fígado e o cérebro, e o seu papel na regulação do metabolismo tem vindo a ser alvo de grande atenção. Sabe-se que a SIRT2 regula processos tais como a adipogénese, gliconeogénese, oxidação de ácidos gordos, lipogénese e sensibilidade à insulina. Além disso, a expressão da SIRT2 é modulada pela disponibilidade energética, sendo suprimida em condições de excesso de energia e estimulada em situações de energia limitada. Tendo em conta o conhecimento até agora adquirido, o objectivo deste estudo é avaliar o papel da SIRT2 na regulação do metabolismo in vivo. Este é o primeiro estudo a revelar a caracterização metabólica de murganhos knockout para SIRT2 (SIRT2-KO). Estes murganhos não desenvolveram um fenótipo metabólico significativo quando alimentados com uma dieta normal. No entanto, quando sujeitos a uma condição de stresse causada por uma dieta rica em lípidos durante 4 semanas, estes animais desenvolveram alterações metabólicas, tais como ganho de peso e consumo energético aumentados, hiperglicemia, hipertrigliceridemia, intolerância à glucose e resistência à insulina. Assim, a SIRT2 parece desempenhar um papel importante durante situações de stresse metabólico. Os resultados aqui apresentados sugerem a modulação da SIRT2 como uma estratégia promissora na prevenção da disfunção metabólica associada à fase inicial de desenvolvimento de doenças metabólicas

Palavras-chave: Sirtuína 2, Obesidade, Resistência à Insulina, Dieta Rica em Gordura

Chapter I

Introduction

1. Sirtuin family

1.1 Mammalian sirtuins

Sirtuins are a highly conserved family of NAD^+-dependent deacetylases, composed by seven members (SIRT1-7). Sirtuin family members are homologues of the silent information regulator 2 (Sir2), an important regulator of transcriptional silencing in *Saccharomyces cerevisiae* (Rine *et al.*, 1979). Sir2 was found to play important roles in metabolism and aging in *S. cerevisiae*, *C. elegans* and *Drosophila* (Rine *et al.*, 1979; Kaeberlein *et al.*, 1999; Tissenbaum and Guarente, 2001). The enzymatic activity of Sir2 is dependent on the cofactor NAD^+ and was linked to lifespan extension by calorie restriction (Lin et al., 2000). However, this role of Sir2 in longevity has been challenged, casting doubt on the robustness of previous findings (Burnett *et al.*, 2011). Nevertheless, sirtuins have been reported to play a role in aging, protecting against age-related diseases, such as metabolic syndrome, neurodegeneration and cancer (Herranz *et al.*, 2010; Kanfi *et al.*, 2012; Hall *et al.*, 2013; Satoh *et al.*, 2013). Particularly, SIRT1, 2 and 6 were recently implicated in lifespan regulation (Mostoslavsky *et al.*, 2006; Herranz *et al.*, 2010; Kanfi *et al.*, 2012; Satoh *et al.*, 2013; North *et al.*, 2014). Although it is not definitely resolved whether sirtuins play important roles in longevity, they certainly are relevant nutrient and stress sensors. The deacetylase activity of sirtuins (Fig. 1) is dependent on NAD^+ levels, which is an indicator of the energy status of the cell, providing an important link between sirtuin activity and the cellular metabolic state.

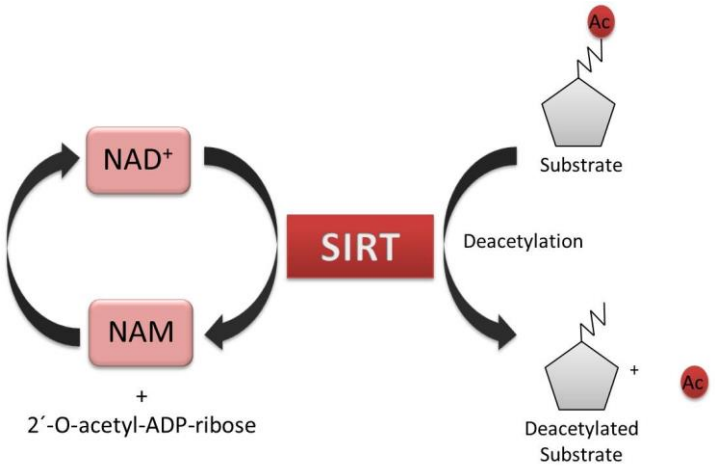

Figure 1. NAD^+-dependent deacetylase activity of sirtuins. Sirtuins (SIRT) remove acyl groups (Ac) from different substrates and cleave the cofactor nicotinamide adenine dinucleotide (NAD^+) in nicotinamide (NAM) and 2'-O-acetyl-ADP-ribose.

The seven mammalian sirtuins target multiple histones and non-histone proteins, thus regulating important cellular processes such as apoptosis, DNA repair, cell cycle progression and stress response (Houtkooper *et al.*, 2012). Sirtuins have different subcellular localizations, activities and targets (Table 1). Although sirtuins were initially described as deacetylases, it is now known that some isoforms possess weak deacetylase activity, being able to remove other acyl groups, or to transfer an ADP-ribose moiety.

SIRT1 is mainly located in the nucleus, but can also be in the cytosol (Tanno *et al.*, 2007). On the contrary, SIRT2 is mainly cytosolic but can also be present in the nucleus (Vaquero *et al.*, 2006). SIRT3, 4 and 5 are mitochondrial sirtuins (Huang *et al.*, 2010) and SIRT6 and 7 can be found in the nucleus and nucleolus, respectively.

Table 1. Sirtuins subcellular localization, activities and targets.

Sirtuin	Subcellular localization	Activity	Key Targets	References
SIRT1	Nucleus, cytosol	Deacetylation	PPAR-γ, PGC-1α, p53, FOXO	(Vaziri *et al.*, 2001; Brunet *et al.*, 2004; Lagouge *et al.*, 2006)
SIRT2	Cytosol, nucleus	Deacetylation, demyristoylation	PGC-1α , α-Tubulin, PEPCK, FOXO, NF-κB, G6PD	(North *et al.*, 2003; Jing *et al.*, 2007; Wang and Tong, 2009; Zhao *et al.*, 2010; Pais *et al.*, 2013; Wang *et al.*, 2014)
SIRT3	Mitochondria	Deacetylation	UCP1, LCAD, SOD2	(Shi *et al.*, 2005; Hirschey *et al.*, 2010; Qiu *et al.*, 2010)
SIRT4	Mitochondria	ADP-ribosylation	GDH	(Haigis *et al.*, 2006)
SIRT5	Mitochondria	Deacetylation, demalonylation, desuccinylation,	CPS1	(Nakagawa *et al.*, 2009)
SIRT6	Nucleus	Deacetylation, ADP-ribosylation	H3K9, H3K56	(Schwer *et al.*, 2010)
SIRT7	Nucleolus	Deacetylation	H3K18Ac	(Barber *et al.*, 2012)

CPS1, carbamoyl phosphate synthase 1; FOXO, forkhead box class O; GDH, glutamate dehydrogenase; G6PD, glucose-6-phosphatase dehydrogenase; LCAD, long-chain acyl CoA dehydrogenase; NF-κB, nuclear factor-κB; PEPCK1, phosphoenolpyruvate carboxykinase 1; PGC-1α, peroxisome proliferator-activated receptor α coactivator-1 α; PPAR-γ, peroxisome proliferator-activated receptor ϒ; SOD2, superoxide dismutase 2; UCP1, uncoupling protein 1.

SIRT1 is the closest homolog to yeast Sir2 and is the best characterized member of the family. SIRT1 is activated by energy stress, such as fasting (Rodgers *et al.*, 2005; Gerhart-Hines *et al.*, 2007), exercise (Canto *et al.*, 2009) or low glucose availability (Fulco et al., 2008) and deacetylates a large number of substrates, such as peroxisome proliferator-activated receptor γ (PPAR-γ), PPAR-γ coactivator-1α (PGC-1α) (Lagouge *et al.*, 2006), p53 (Vaziri *et al.*, 2001), nuclear factor-κB (NF-κβ) and forkhead proteins (FOXO) (Brunet *et al.*, 2004), key metabolic regulators. In the liver, SIRT1 promotes gluconeogenesis through PGC-

1α deacetylation (Rodgers *et al.*, 2005). Beyond the liver, PGC-1α-mediated SIRT1 action stimulates energy expenditure in brown adipose tissue (BAT) and skeletal muscle. SIRT1-activated PPAR-γ inhibits fat accumulation in white adipose tissue (WAT) (Picard *et al.*, 2004).

SIRT3 has been linked to thermogenesis and mitochondrial function (Shi *et al.*, 2005; Bellizzi *et al.*, 2007). In mice, SIRT3 expression is induced in WAT and BAT adipose tissue by calorie restriction and in BAT upon cold exposure. SIRT3 activates PGC-1α and uncoupling protein 1 (UCP1), suggesting an important role in thermogenesis (Shi *et al.*, 2005). During prolonged fasting, SIRT3 acts on long-chain acyl CoA dehydrogenase (LCAD), a protein involved in fatty acid oxidation, resulting in the activation of fatty acid breakdown (Hirschey *et al.*, 2010). Calorie restriction seems to reduce cellular ROS levels by SIRT3-dependent superoxide dismutase (SOD2) activation (Qiu *et al.*, 2010). SIRT4 and SIRT6 have ADP-ribosylation activity (Liszt *et al.*, 2005; Haigis *et al.*, 2006). SIRT4 inhibits glutamate dehydrogenase (GDH) decreasing amino acid-dependent insulin secretion (Haigis *et al.*, 2006). SIRT6 is suggested to control genomic stability and DNA repair (Mostoslavsky *et al.*, 2006). It not only transfers ADP-ribose moieties but also deacetylates histones H3K9 and H3K56 (Schwer *et al.*, 2010). SIRT5 has the ability to remove malonyl and succinyl groups from carbamoyl phosphate synthase 1 (CPS1) (Du *et al.*, 2011), activating ammonia detoxification through urea cycle, during fasting (Nakagawa *et al.*, 2009). SIRT7 activates RNA polymerase I transcription, suggested to be required for cell viability (Ford *et al.*, 2006). This protein was recently associated with the stabilization of the transformed state of cancer cells, by deacetylation of histone H3K18Ac (Barber et al., 2012).

SIRT2 is one of the least studied sirtuins and was initially found to be a bona fide tubulin deacetylase (North et al., 2003). Further research has shown that SIRT2 acts on multiple histone and non-histone proteins. Besides its deacetylase activity, SIRT2 has also the capacity to efficiently remove myristoyl groups (Teng *et al.*, 2015). Although SIRT2 is ubiquitously expressed, it has been detected particularly in metabolic relevant tissues, such as brain, liver, skeletal muscle and adipose tissue (Wang *et al.*, 2007; Kim *et al.*, 2011; Maxwell *et al.*, 2011). Interestingly, SIRT2 is the most abundant sirtuin in adipocytes (Jing *et*

al., 2007). In the brain, SIRT2 is highly expressed in the hippocampus, striatum, cortex and spinal cord (Maxwell *et al.*, 2011). Recent results from our group demonstrated that SIRT2 is also expressed in the hypothalamus, a key brain region regulating energy homeostasis (Santos, 2015; unpublished data). Moreover, hypothalamic SIRT2 expression is downregulated in *in vivo* models of high fat diet-induced obesity and *in vitro* models of palmitate-induced insulin resistance (Santos, 2015; unpublished data). SIRT2 expression was found to be downregulated in WAT of human obese subjects and mice fed a HFD (Krishnan *et al.*, 2012). By contrast, long-term calorie restriction in mice increases SIRT2 protein levels in WAT and kidney (Wang *et al.*, 2007). Short-term food deprivation also stimulates SIRT2 expression in WAT (Wang and Tong, 2009). In human overweight subjects, hypocaloric diet increases SIRT2 gene expression in peripheral blood mononuclear cells (Crujeiras *et al.*, 2008). Overall, these studies indicate that SIRT2 expression, and possibly activity, is modulated by energy availability, being increased in low-energy conditions and downregulated upon energy excess.

1.2 SIRT2 actions

Although SIRT2 is mainly present in the cytoplasm, it shuttles to the nucleus during the G2/M transition of the cell cycle, where it regulates chromosomal condensation and act as a checkpoint protein (Dryden *et al.*, 2003; Vaquero *et al.*, 2006; Inoue *et al.*, 2007). SIRT2 interacts with several mitotic structures to ensure the normal cell division (North and Verdin, 2007). Recently, it was shown that SIRT2 is able to deacetylate the mitotic checkpoint kinase BubR1, mediating its role in lifespan extension (North *et al.*, 2014).

The role of SIRT2 in tumorigenesis has been conflicting. It was shown that SIRT2 acts as a tumor suppressor, preventing chromosomal instability (Kim *et al.*, 2011). Moreover, a recent study showed that loss of SIRT2 in cancer cells leads to metabolic reprograming that favors carcinogenesis (Park *et al.*, 2016) However, a pro-tumorigenic role has also been proposed for SIRT2 (Lain *et al.*, 2008; Chen *et al.*, 2013). Further studies will be required to clarify the role of this protein in tumorigenesis.

In contrast with the apparent beneficial role of SIRT2 activation as a therapeutic strategy to reverse age-related diseases, studies using models of neurodegenerative diseases, such as Parkinson´s and Huntington's diseases, have suggested that SIRT2 inhibition has protective roles instead (Luthi-Carter *et al.*, 2010; de Oliveira *et al.*, 2012; Donmez and Outeiro, 2013; Chen *et al.*, 2015). However, these studies were performed in cellular and invertebrate models, and a recent mouse study showed that SIRT2 inhibition does not have any beneficial effects on Huntington's disease progression (Bobrowska *et al.*, 2012). Further *in vivo* studies are needed to clarify the role of SIRT2 in neurodegeneration.

1.2.1 Metabolic roles

1.2.1.1 Glucose metabolism

Gluconeogenesis

The liver is responsible for maintaining glucose homeostasis during states of limited energy availability, such as fasting and calorie restriction. Gluconeogenesis is the process by which the liver generates glucose from non-carbohydrate carbon sources, such as lactate and pyruvate. FOXO1 and PGC-1α are hepatocyte nuclear factors that can increase the transcription of gluconeogenic enzyme genes, such as glucose-6-phosphatase (G6Pase), fructose-1,6-biphosphatase (FBPase) and phosphoenolpyruvate carboxykinase (PEPCK1) (Puigserver *et al.*, 2003). SIRT2 is able to deacetylate FOXO1 and PGC-1α (Jing *et al.*, 2007; Wang and Tong, 2009), suggesting that this protein can modulate gluconeogenesis through distinct mechanisms. PEPCK1 converts oxaloacetate into phosphoenolpyruvate, the first rate-limiting step of gluconeogenesis. It was proposed that PEPCK1 acetylation promotes its degradation under high-glucose conditions (Zhao *et al.*, 2010). In glucose deprivation conditions, SIRT2 deacetylates and stabilizes PEPCK1, enhancing gluconeogenesis (Jiang *et al.*, 2011).

Insulin sensitivity

Insulin binding to its receptor on the cellular membrane leads to the activation of two main pathways. The phosphatidylinositol 3-kinase (PI3-K)-Akt pathway is responsible for the metabolic actions of insulin and the Ras-mitogen-activated protein kinase (MAPK) pathway for cell growth and differentiation (Taniguchi *et al.*, 2006). When activated, Akt phosphorylates several downstream substrates such as other protein kinases, signaling proteins and transcription factors. The first target identified was glycogen synthase kinase-3 (GSK3), whose phosphorylation and inactivation by Akt stimulates glycogen synthesis (Cross *et al.*, 1995). Akt also phosphorylates FOXO1, AS160 and mTOR, leading to inhibition of gluconeogenesis, increased glucose uptake and protein synthesis, respectively (Taniguchi *et al.*, 2006).

There is some evidence that SIRT2 modulates insulin-activated PI3K-Akt pathway. Several studies have reported that the FOXO transcription factors, major direct substrates of Akt, are deacetylated by SIRT2 (Jing *et al.*, 2007; Wang *et al.*, 2007; Wang and Tong, 2009). Two recent studies showed that SIRT2 binds to and modulates Akt in insulin-responsive cells, such as human embryonic kidney cells (HEK-293t) and adipocytes (3T3-L1) (Chen *et al.*, 2013; Ramakrishnan *et al.*, 2014). SIRT2 deacetylase activity was reported to be crucial for Akt activation. SIRT2 inhibition blocked Akt activation and, conversely, its overexpression sensitized cells to insulin-induced Akt activation (Ramakrishnan *et al.*, 2014). In contrast, SIRT2 is overexpressed in insulin-resistant C2C12 skeletal muscle cells, and its pharmacological inhibition ameliorates insulin sensitivity by increasing glucose uptake and Akt phosphorylation (Arora and Dey, 2014). This study suggests that SIRT2 may negatively affect insulin sensitivity in skeletal muscle cells. Recent findings have also reported that SIRT2 binds and deacetylates TUG, a protein involved in GLUT4 exocytosis and therefore glucose uptake. TUG was shown to be highly acetylated in the liver of SIRT2-KO mice, whose insulin sensitivity was enhanced (Belman *et al.*, 2015). These studies suggested an Akt-independent role of insulin on TUG and glucose uptake, which is consistent with previous results (Xu *et al.*, 2011). Further research is necessary to clarify the role of SIRT2 in insulin

sensitivity, and it is possible that this protein display different and even opposite roles in distinct cells and tissues.

1.2.1.2 Lipid metabolism

Adipogenesis

Adipose tissue is able to differentiate new mature adipocytes from undifferentiated preadipocytes, through a process called adipogenesis. Adipocytes increase in number and size upon increased needs for fat accumulation.

FOXO1, a known SIRT2 target, has emerged as an important regulator of adipogenesis, besides the well-known regulators of this process, PPAR-γ and CCAAT/enhancer-binding proteins (C/EBPs). FOXO1 was reported to inhibit adipogenesis at an early phase of adipocyte differentiation (Nakae *et al.*, 2003). SIRT2 acts by decreasing the levels of FOXO1 acetylation, which in turn decreases its phosphorylation. Therefore, FOXO1 remains in the nucleus, allowing the interaction of FOXO1 with the PPAR-γ promoter, repressing PPAR-γ transcription and consequently adipocyte differentiation (Jing *et al.*, 2007). In agreement with this study, SIRT2 was subsequently reported to respond to nutrient deprivation and energy expenditure, promoting lipolysis and inhibiting adipocyte differentiation (Wang and Tong, 2009).

Lipogenesis

Lipid synthesis occurs in nutrient-rich conditions, upon high levels of insulin or excessive carbohydrates, and consists in the process of *de novo* fatty acid synthesis (Strable and Ntambi, 2010). Lipogenesis is a complex process regulated by transcription factors such as liver X receptor (LXR), sterol regulatory element-binding protein-1c (SREBP-1c), and carbohydrate response element binding protein (ChREBP) (Strable and Ntambi, 2010). ATP-citrate lyase (ACLY) is the lipogenic enzyme that converts cytosolic glucose-derived citrate into acetyl coenzyme CoA (acetyl-CoA), the building block for steroids and fatty acids. Interestingly, ACLY activity is able to link the metabolic cellular state with histone

acetylation and gene expression (Wellen *et al.*, 2009). SIRT2 was reported to decrease lipogenesis due to ACLY deacetylation, leading to its ubiquitylation and degradation (Lin *et al.*, 2013). Under high-glucose conditions, ACLY is acetylated in cells and mice liver, being stable to allow lipid synthesis (Lin *et al.*, 2013). In an ACLY-dependent manner, SIRT2 seems to be able to reduce lipogenesis under nutrient excess conditions. Liver-specific ACLY abrogation, in leptin receptor-deficient mice, inhibits hepatic lipogenesis, improving systemic glucose metabolism and hepatic steatosis (Wang *et al.*, 2009). Therefore, hepatic SIRT2-dependent inhibition of ACLY may be a potential therapeutic strategy to treat metabolic disorders.

SIRT2 seems to play an important role in cholesterol biosynthesis but its role has been conflicting (Luthi-Carter *et al.*, 2010; Bobrowska *et al.*, 2012). Initially, studies in cellular and invertebrate models reported that SIRT2 inhibition protects against Huntington's disease (HD) by decreasing sterol biosynthesis (Luthi-Carter *et al.*, 2010; Taylor *et al.*, 2011). The retention of SREBP-2 in the cytoplasm, mediated by SIRT2 inhibition, was proposed to result in the downregulation of key enzymes in the cholesterol biosynthesis (Luthi-Carter *et al.*, 2010). A brain-permeable SIRT2 inhibitor was able to inhibit neuronal cholesterol biosynthesis, supporting the initial findings (Taylor *et al.*, 2011). However, a subsequent *in vivo* study has proposed that genetic and pharmacological reduction or loss of SIRT2 has no effect on cholesterol biosynthesis and on HD progression (Bobrowska *et al.*, 2012).

Fatty acid oxidation

Fatty acid oxidation is promoted by nutrient deprivation and occurs in the mitochondrial matrix. Peroxisome proliferator-activated receptors (PPARs) and PGC-1α are the main transcriptional regulators of genes involved in this metabolic pathway (Vega et al., 2000). Hypoxia-inducible factor 1 α (HIF-1α) activation in WAT is critical to the development of dietary-induced obesity and associated pathologies, such as insulin resistance. HIF-1α was proposed to have the capacity to suppress fatty acid oxidation by repressing PGC-1α in a SIRT2-dependent manner. Krishman and colleagues showed that inactivation of HIF-1α leads to the accumulation of SIRT2 and reduction of PGC-1α acetylation, promoting fatty

acid oxidation, energy expenditure and mitochondrial biogenesis in WAT (Krishnan *et al.*, 2012). Thus, obesity-induced hypoxia activates HIF-1α, which consequently inhibits SIRT2 deacetylase activity in WAT. By decreasing PGC-1α deacetylation, SIRT2 inhibition leads to its hyperacetylated and suppressed state, which inhibits the expression of genes associated with fatty acid oxidation (Krishnan *et al.*, 2012).

Table 2. Metabolically relevant targets of Sirtuin 2.

Tissue	Target	Biological relevance	References
Adipose tissue	**PGC-1α**	Fatty acid oxidation	(Krishnan *et al.*, 2012)
	FOXO1	Adipogenesis Lipolysis	(Jing *et al.*, 2007; Wang and Tong, 2009)
	Akt	Insulin sensitivity	(Ramakrishnan *et al.*, 2014)
Liver	**TUG**	Insulin sensitivity	(Belman *et al.*, 2015)
	PEPCK1	Gluconeogenesis	(Jiang *et al.*, 2011)
Brain	**SREBP-2**	Cholesterol biosynthesis	(Luthi-Carter *et al.*, 2010)
Mammalian Cells	**ACLY**	Lipogenesis	(Lin *et al.*, 2013)

ACLY, ATP-citrate lyase; Akt, Protein kinase B; FOXO, Forkhead box class O; PEPCK1, Phosphoenolpyruvate carboxykinase 1; PGC-1α, peroxisome proliferator-activated receptor α coactivator-1 α; SREBP-2, Sterol regulatory element-binding protein-1c.

1.2.2 Other relevant roles

Inflammation

Inflammatory processes have been increasingly linked to metabolic disorders. Chronic inflammation seems to play a crucial role in metabolic disorders such as obesity and type 2 diabetes (Wellen and Hotamisligil, 2005; Shoelson *et al.*, 2006; Osborn and Olefsky, 2012). NF-κB and MAPKs are the main regulators of the expression of genes involved in

inflammatory processes. The inflammatory condition reported in peripheral tissues and brain upon metabolic diseases was linked to NF-κB and MAPKs activation (Zhang *et al.*, 2008; Lumeng and Saltiel, 2011). SIRT2 was reported to inhibit inflammation by directly binding and deacetylating the NF-κB p65 subunit, decreasing the expression of NF-κB-regulated inflammatory genes (Rothgiesser *et al.*, 2010). Subsequent studies linked SIRT2 activity to inflammatory protection in brain inflammation (Pais *et al.*, 2013), colitis (Lo Sasso *et al.*, 2014) and arthritis (Lin *et al.*, 2013). Further studies are necessary to clarify the role of SIRT2 in inflammation in the context of metabolic diseases.

Oxidative Stress

Oxidative stress occurs when the production of reactive oxygen species (ROS) overcomes the antioxidant defenses of the cell. Enzymes such as manganese superoxide dismutase (MnSOD) and catalase protect cells against damage caused by ROS. Oxidative stress has been linked to metabolic disorders and aging (Frisard and Ravussin, 2006). Sirtuins play important roles in ROS defense mechanisms, since some of their target proteins, such as FOXO3a, glucose 6-phosphate dehydrogenase (G6PD), phosphoglycerate mutase (PGAM2) and NF-κB, are important regulators of the cellular redox homeostasis (Webster *et al.*, 2012). SIRT2 has been shown to deacetylate and activate FOXO3a, G6PD and PGAM2 in response to oxidative stress, thereby reducing cellular ROS levels (Wang *et al.*, 2007; Wang *et al.*, 2014; Xu *et al.*, 2014). FOXO3a is a transcriptional activator of the SOD2 gene, encoding the mitochondrial MnSOD antioxidant protein (Chen *et al.*, 2013). G6PD is an important enzyme of the pentose phosphate pathway (PPP) and plays an essential role in the oxidative stress response by producing NADH, the main cellular reductant (Wang *et al.*, 2014). Some NF-κB-regulated genes also play important roles in regulating intracellular ROS levels (Morgan and Liu, 2011). In conclusion, SIRT2 may protect against metabolic disorders by enhancing the response to cellular oxidative stress.

2. Metabolic disorders

2.1 Type 2 diabetes

In 2000, 171 million individuals were estimated to have diabetes, and this is expected to increase to 366 million in 2030, being the 7th leading cause of death (Wild et al., 2004). In 1998, type 2 diabetes was already considered to be a major risk factor for cardiovascular diseases and the main cause of lower limb amputations, blindness and renal failure (Kahn, 1998). Insulin resistance is known to be one of the conditions developed during the onset of type 2 diabetes. When insulin resistance is combined with defects in the response of insulin secretion by pancreatic β-cells upon glucose stimulation, this can result in impaired glucose tolerance, hyperglycemia and type 2 diabetes.

2.2 Obesity

Since the beginning of the 20th century, the prevalence of obesity has been increasing at an alarming rate, emerging as a serious health problem worldwide (Newburgh and Johnston, 1930; Koehler, 1934; Lambie, 1935). Between 1980 and 2014, obesity has more than doubled (World Health Organization, 2015) and, by 2030, the number of obese adults was projected to be 573 million (Kelly et al., 2008). Obesity can be defined as a disorder in which occurs an excessive accumulation of fat, leading to severe health problems. Obesity and overweight are important risk factors for metabolic disorders such as diabetes, cardiovascular diseases, cancer and premature death (Kopelman, 2000). In obese individuals, adipose tissue releases increased amounts of fatty acids (Roden *et al.*, 1996), hormones (Scherer, 2006), proinflammatory cytokines (Uysal *et al.*, 1997; Hirosumi *et al.*, 2002) and other factors that are known to be involved in the development of insulin resistance (Kolterman *et al.*, 1980; Kahn *et al.*, 2006).

2.3 Metabolic dysfunction induced by acute HFD feeding

There are several animal models of type 2 diabetes and obesity (King, 2012; Lutz and Woods, 2012), but most of them develop severe phenotypes. Genetic manipulation to create *ob/ob* obese mice and chemically-induced β-cells destruction to mimic diabetes are examples of models with low clinical relevance (King, 2012; Lutz and Woods, 2012). It is important to use reliable and clinically relevant experimental models to allow the study of metabolic diseases in a progressive manner.

HFD-induced models are thought to mimic the development of human metabolic dysfunction more accurately since they consist on an environmental manipulation rather than a genetic or chemical one. The first model of HFD feeding to C57BL/6 mice was described in 1988 (Surwit *et al.*, 1988). This model has been shown to be more efficient in C57BL/6 compared with other strains; thus, it should be noted that the genetic background of the mice can influence the susceptibility to diet-induced metabolic dysfunctions (West *et al.*, 1992; Surwit *et al.*, 1995; Almind and Kahn, 2004). Dietary fat composition seems to have a major role in determining the effects of the HFD (Riccardi *et al.*, 2004; Buettner *et al.*, 2006; El Akoum *et al.*, 2011). Saturated fatty acids (SFA), such as palmitic acid, are more deleterious since they are poorly used for energy production, remaining to be processed into triglycerides (TAG) and other lipid metabolites (such as diacylglycerol and ceramides), whereas unsaturated fatty acids are readily used for energy production (DeLany *et al.*, 2000; Storlien *et al.*, 2001).

Upon long-term feeding (more than 12 weeks), diets rich in saturated fatty acids have long been reported to cause severe metabolic dysfunction, such as atherosclerosis, diabetes and obesity (Buettner *et al.*, 2007). However, for several years, the impact of short-term HFD feeding has remained unknown. Recent studies have reported that acute HFD feeding is able to cause increased adiposity, hyperglycemia, impaired glucose tolerance, insulin and leptin resistance, hyperphagia and hyperleptinemia (Ziotopoulou *et al.*, 2000; Wang *et al.*, 2001; Lee *et al.*, 2011; Turner *et al.*, 2013). These findings were based on hyperinsulinemic-euglycemic clamp technique, measurements of glucose, insulin and leptin levels, glucose

and insulin tolerance tests, assessment of hypothalamic neuropeptide expression and hepatic insulin signaling. These studies highlight that metabolic defects occur before the establishment of severe diseases, such as obesity and type 2 diabetes, and do not require extensive periods of HFD feeding. Short-term HFD studies are able to mimic the early onset of severe metabolic disorders, allowing us to unravel their features and possibly design novel therapeutic approaches.

2.3.1 Insulin resistance

Several studies reported that acute lipid overload to peripheral tissues contributes to the accumulation of FFA and lipid metabolites, such as ceramides and diacylglycerol (DAG). These compounds are able to block the insulin signaling pathway, leading to an insulin resistant state, characterized by hyperglycemia and hyperinsulinemia (Goldstein, 2002; Wilcox, 2005). Insulin resistance is a condition in which a normal concentration of insulin is not able to efficiently perform its physiological effects. This disorder is of great importance because it is considered the pathogenetic link underlying different metabolic abnormalities, such as hypertension, dyslipidemia, type 2 diabetes, atherosclerosis, cardiovascular disease and polycystic ovaries syndrome. Insulin is the most potent anabolic hormone in human body, regulating carbohydrate, lipid and protein metabolism. Upon increased blood glucose levels as a result of food ingestion, insulin increases glucose uptake in skeletal muscle and adipose tissue and decreases hepatic glucose production (HGP) and lipolysis in adipose tissue (Macleod, 1922; Saltiel and Kahn, 2001). Saturated fatty acids, such as palmitate and stearate, but not monounsaturated (oleate and palmitoleate) block insulin-mediated Akt/PKB activation while promoting the accumulation of ceramide and DAG (Chavez *et al.*, 2003).

DAG has been shown to accumulate in insulin-resistant liver (Puri *et al.*, 2007; Kotronen *et al.*, 2009) and to play an important role in the development of insulin resistance (Magkos *et al.*, 2012). DAG activation of the protein kinase C (PKC) has been associated with hepatic insulin resistance. Knocking down PKCε expression in liver protected mice from lipid-induced insulin resistance (Samuel *et al.*, 2007). DAG-activated PKCs inhibit insulin receptor

substrates (IRS) through phosphorylation of serine residues, preventing their activation by insulin through tyrosine residues phosphorylation (Turban and Hajduch, 2011; Jornayvaz and Shulman, 2012). However, it seems that DAG may not play an important role in the onset of insulin resistance in other tissues, such as muscle (Amati *et al.*, 2011; Coen *et al.*, 2013).

Ceramides are mainly produced from long chain fatty acyl-CoAs (Holland and Summers, 2008) and have been reported to be toxic when accumulated in tissue (Adams *et al.*, 2004; Lipina and Hundal, 2011). One recent study reported that ceramides induce hypothalamic lipotoxicity and endoplasmic reticulum (ER) stress, leading to sympathetic inhibition, reduced BAT thermogenesis, and weight gain (Contreras *et al.*, 2014). Increased ceramide levels were detected in mice fed a HFD (Samad *et al.*, 2006). The inverse relationship between ceramides and insulin sensitivity was reported *in vitro* in C2C12 and L6 myotubes and in adipocytes (Summers *et al.*, 1998; Schmitz-Peiffer *et al.*, 1999; Hajduch *et al.*, 2001). Cells treated with SFAs, such as palmitate, have increased levels of ceramides and decreased insulin sensitivity (Schmitz-Peiffer *et al.*, 1999; Powell *et al.*, 2004). Additionally, cells incubated with analogues of ceramides suffer from similar insulin resistance and inhibited glucose transport (Summers *et al.*, 1998; Hajduch *et al.*, 2001). Ceramides were reported to attenuate insulin-stimulated glucose transport and glycogen synthesis by suppressing the insulin-induced phosphorylation of IRS-1 (Kanety *et al.*, 1996) and PI3K (Zundel and Giaccia, 1998). However, some studies showed that ceramides had no effects on the activation of these proteins (Summers *et al.*, 1998; Hajduch *et al.*, 2001). Ceramides were also associated with defects in Akt activation (Teruel *et al.*, 2001; Bourbon *et al.*, 2002; Stratford *et al.*, 2004). Akt activation was shown to be attenuated in muscle cells treated with FFA, being required the conversion of palmitate into ceramides (Schmitz-Peiffer *et al.*, 1999; Chavez and Summers, 2003; Powell *et al.*, 2004). Ceramides inhibit Akt activity either by promoting its dephosphorylation (Salinas *et al.*, 2000; Cazzolli *et al.*, 2001) or by activating atypical PKC (aPKCs), which attenuates the recruitment of Akt to the plasma membrane (Mao *et al.*, 2000; Powell *et al.*, 2003).

2.3.2 Dyslipidemia and oxidative stress

Dyslipidemia, an excessive amount of lipids (e.g. triglycerides and cholesterol) in blood caused by an abnormal lipid metabolism, is known to be induced by HFD feeding (Onody et al., 2003; Yang et al., 2008). Dyslipidemia has been reported to increase oxidative stress and to decrease antioxidant enzyme activity (Slim et al., 1996), and is a major risk factor for cardiovascular disease and obesity. It was reported that the expression of genes for hepatic enzymes involved in fatty acid oxidation were significantly lower in mice fed a HFD for 6 weeks (Yang *et al.*, 2008). Interestingly, the expression of genes for cholesterol biosynthesis was significantly decreased in HFD feeding mice, compared with control mice. Previous studies have been contradictory regarding the levels of triglycerides and cholesterol in blood, upon HFD feeding. Whereas some studies showed that HFD feeding for 6 weeks or cholesterol-rich diets cause hyperlipidemia (Onody *et al.*, 2003; Yang *et al.*, 2006; Yang *et al.*, 2008), several other studies reported decreased levels of triglycerides in mice fed a HFD (Biddinger et al., 2005; Guo et al., 2009; Williams et al., 2014). Oosterveer found that HFD feeding could decrease VLDL production, leading to a decrease in the TG levels carried by these VLDL particles (Oosterveer et al., 2009).

HFD feeding results in significant plasma and hepatic oxidative damage, as characterized by increased plasma malondialdehyde (MDA) concentration and decreased total antioxidant capacity (TAC) and plasma superoxide dismutase (SOD) activities (Yang *et al.*, 2008). Gene transcript levels of ROS scavengers were significantly decreased in HFD mice compared with the controls (Yang *et al.*, 2008). It has been reported that the activation of antioxidant enzymes or treatment with antioxidant agents ameliorate HFD-induced dyslipidemia (Bursill and Roach, 2006; Yang et al., 2006).

2.3.3 Inflammation

HFD feeding induces inflammation in the adipose tissue, liver, skeletal muscle and brain, mainly by activating Toll-like receptor 4 (TLR4) (Fessler *et al.*, 2009; Ji *et al.*, 2012; Wiedemann *et al.*, 2013; Lee *et al.*, 2015; Waise *et al.*, 2015). One previous study revealed

that saturated, rather than unsaturated, fatty acids induce NF-κB activation and expression of COX-2 through TLR4 (Lee *et al.*, 2001). Activation of the canonical NF-κB pathway in liver, mediating inhibitory crosstalk at various steps in insulin signaling pathway, causes hepatic insulin resistance (Baker *et al.*, 2011). In hepatocytes, intracellular accumulation of palmitate and activation of PKCs were reported to activate NF-κB pathway and impact insulin sensitivity (Van Beek et al., 2012). Adipose tissue regulates energy storage and release pro-inflammatory cytokines to report overnutrition to other tissues. Adipocytes from obese mice produce chemokines and cytokines such as IL-6 and TNF-α (Kern *et al.*, 2001). TNF-α is known to impair insulin receptor signaling by impairing its tyrosine kinase activity, thereby decreasing tyrosine phosphorylation of IRS-1, a major endogenous substrate for the activated insulin receptor (Hotamisligil *et al.*, 1996; Peraldi *et al.*, 1996). In contrast, another report indicated that TNF-α decreases expression of IRS-1 and GLUT4 without affecting tyrosine kinase activity of insulin receptor (Stephens *et al.*, 1997). TNF-α also inhibits lipoprotein lipase (LPL) and stimulates lipolysis in adipocytes (Ruan *et al.*, 2002; Ruan *et al.*, 2003) and the resulting increase in circulating FFA would be expected to contribute to insulin resistance.

The inflammatory state induced by HFD feeding plays a critical role in the long-term development of metabolic disorders (Wellen and Hotamisligil, 2005; Lumeng and Saltiel, 2011). However, the role of inflammation on the metabolic dysfunction caused by short-term HFD feeding has been controversial. It was reported that the initial state of HFD-induced insulin resistance is independent on inflammation (Lee *et al.*, 2011). However, recent studies showed that the inflammation occurs early on the course of HFD-induced metabolic dysfunction, in adipose tissue (Ji *et al.*, 2012; Wiedemann *et al.*, 2013) and hypothalamus (Thaler *et al.*, 2012; Waise *et al.*, 2015).

2.3.4 Endoplasmic reticulum stress

Defects in the normal function of the endoplasmic reticulum (ER), and subsequent ER stress, in the hypothalamus have been linked to HFD-induced metabolic stress. ER is responsible for the synthesis, maturation and folding of almost all the signaling proteins

used by cells. ER stress consists on the impaired ER function, which activates the unfolded protein response (UPR), a signaling pathway that promotes decreased protein synthesis and degradation of misfolded proteins (Schroder and Kaufman, 2005; Marciniak and Ron, 2006; Ron and Walter, 2007) Several studies have demonstrated that ER stress plays a key role in the obesity-induced impairment of energy and glucose metabolism in peripheral tissues (Ozcan *et al.*, 2004; Fu *et al.*, 2012). Interestingly, recent studies have reported that HFD-induced inflammation causes ER stress in the hypothalamus, leading to insulin resistance and body weight gain (Hosoi *et al.*, 2008; Zhang *et al.*, 2008; Won *et al.*, 2009; Ropelle *et al.*, 2010).

The precise mechanisms that cause metabolic dysfunction upon short-term HFD feeding remain highly debatable. Initially, several studies suggested that metabolic dysfunction induced by acute HFD feeding is largely due to fat accumulation and insulin resistance in the liver (Samuel *et al.*, 2004; Kleemann *et al.*, 2010; Turner *et al.*, 2013). The role of other peripheral tissues, such as adipose tissue and muscle, was unknown for years, but has been highlighted in some recent studies (Lanthier *et al.*, 2010; Ji *et al.*, 2012). Time- and tissue-specific mechanisms seem to be involved (Kim *et al.*, 2001; Kleemann *et al.*, 2010). Further studies will be required to explore if the acute dietary lipid overload *per se* is responsible for the observed metabolic changes, or if other processes, such as inflammation, are involved and how they work in an integrated manner.

3. Regulation of energy and glucose metabolism

Energy homeostasis is critical for survival and is dependent on the balance between energy consumed and energy expended. Therefore, multiple and complex mechanisms are involved in the regulation of energy intake and energy expenditure to maintain body weight. Energy intake occurs by feeding and energy expenditure in the form of physical activity, basal metabolism and adaptive thermogenesis (Spiegelman and Flier, 2001). Physical activity refers to voluntary movements, while basal metabolism involves the necessary processes to sustain life. Adaptive thermogenesis is the process which dissipates

energy in the form of heat to maintain the body temperature in response to environmental changes, such as cold exposure and HFD feeding (Spiegelman and Flier, 2001). When energy intake exceeds the total energy expenditure in a chronic manner leads to obesity and obesity-associated disorders. The central nervous system plays a crucial role in the regulation of energy metabolism. The central sensing and integration of peripheral nutritional, hormonal and neural signals is a highly complex process and involves several brain regions, from cortex to brain stem (Sandoval *et al.*, 2008). However, the hypothalamus is the primarily responsible for energy homeostasis (Morton *et al.*, 2014).

3.1 Regulation of food intake

Food intake is regulated by the hypothalamus, which responds to whole-body signals. The arcuate nucleus of the hypothalamus (ARC) is close to the circumventricular organ median eminence (ME), a region characterized by an extensive vasculature and weakened blood-brain barrier (BBB). Thus, ARC is the hypothalamic nucleus that is able to primarily sense periphery-derived circulating hormones and nutrients (Broadwell and Brightman, 1976). The ARC regulates food intake through the production of two different types of neuropeptides. Orexigenic neurons produce neuropeptide Y (NPY) and agouti-related peptide (AgRP) and anorexigenic neurons produce proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcripts (CART) (Fig. 2).

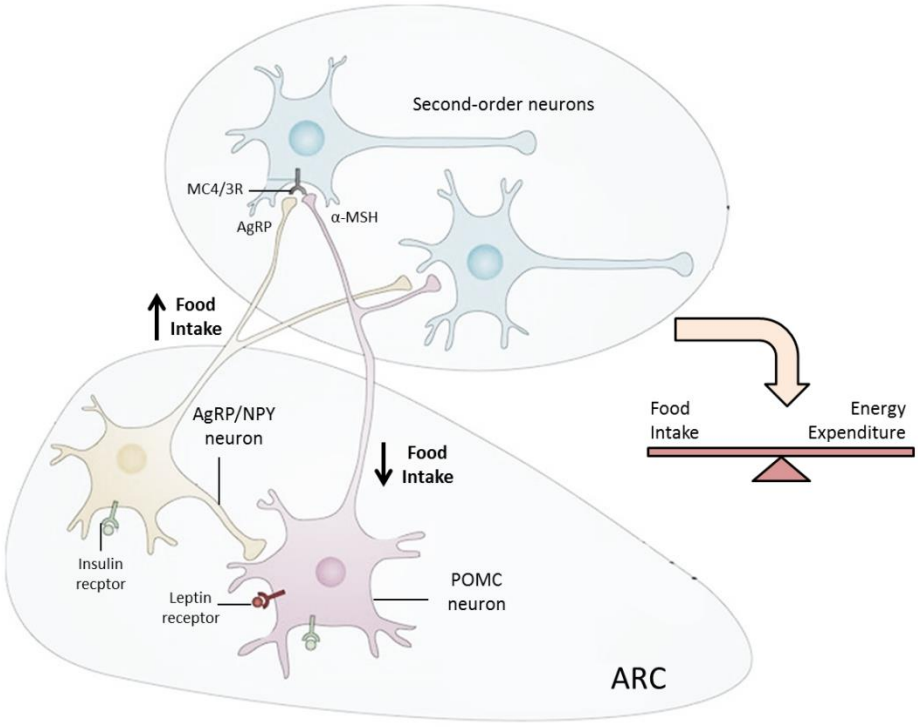

Figure 2. Regulation of energy balance by hypothalamic ARC neurons. Orexigenic neurons produce neuropeptide Y and agouti-related peptide (AgRP) and anorexigenic neurons produce proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcripts (CART). AgRP competes with the POMC cleavage product, α-melanocyte-stimulating hormone (α-MSH), to bind melanocortin-3 and -4 receptors (MC3R and MC4R). ARC neurons sense peripheral signals, such as leptin and insulin, and act on second-order neurons to modulate food intake and energy expenditure. Adapted from Grayson et al. (2013).

Peripheral hormones, such as insulin, ghrelin and leptin primarily act on POMC neurons (Schwartz *et al.*, 2000). POMC neurons project to neurons located in other hypothalamic nuclei, such as the paraventricular nucleus (PVN), lateral hypothalamus (LH) and ventromedial hypothalamus (VMH) and to neurons of other brain regions such as brain stem and spinal cord (Bouret *et al.*, 2004). These second-order neurons have melanocortin-3 and -4 receptors (MC3R and MC4R), the binding site for the POMC cleavage product, α-melanocyte-stimulating hormone (α-MSH). α-MSH acts on MC3R and MC4R receptors to suppress food intake and increase energy expenditure (Balthasar *et al.*, 2005). Inactivation of MC4R by gene targeting resulted in hyperphagia, hyperinsulinemia, hyperglycemia and obesity (Huszar *et al.*, 1997). In human, mutations in MC4R are also associated with obesity

(Tao, 2005). These studies suggested a pivotal role for the melanocortin system in the regulation of body weight (Fan *et al.*, 1997; Benoit *et al.*, 2002). The orexigenic neuropeptide AgRP, produced by NPY/AgRP neurons, compete with α-MSH for MC3R and MC4R binding, counteracting the α-MSH-induced food intake suppression (Ollmann *et al.*, 1997). Moreover, within the ARC, orexigenic neurons provide inhibitory GABAergic projections to anorexigenic POMC neurons (Cowley *et al.*, 2001). The postembryonic AgRP/NPY neurons deficiency leads to hypophagia and lean mice phenotype (Bewick *et al.*, 2005). NPY is also one important regulator of feeding behavior and energy homeostasis (Yulyaningsih *et al.*, 2011). Thus, NPY/AgRP neurons seem critical to induce food intake and body weight gain.

Second-order neurons from the PVN are involved in the regulation of peripheral metabolism, such as the fatty acid oxidation and lipolysis (Foster *et al.*, 2010). These actions are mediated by the production of neuropeptides and through sympathetic innervation.

The brain stem is a key brain region linking peripheral metabolism and the hypothalamic regulation of food intake. The nucleus tractus solitarius (NTS) receive signals from the gastro-intestinal tract through the vagus nerve, the main responsible for the gut-brain axis. The hypothalamus is intimately connected with the brain stem through projections from the PVN to NTS, and vice versa (Geerling *et al.*, 2010). In conclusion, both the hypothalamus and the brain stem sense and integrate peripheral metabolic signals to regulate food intake and body weight.

3.2 Regulation of energy expenditure

Whole-body energy expenditure occurs by basal metabolic rate, physical activity and adaptive thermogenesis. Thermogenesis occurs mainly in BAT upon cold exposure to dissipate energy as heat and upon HFD feeding to regulate body weight (Westerterp, 2004). BAT is innervated by the sympathetic nervous system (SNS) and the SNS-derived noradrenaline further activates G-protein-coupled β-adrenergic receptors that lead to the activation of UCP1 (Sell *et al.*, 2004). UCP1 is present in the inner membrane of the mitochondria where it uncouples the mitochondrial proton motive force from ATP

production to energy release as heat (Spiegelman and Flier, 2001). White adipocytes can be converted into brown-like adipocytes through a process called WAT browning that increases energy expenditure. Insulin and leptin act on hypothalamic POMC neurons to promote WAT browning, and consequently body weight loss (Dodd *et al.*, 2015). Prdm16 is a transcriptional coregulator involved in the normal development of BAT and its transgenic expression in WAT adipocytes induces browning, showing that Prdm16 is a critical mediator of adaptive thermogenesis in WAT. Prdm16 transgenic mice fed a HFD showed increased energy expenditure, reduced weight gain and improved glucose tolerance (Seale et al., 2011). Hypothalamic regions also modulate thermogenesis through the SNS (Morrison *et al.*, 2014; Seoane-Collazo *et al.*, 2015). The hypothalamus also regulates physical activity (Spiegelman and Flier, 2001). The transforming growth factor-α (TGF-α) was identified as an inhibitor of locomotion. This action was reported to be dependent on hypothalamic epidermal growth factor (EGF) receptors (Kramer *et al.*, 2001). LH neurons-derived orexin was suggested to promote locomotor activity (Samson *et al.*, 2010). POMC neurons were also reported to stimulate physical activity upon leptin stimulation (Huo *et al.*, 2009).

3.3 Regulation of glucose metabolism

Plasma glucose concentration is normally tightly regulated by hormonal and neuronal signals to achieve glucose homeostasis. In mammalian cells, glucose metabolism is the main responsible for energy production and substrate storage (Bouche et al., 2004). Then, a continuous supply of glucose is necessary, especially for the nervous system that fails to store glucose or use other substrates as fuel (Mergenthaler *et al.*, 2013).

Blood glucose is derived from intestinal absorption from diet or from endogenous production in liver and kidney (Bouche *et al.*, 2004) and both insulin-dependent and insulin-independent processes are responsible for blood glucose levels regulation. Glucose transporters (GLUT) are required to facilitate the glucose entry into cells. GLUT4 mediates insulin-dependent glucose uptake by insulin-sensitive tissues such as skeletal muscle and adipose tissue. In contrast, insulin-independent glucose transporters (e.g. GLUT1, 2 and 3)

are responsible for glucose translocation into insulin-independent organs, such as liver and brain.

Once inside the cell, glucose can undergo storage in the form of glycogen, oxidation or conversion into other substrates with metabolic relevance (such as acetyl-CoA, to produce fatty acids through lipogenesis). Glycogen synthesis (glycogenesis) is the main form of glucose storage and occurs mainly in the liver and skeletal muscle. The liver is able to export glycogen to maintain constant blood glucose levels and in the muscle, glycogen provides energy for contraction. Glucose oxidation into pyruvate (glycolysis) occurs in all living cells and pyruvate can be further oxidized in the Krebs Cycle, under aerobic conditions, or undergo gluconeogenesis. Gluconeogenesis is the process of glucose production from non-carbohydrate carbons, such as lactate and pyruvate, and occurs mainly in the liver to restore glucose levels during fasting. Glycogenolysis (glycogen breakdown) in the liver also provides glucose during fasting or exercise, on the other hand, muscle glycogen just fuels muscle cells.

Insulin and glucagon, both produced in the pancreas by islets of Langerhans, are the main regulators of glucose metabolism (Triplitt, 2012). Insulin, produced by β-cells, reduces blood glucose levels by enhancing glucose uptake in insulin-sensitive tissues, suppressing HGP and promoting glycogen synthesis and lipogenesis. Glucagon, produced by α-cells, is produced in response to low levels of blood glucose and induces an increase in glucose levels by promoting glycogenolysis and gluconeogenesis (Triplitt, 2012).

The hypothalamic regulation of glucose metabolism was established by the discovery of glucose-sensing neurons in the hypothalamus (Anand *et al.*, 1964; Oomura *et al.*, 1969). Glucose-sensing neurons are excitable upon changes in extracellular glucose concentrations. Glucose-excited neurons are stimulated by increased levels of glucose and (Dunn-Meynell *et al.*, 1998) and glucose-inhibited neurons respond to a fall in the extracellular glucose levels (Routh, 2002). Apart from the hypothalamus, also the brain stem plays an important role in the glucose homeostasis, containing both glucose-excited and –inhibited neurons (Mizuno and Oomura, 1984; Funahashi and Adachi, 1993; Yettefti *et al.*, 1997). Interestingly, these two brain regions are also crucial in the regulation of energy

balance, as have been shown above. Several other nutritional and hormonal signals, such as insulin, leptin and fatty acids, are sensed by the brain to modulate the energy and glucose balance, in order to respond to the whole-body needs.

4. Objectives

SIRT2 has emerged as an important regulator of mammalian metabolism, being particularly expressed in metabolically relevant tissues, such as adipose tissue, liver and brain. SIRT2 targets several proteins involved in key processes of lipid and glucose metabolism, such as adipogenesis, insulin sensitivity and gluconeogenesis. Previous studies in obese subjects and mice fed a high fat diet (HFD) have reported that SIRT2 is downregulated in WAT (Krishnan *et al.*, 2012). In contrast, calorie restriction in mice was demonstrated to increase SIRT2 levels in WAT and kidney (Wang *et al.*, 2007). In humans, a hypocaloric diet increased SIRT2 expression in peripheral blood mononuclear cells (Crujeiras *et al.*, 2008). Previous studies from our group showed that SIRT2 is also expressed in the hypothalamus, the main central regulator of energy and glucose metabolism. Hypothalamic SIRT2 expression was downregulated in a mouse model of HFD-induced obesity and after administration of palmitate. Thus, SIRT2 expression seems to play an important role during metabolic stress conditions, being modulated by energy availability.

Taking into account these findings, the major aim of the present work was to study the physiological role of SIRT2 in whole-body metabolic homeostasis. For that purpose, we investigated the effect of SIRT2 deficiency on whole-body energy and glucose metabolism, by using a whole-body SIRT2-KO mouse model. The metabolic characterization was performed under a normal condition of chow diet (CD) feeding and in a condition of metabolic stress induced by short-term HFD feeding. We were interested in studying the role of SIRT2 in the early onset of metabolic disorders (e.g. obesity and type 2 diabetes) in order to identify potential therapeutic targets to counteract the progression of these disorders. To achieve the above mentioned goals, this work was divided in two main aims:

- Metabolic characterization of SIRT2-KO mice under a normal condition of CD feeding;
- Metabolic characterization of SIRT2-KO mice under a condition of metabolic stress induced by short-term HFD feeding.

Chapter II

Materials and Methods

1. Experimental animals and diets

Seven week-old male wild-type (WT) C57BL/6 mice were purchased from Charles River Laboratories. B6.129-Sirt2$^{tm1.1Fwa}$ (SIRT2-KO) mice were purchased from The Jackson Laboratory (Ref. 012772). SIRT2-KO mice were generated in B6.129 genetic background and backcrossed to C57BL/6 for 8 generations to produce homozygous SIRT2$^{-/-}$ mice. A colony of these SIRT2-KO mice was established at the Centre for Neuroscience and Cell Biology of the University of Coimbra. WT and SIRT2-KO mice were age- and sex-matched.

Animals were maintained in a temperature-controlled room with 12h light/12h dark cycles. Mice were housed in groups of 2-4 animals and had free access to food and water. The experiments were carried out in accordance with the European Community directive (2010/63/EU) for the care and use of laboratory animals. Animal experiments were guided by researchers who received adequate training (FELASA certified course) and certification from Portuguese authorities (Direção Geral de Veterinária).

SIRT2-KO mice were subjected to a standard chow diet (CD) (4RF21, Mucedola, Italy) until the beginning of the dietary protocol. WT mice were fed the same CD for a 10 days acclimation period. During the 31-days dietary protocol mice were fed either a CD or HFD (Table 3), the most widely used to induce obesity and metabolic dysfunction (D12492, Research Diets, US). The HFD is composed of 60% Kcal from fat and lard-enriched.

Table 3. Normal chow diet (CD) and high fat diet (HFD) composition.

Content			**CD**	**HFD**
Proteins			18.5%	20%
Carbohydrates			54%	20%
Fats	Soybean oil		3%	5%
	Lard		-	55%
	of which	Saturated FA	21%	32%
		Unsaturated FA	79%	68%

Energy (Kcal/g)	2.67	5.24

2. Metabolic phenotyping

Mice began the 31-days dietary protocol with, on average, 8 weeks of age. The intraperitoneal glucose and insulin tolerance tests (ipGTT and ipITT) were performed on days 24 and 28, respectively. Tests were separated at least 3 days to allow mice to recover. After the final test (ipITT), mice were allowed to recover for 3 days and subsequently sacrificed for blood and tissue collection.

2.1 Body weight and food intake assessment

Food intake and body weight were assessed twice a week during the 31 days of the study. However, only the records corresponding to the first 23 days are presented, since the procedures performed in the rest of the study had an impact on body weight. Food intake per mouse was calculated dividing food intake by the number of mice in the cage.

2.2 Intraperitoneal glucose tolerance test (ipGTT)

Mice were fasted overnight for 15-16 hours (6 p.m to 9/10 a.m) by transferring them to clean cages without food or faeces. Basal blood glucose levels were measured from the tail vein before intraperitoneal glucose injection (1.5 g D-glucose/Kg of body weight). Blood glucose levels were measured at minutes 15, 30, 60, 90 and 120 following glucose load, and glucose concentration was determined with a glucose meter (Free Style Precision Neo glucometer, Abbott). During ipGTT mice were singly housed. Area under the curve (AUC) of the ipGTT was calculated using the trapezoidal rule.

2.3 Intraperitoneal insulin tolerance test (ipITT)

Mice were fasted for 5-6 hours (9 a.m to 2/3 p.m) and singly housed for ipITT. Blood glucose levels were measured before insulin injection (0.75 U/Kg of body weight) (Humalog, Lilly), and at minutes 15, 30, 60, 90 and 120 after injection, using the Free Style Precision Neo glucometer (Abbott). Area under the curve (AUC) was calculated by using the trapezoidal rule.

2.4 Insulin stimulation and tissue collection

Mice were fasted overnight and injected intraperitoneally with insulin (2U/Kg of body weight) (Humalog, Lilly) or saline. Mice were anesthetized with isoflurane and sacrificed by cervical dislocation, 30 minutes after insulin stimulation. Tissues were rapidly dissected, weighed and immediately frozen in liquid nitrogen. Tissues were stored at -80ºC for later analysis.

2.5 Cholesterol and triglycerides quantification

After sacrifice, trunk blood was collected into tubes without anticoagulant (BIOplastics B74085), for serum separation. Serum cholesterol and triglyceride levels were quantified by Integra 800 (Roche) in a clinical analysis laboratory (Aeminium, Coimbra).

2.6 Insulin signaling

2.6.1 Protein extraction and quantification

Tissues were lysed with radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 5 mM EDTA; 1% (v/v) Triton X-100; 0.5 % (w/v) deoxycholate; 0.1 % (w/v) sodium dodecyl sulphate (SDS); protease inhibitors cocktail (Roche Diagnostics); 1 mM sodium orthovanadate; 1 mM dithiothreitol (DTT); 10 mM sodium fluoride (NaF); 200 µM phenylmethylsulfonylfluoride (PMSF)). Tissue lysates were prepared by mechanical

disruption, using plastic potters, and followed by sonication (4 pulses of 3 seconds each). Lysates were centrifuged at 15.700 rpm during 20 minutes at 4°C. Protein concentration was determined by the bicinchoninic acid (BCA) method. The samples were denatured with 6x concentrated sample buffer (0.5 M Tris-HCl, pH 6.8; 30% (v/v) glycerol; 10.4 % (w/v) SDS; 0.6 M DTT; 0.012% (w/v) bromophenol blue) and heated at 95ºC for 5 min.

2.6.2 Western blotting

Proteins were resolved by SDS–PAGE using 10% polyacrylamide gels. Electrophoresis was run on a Tris-Bicine buffer (25 mM Tris; 25 mM Bicine; 1% (w/v) SDS; pH 8.3), at 70 V in the first 10 minutes and at 120 V until an appropriate separation of the molecular weight standards. Proteins were electro-transferred to PVDF membrane in CAPS transfer buffer (10 mM CAPS, pH 11.0; 10% (v/v) methanol) upon a constant current of 750 mA, during 1.5 hours. Membranes were blocked for 1 hour at room temperature with blocking buffer (5% non-fat dry milk or 5% bovine serum albumin in Tris buffered saline (20 mM Tris; 137 mM NaCl; pH7.6) with 0.1% TWEEN-20. Membranes were incubated overnight at 4ºC with the primary antibody: rabbit polyclonal anti-pAKT (Ser 473) (1:1000; Cell Signalling 9271) or rabbit polyclonal anti-Akt (1:1000; Cell Signalling 9272), followed by 1-hour incubation with the corresponding alkaline phosphatase-linked secondary goat anti-rabbit antibody. Proteins were visualized with enhanced chemifluorescence substrate (ECF, GE Healthcare) and scanned by VersaDoc Imaging System Model 3000 (BioRad). Bands quantification was performed using Quantity One Version 4.6.3 (Bio Rad).

2.7 Statistical analysis

Results are expressed as mean ± SEM. Statistical analyses were performed with unpaired Student's t-test. Statistical analysis was performed using GraphPad Prism Software. Statistical significance is displayed as * ($p \leq 0.05$), ** ($p \leq 0.01$).

Chapter III

Results

1. Metabolic phenotyping of SIRT2-KO mice fed a chow diet (CD)

1.1 SIRT2-KO mice exhibit a similar body weight gain as WT mice

Taking into account the increasing evidence that SIRT2 plays an important role in metabolically relevant tissues, we aimed to study the effect of SIRT2 deficiency on whole-body metabolic homeostasis using SIRT2-KO mice (subsequently referred to as KO).

In order to assess the effect of SIRT2 deficiency on the energy and glucose homeostasis upon a normal condition of CD feeding, WT and KO mice were subjected to a metabolic phenotyping protocol during 31 days (Fig. 3A). The average initial weight of WT (23.61 ± 0.62 g) and KO (23.03 ± 0.99 g) mice was similar (p=0.6272). The body weight gain curve of KO mice was identical to that of WT mice (Fig. 3B). Consistently, no differences were observed in the total body weight gain after 23 days of CD feeding (Fig. 3C).

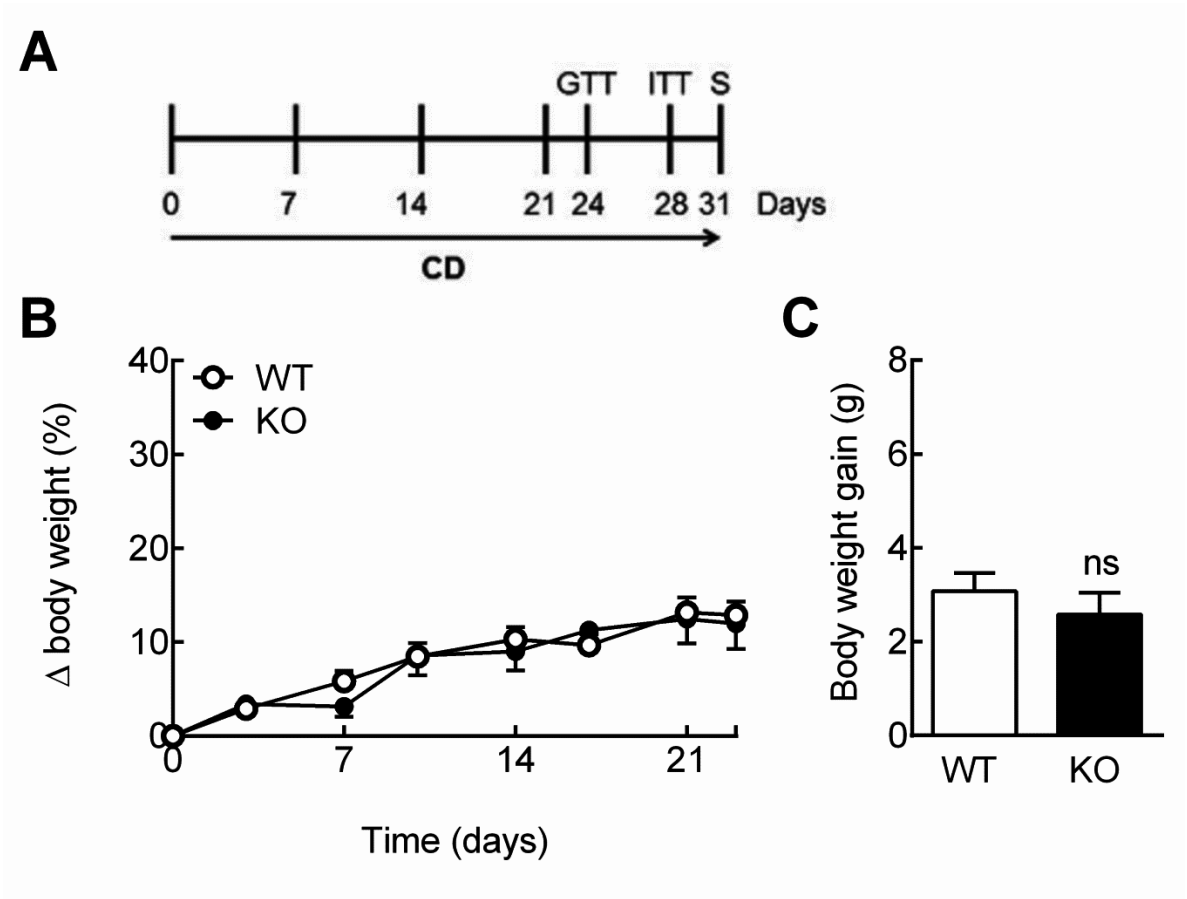

Figure 3. Body weight evolution of WT and KO mice during 23 days of Chow diet (CD). **(A)** Experimental schedule of the metabolic phenotyping under CD. Mice began the dietary protocol with 8 weeks of age. GTT: Intraperitoneal glucose tolerance test, ITT: intraperitoneal insulin tolerance test, S: Sacrifice. **(B)** Curve of body weight gain (%) during 23 days of CD feeding. Results were normalized to the initial body weight. **(C)** Total body weight gain (g) after 23 days of CD feeding. Results are presented as mean ± SEM; n = 6-8/group. Statistical significance was determined by the unpaired Student's t-test (ns=non significant compared to WT).

1.2 Weight of adipose tissue depots of SIRT2-KO mice is similar to WT mice

After 31 days of CD feeding, mice were sacrificed and metabolic tissues were collected and weighed. In line with the results of the body weight gain, no significant differences were observed in the weight of gonadal white adipose tissue (gWAT) between WT and KO mice (Fig. 4). Brown adipose tissue (BAT) had also similar weight in both genotypes (Fig. 4). The average liver weight of KO mice weighed 30.0 ± 5.1 % less than that of WT mice (Fig. 4). These findings showed that SIRT2 deficiency did not affect adiposity.

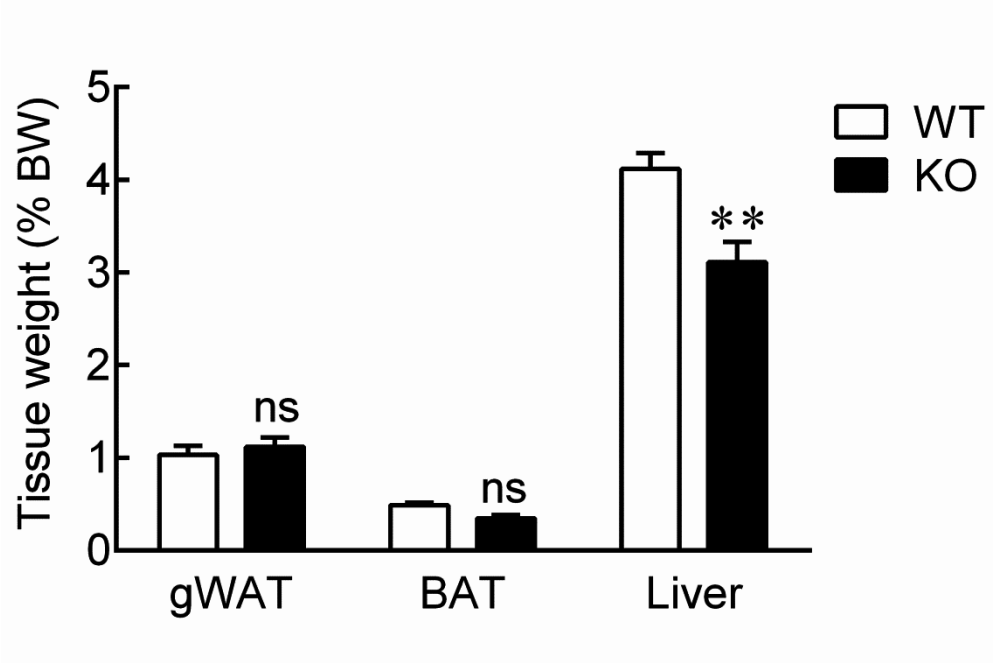

Figure 4. Tissues weight of WT and KO mice, after 31 days of CD feeding. Tissue weight was normalized to body weight (% BW). gWAT: gonadal weight adipose tissue, BAT: brown adipose tissue. Results are presented as mean ± SEM; n = 6-8/group. Statistical significance was determined by the unpaired Student's t-test (** $p \leq 0.01$; ns=non significant compared to WT).

1.3 Food and energy intake of SIRT2-KO mice are similar to WT mice

To investigate the role of SIRT2 on food and energy intake we monitored the consumption of CD over 23 days in WT and KO mice. The cumulative energy intake of KO mice over time was indistinguishable from that of WT mice (Fig. 5A). No significant differences were observed in the total energy intake of WT and KO mice (Fig. 5B), since both groups showed similar food intake (Fig. 5C).

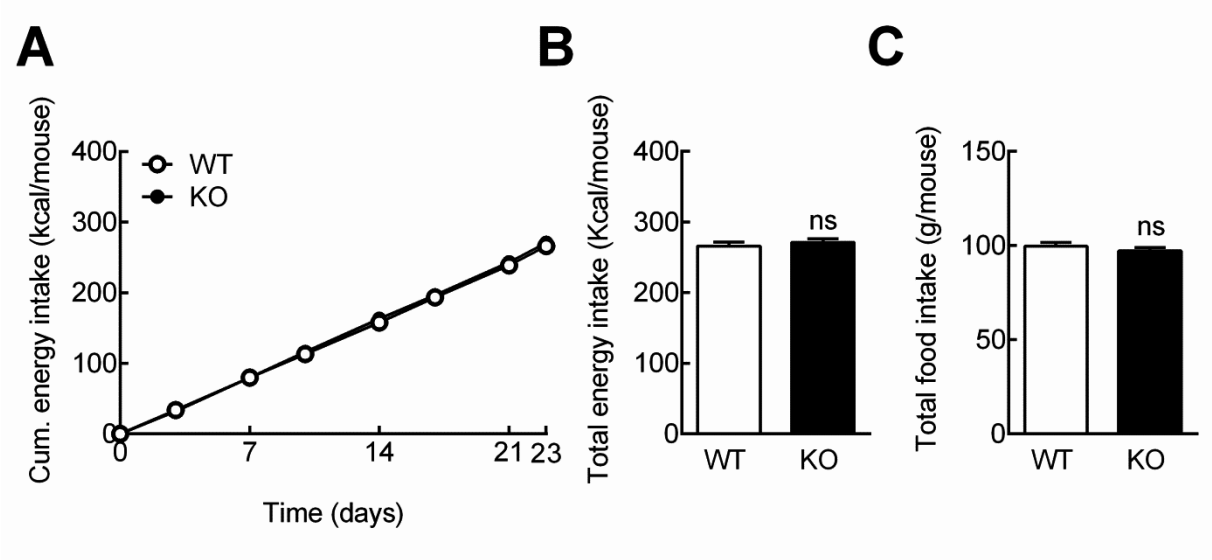

Figure 5. Food and energy intake of WT and KO mice during 23 days of CD feeding. **(A)** Cumulative (Cum) energy intake over 23 days. **(B)** Total energy intake (Kcal) and **(C)** total food intake (g) after 23 days of CD feeding. Results are presented as mean ± SEM; n = 6-8/group. Statistical significance was determined by the unpaired Student's t-test (ns=non significant compared to WT).

1.4 SIRT2-KO mice have fasting blood glucose levels similar to WT mice

In order to evaluate the role of SIRT2 in glucose metabolism in a normal condition of CD feeding, we measured the blood glucose levels of mice fasted for 15 hours. Fasting blood glucose levels were similar in both genotypes fed a CD (Fig. 6).

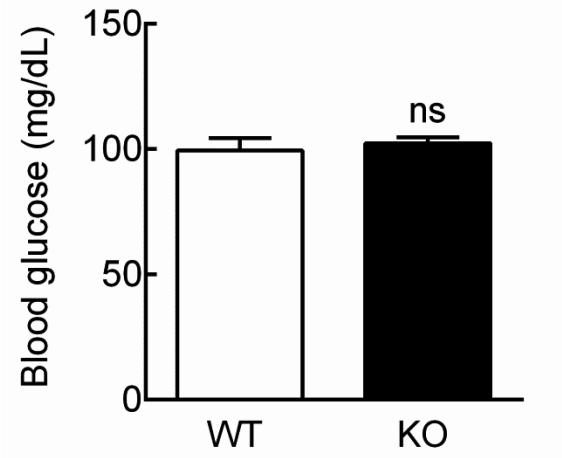

Figure 6. Blood glucose levels of WT and KO mice after 24 days of CD feeding. Glucose levels were measured after 15 hours of fasting. Results are presented as mean ± SEM; n = 6-8/group. Statistical significance was determined by the unpaired Student's t-test (ns=non significant compared to WT).

1.5 SIRT2 KO mice have similar glucose tolerance as WT mice

To further evaluate the role of SIRT2 in glucose metabolism of mice fed a CD, we performed an ipGTT in WT and KO mice, in order to assess their glucose clearance rates. KO mice responded to the glucose injection similarly to control mice (Fig. 7A). Consistently, the area under the curve (AUC) showed similar values in animals of both genotypes (Fig. 7B). These findings indicate that SIRT2 deficiency does not affect blood glucose clearance of mice fed a CD. These data is consistent with the blood glucose levels, showing that SIRT2 deficiency does not affect glucose homeostasis under a normal condition of CD feeding.

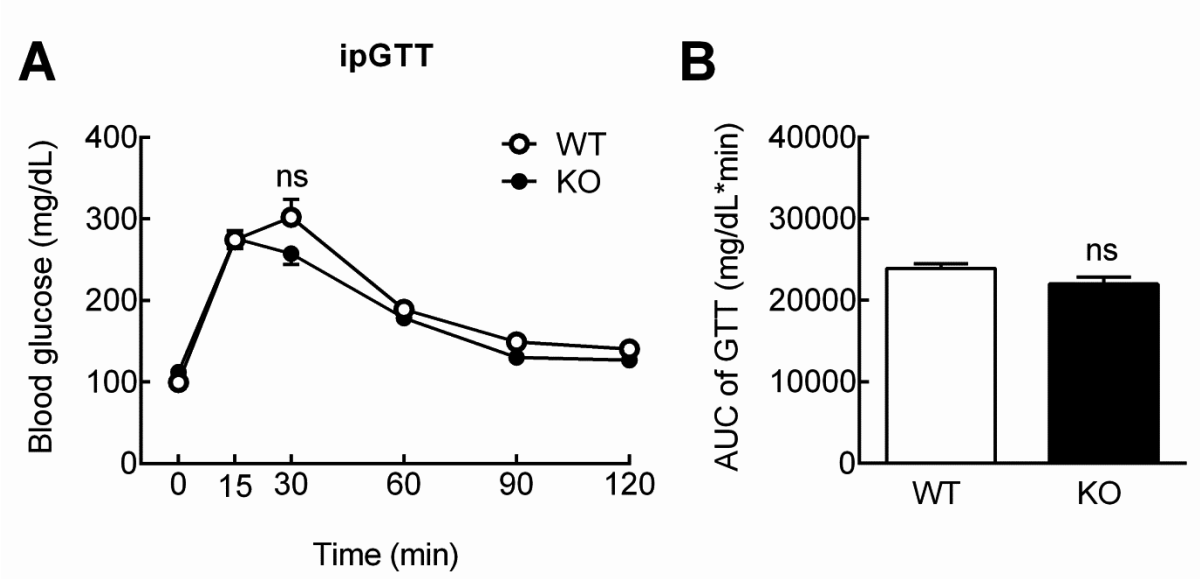

Figure 7. Intraperitoneal glucose tolerance test (ipGTT) was conducted in WT and KO mice after 24 days of CD feeding, and the area under the curve (AUC) calculated. (A) Blood glucose levels of WT and KO mice at different time points after intraperitoneal injection of D-glucose (1.5 g glucose/kg body weight). (B) Average AUC of the ipGTT curves. AUC was calculated using the trapezoidal rule. Results are presented as mean ± SEM; n = 6-8/group. Statistical significance was determined by the unpaired Student's t-test (ns=non significant compared to WT).

1.6 Insulin sensitivity is affected in SIRT2-KO mice

To assess the impact of SIRT2 deficiency on the response to insulin, an ipITT was performed and the rates of insulin-induced glucose excursion after an insulin injection evaluated. KO mice showed a slight trend towards insulin resistance compared to WT mice during the ipITT, although only the 15 minutes time point reached statistical significance (p=0.014) (Fig. 8A). Consistently, no difference was observed in the area under the curve (AUC) of the complete ipITT curves between genotypes (Fig. 8B). However, the AUC of the initial phase (first 60 minutes) of the ipITT test revealed significantly increased values in KO mice compared to control mice (Fig. 8C). These data suggest a lower rate of glucose excursion in KO mice fed a CD, indicating that SIRT2 deficiency may lead to insulin resistance.

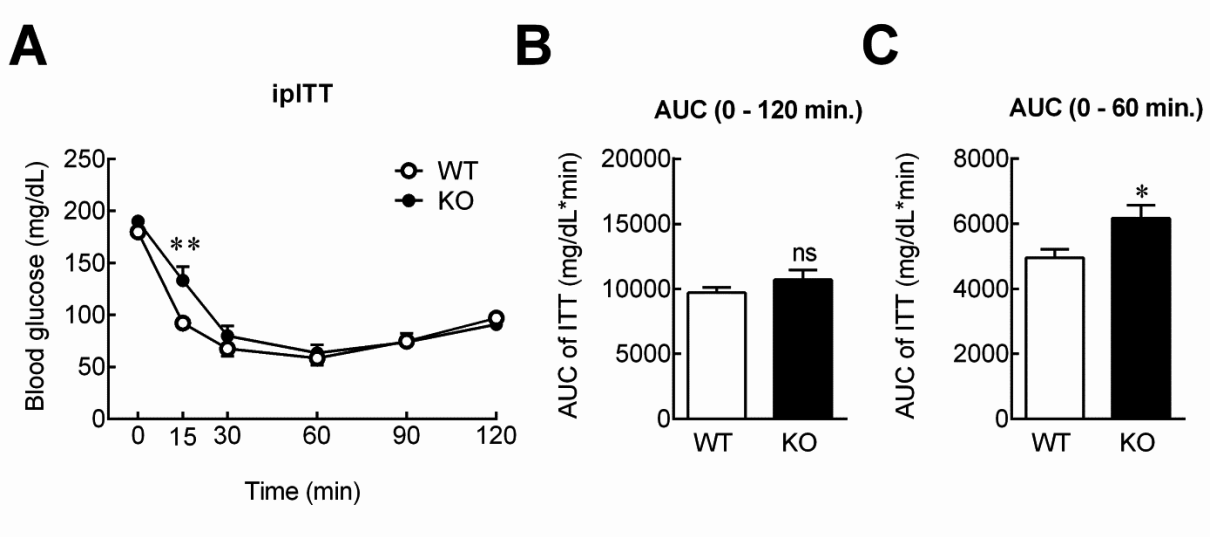

Figure 8. WT and KO mice were subjected to intraperitoneal insulin tolerance test (ipITT) after 28 days of CD feeding and the area under the curve (AUC) calculated. **(A)** Blood glucose levels of WT and KO mice at different time points after i.p injection of human insulin (0.75 U per kg of body weight). **(B)** Average AUC of the whole ipITT curves (from 0 to 120 minutes time point). **(C)** Average AUC of the initial 60 minutes of the ipITT curves. AUC was calculated using the trapezoidal rule. Results are presented as mean ± SEM; n = 7-8/group. Statistical significance was determined by the unpaired Student's t-test (ns=non significant; * $p \leq 0.05$; ** $p \leq 0.01$ compared to WT).

1.7 SIRT2-KO mice have normal liver insulin signaling

To investigate the effect of SIRT2 deficiency on insulin signaling, we examined changes of the insulin signaling pathway in the liver of WT and KO mice fed a CD. To this end, we assessed Akt phosphorylation at serine 473 (P-Akt^{473}) in tissues obtained from mice fasted for 15 hours and injected with 0.9% saline or insulin (2U/Kg of body weight) for 30 minutes (Fig. 9A). We found a 12-fold increase in P-Akt in livers from mice stimulated with insulin, in comparison with livers from saline-administered animals. However, the insulin-induced Akt activation in KO mice was indistinguishable from that observed in control mice (Fig. 9B). These results suggest that SIRT2 deficiency has no impact on the molecular response to insulin, at least in the liver.

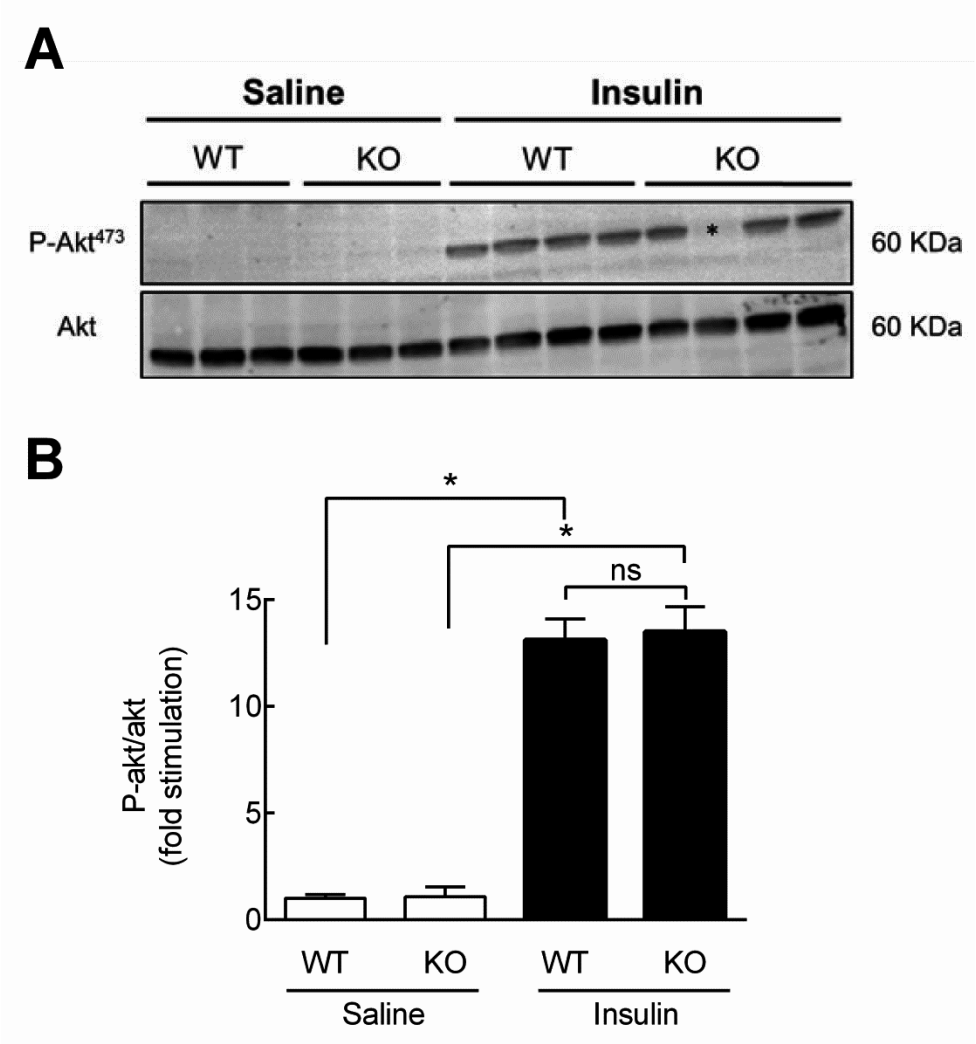

Figure 9. Liver insulin signaling in WT and KO mice fed a CD for 31 days. P-Akt phosphorylation was used as a readout for insulin signaling pathway activation **(A)** Representative immunoblot showing Akt phosphorylation in the liver lysates of WT and KO mice upon saline or insulin injection. **(B)** Quantification of P-Akt/Akt ratios. Levels of P-Akt were normalised to total Akt protein in the same lane. All P-Akt/Akt ratios were normalised to the value from WT mice injected with saline. Mice were fasted for 15 hours, intraperitoneally injected with 0.9% saline or human insulin (2U/Kg body weight) and sacrificed after 30 minutes. Each lane correspond to a distinct animal. * This lane was not quantified. Results are presented as mean ± SEM; n = 3-4/group. Statistical significance was determined by the unpaired Student's t-test (ns=non significant compared to WT; * $p \leq 0.05$ compared to saline).

1.8 SIRT2-KO mice have cholesterol levels similar to WT mice

In order to evaluate the role of SIRT2 in lipid metabolism under a normal condition of CD feeding, we measured serum cholesterol levels of mice fasted for 15 hours. Fasting cholesterol levels were similar in both genotypes fed a CD (Fig. 10).

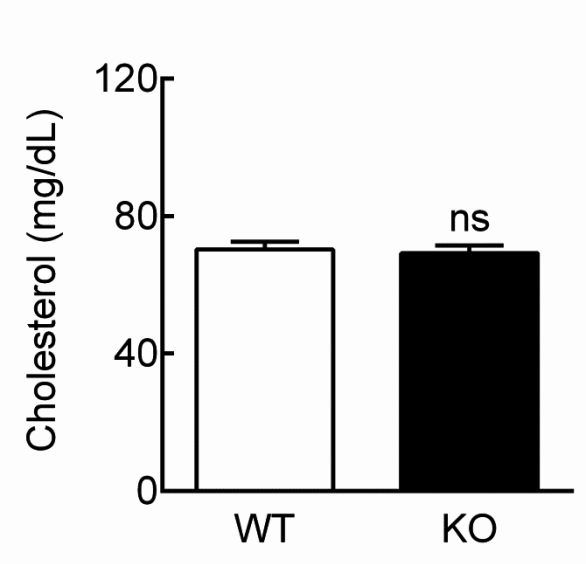

Figure 10. Serum cholesterol levels of WT and KO mice after 31 days of CD feeding. Cholesterol levels were measured after 15 hours of fasting. Results are presented as mean ± SEM; n = 8/group. Statistical significance was determined by the unpaired Student's t-test (ns=non significant compared to WT).

1.9 SIRT2-KO mice have triglyceride levels similar to WT mice

To assess the impact of SIRT2 deficiency in triglyceride levels under a normal condition of CD feeding, we measured serum triglyceride levels. Serum triglyceride levels were similar in both genotypes (Fig. 11), what is consistent with the serum cholesterol levels suggesting that SIRT2 does not play an important role in lipid metabolism under a CD feeding.

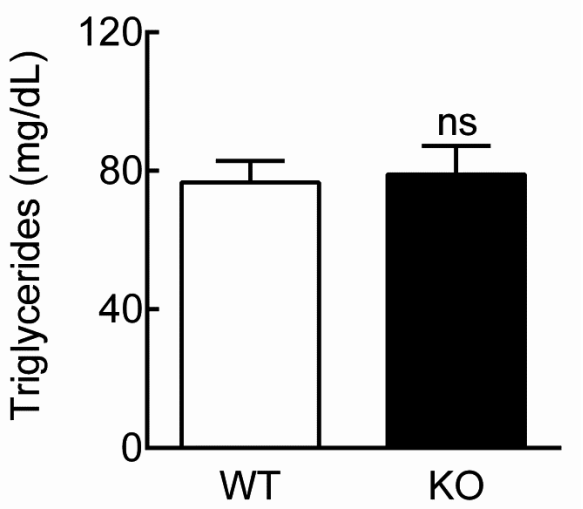

Figure 11. Serum triglyceride levels of WT and KO mice after 31 days of CD feeding. Triglyceride levels were measured after 15 hours of fasting. Results are presented as mean ± SEM; n = 3-4/group. Statistical significance was determined by the unpaired Student's t-test (ns=non significant compared to WT mice).

2. Metabolic phenotyping of SIRT2-KO mice under high fat diet (HFD)

2.1 SIRT2 KO mice have higher body weight gain compared to WT mice

Since no marked metabolic phenotype was observed in KO mice fed a CD, we challenged mice with an HFD to investigate the impact of SIRT2 deficiency on the metabolic homeostasis under conditions of metabolic stress. A similar metabolic phenotyping protocol was performed during 31 days of HFD feeding (Fig. 12A). WT mice on a HFD gained significantly more body weight than CD-fed WT mice (Table 4), indicating that HFD feeding for 23 days is able to induce increased body weight gain.

Table 4. Summary of the metabolic phenotyping of WT mice fed a chow diet (CD) or a high fat diet (HFD).

Metabolic phenotyping of WT mice		
Parameter	**CD**	**HFD**
Body weight gain (g)	3.08 ± 0.39	4.73 ± 0.52*
gWAT weight (g)	0.26 ± 0.03	0.52 ± 0.09*
Liver weight (g)	1.04 ± 0.06	0.86 ± 0.03*
Total energy intake (Kcal/mouse)	266.0 ± 5.2	337.0 ± 0.7**
Total food intake (g/mouse)	99.6 ± 1.9	64.3 ± 0.1**
Fasting blood glucose(mg/dL)	99.4 ± 5.0	116.6 ± 3.7*
AUC GTT (mg/dL*min)	23916 ± 574	27668 ± 1158**
AUC ITT (mg/dL*min)	9700.50 ± 392.85	11308.14 ± 310.20*
Serum cholesterol (mg/dL)	70.3 ± 2.2	95.0 ± 8.2*
Serum triglycerides (mg/dL	76. 7 ± 6.1	84.3 ± 4.5

Parameters were assessed according to the standardized phenotyping protocol. Results are presented as mean ± SEM; n = 6-8/group. Statistical significance was determined by the unpaired Student's t-test (* $p \leq 0.05$;** $p \leq 0.01$ compared to CD).

WT and KO mice had similar initial weights (24.38 ± 0.37 g and 22.91 ± 1.37 g, respectively; p=0.2908). From day 14 onwards, the curve of body weight gain of KO mice was significantly higher than that of control mice (Fig. 12B). After 23 days of HFD feeding the total body weight gain of mice lacking SIRT2 was 50.4 ± 13.3 % higher than that of control mice (Fig. 12C), indicating that SIRT2 deficiency promotes body weight gain in mice fed a HFD.

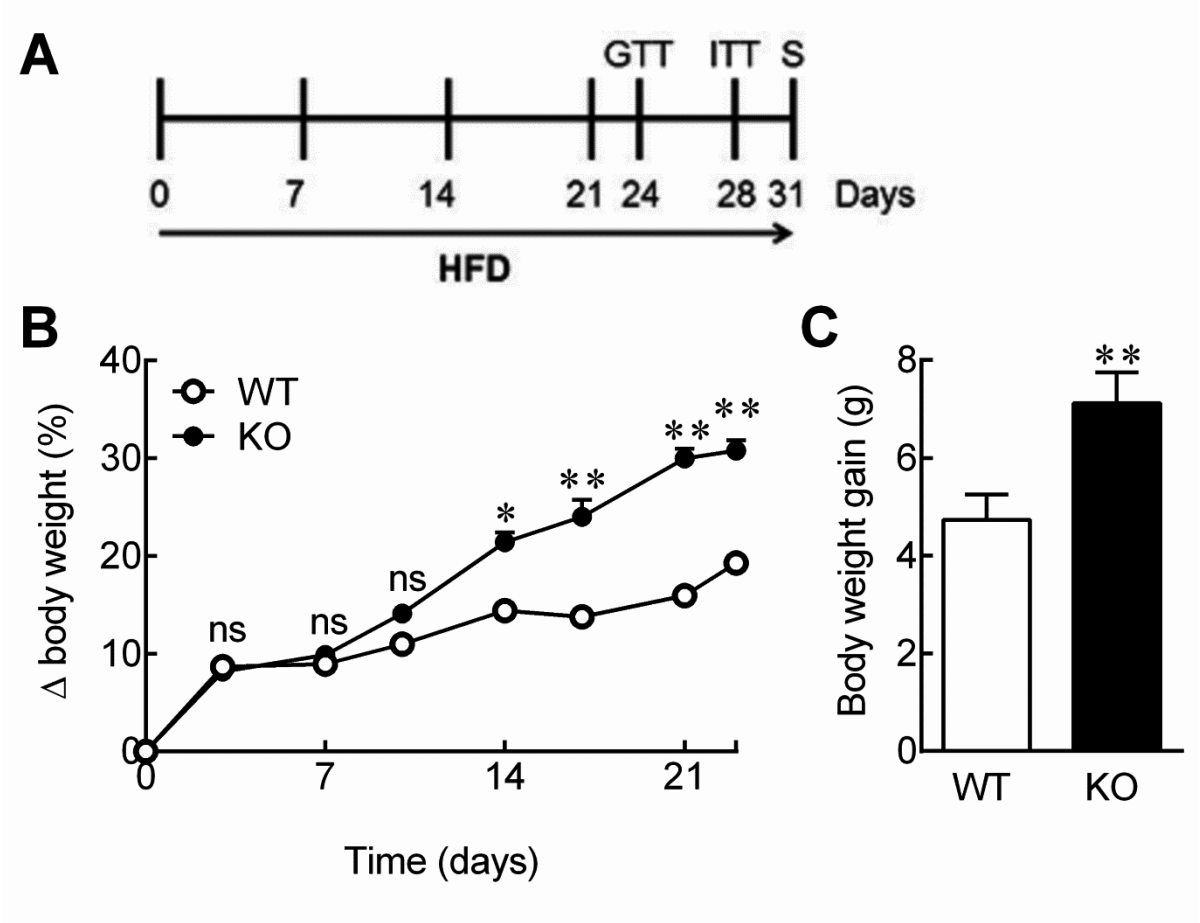

Figure 12. Body weight evolution of WT and KO mice during 23 days of HFD feeding. **(A)** Experimental schedule of the metabolic phenotyping of HFD fed mice. Mice began the dietary protocol with 8 weeks of age. GTT: Intraperitoneal glucose tolerance test, ITT: intraperitoneal insulin tolerance test, S: Sacrifice. **(B)** Change in body weight during 23 days of HFD feeding. Results are represented in percentage of body weight **(C)** Total body weight gain after 23 days of HFD feeding. Results are presented as mean ± SEM; n = 6-8/group. Statistical significance was determined by the unpaired Student's t-test (ns=non significant; * $p \leq 0.05$; ** $p \leq 0.01$ compared to WT).

2.2 Changes in tissue weight of SIRT2-KO mice

The weight of gWAT of WT mice fed a HFD doubled as compared with CD feeding (Table 4), being consistent with the increase in body weight gain. Although the absolute weight of the gWAT of KO mice fed a HFD was 45.7 % higher than that of control mice (0.76 ± 0.14 g and 0.52 ± 0.09 g, respectively), the difference was not statistically significant (Fig. 13). These findings could explain the increased body weight gain in KO mice. We found, however, a significant difference in BAT weight between the WT and the KO mice (Fig. 13).

The liver weight of KO mice was decreased, in comparison with WT mice, both under CD (30.0 ± 5.1 % decrease) and also after 31 days of HFD feeding (19.5 ± 2.8 % decrease)(Fig. 13).

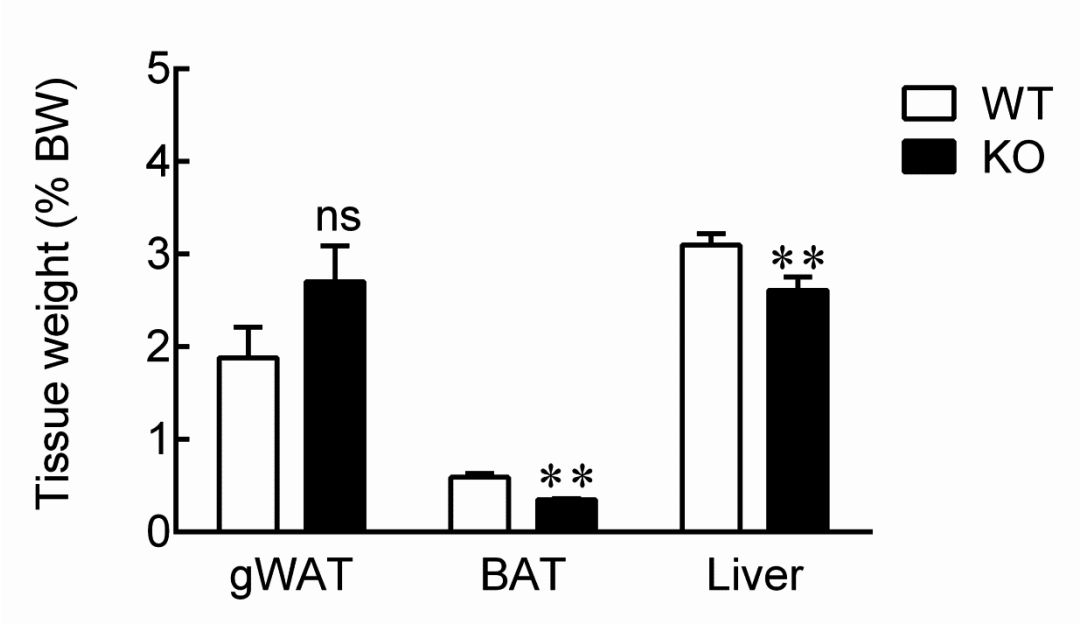

Figure 13. Weight of tissues of WT and KO mice, after 31 days of HFD feeding. Tiessue weight was normalized by the body weight (% BW). gWAT: gonadal white adipose tissue, BAT: brown adipose tissue. Results are presented as mean ± SEM; n = 6-8/group. Statistical significance was determined by the unpaired Student's t-test (ns=non significant; ** $p \leq 0.01$ compared to WT).

2.3 SIRT2-KO mice have increased food and energy intake

To investigate the role of SIRT2 on the energy homeostasis upon metabolic stress conditions, we monitored the consumption of HFD during 23 days. Consistent with previous findings, the energy intake of WT mice fed a HFD was 26.7 ± 0.3 % higher than that of WT mice fed a CD (Table 4). However, the food intake was 35.4 ± 0.1 % decreased in WT mice fed a HFD, compared with WT fed a CD (Table 4). These findings in WT mice show that HFD feeding for 23 days is able to markedly increase energy intake and result in a negative compensatory mechanism that leads to the reduction of food intake. Upon HFD feeding, the cumulative energy intake of KO mice was continuously higher than that of control mice (Fig. 14A). Mice lacking SIRT2 had a total food and energy intake 13.2 ± 1.9 % higher than that of

wild type mice (Fig. 14B and C), suggesting that SIRT2 plays a role in the regulation of food intake in mice fed a HFD.

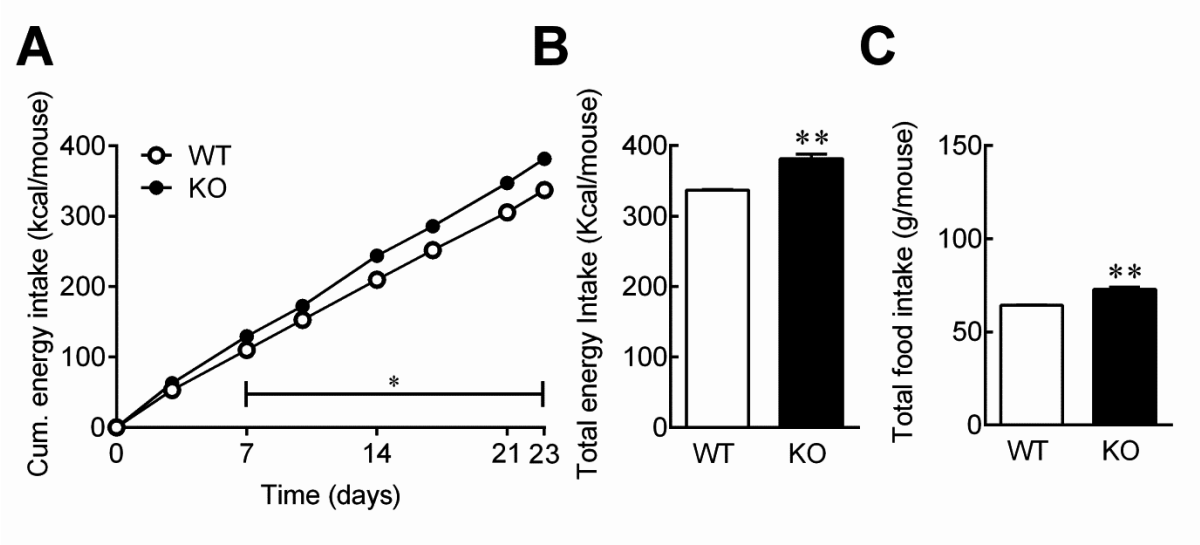

Figure 14. Food and energy intake of WT and KO mice during 23 days of HFD feeding. **(A)** Cumulative energy intake over 23 days. **(B)** Total energy intake (Kcal) and **(C)** total food intake (g) after 23 days of HFD feeding. Results are presented as mean ± SEM; n = 6-8/group. Statistical significance was determined by the unpaired Student's t-test (* $p \leq 0.05$; ** $p \leq 0.01$ compared to WT).

2.4 SIRT2-KO mice have increased fasting glucose levels

To investigate the impact of SIRT2 deficiency on glucose homeostasis upon a stress condition of HFD feeding, fasting blood glucose levels were measured after 15 hours of fasting. The blood glucose levels of WT mice fed a HFD were 17.3 ± 3.7 % higher than that of WT mice fed a CD (Table 4), indicating that 24 days of HFD feeding induces fasting hyperglycemia. KO mice had a significantly elevated (12.2 ± 3.9 %) fasting blood glucose level compared with WT mice fed the same HFD (Fig. 15), suggesting that SIRT2 deficiency affects glucose homeostasis in the fasted state.

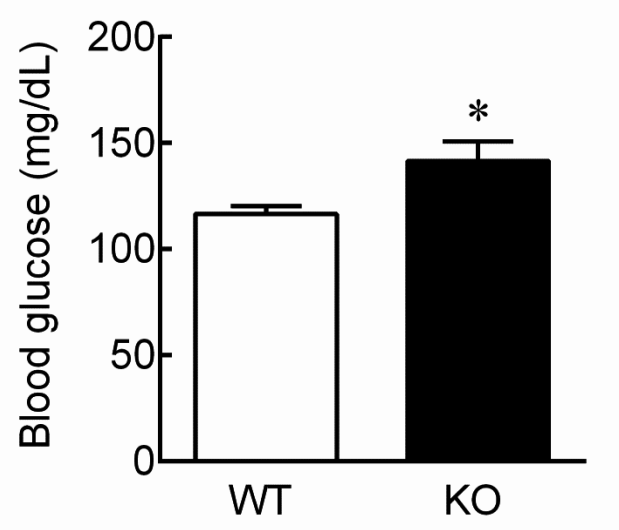

Figure 15. Fasting blood glucose levels of WT and KO mice after 24 days of HFD feeding. Mice were fasted for 15 hours before measurement of glucose levels. Results are presented as mean ± SEM; n = 6-8/group. Statistical significance was determined by the unpaired Student's t-test (* $p \leq 0.05$ compared to WT).

2.5 SIRT2-KO mice may have impaired glucose tolerance

To further evaluate the effect of SIRT2 deficiency on the glucose metabolism of mice fed a HFD, an ipGTT was performed in both WT and KO mice to assess their glucose tolerance. The AUC of the ipGTT curve from WT mice fed a HFD, compared with CD-fed WT mice, revealed the development of glucose intolerance in WT mice subjected to HFD for 24 days (Table 4). KO mice fed a HFD showed a tendency towards glucose intolerance compared to WT mice fed the same diet, although only the 30 minutes time point reached statistical significance (Fig. 16A). Consistently, the AUC of the ipGTT curves of KO mice was not significantly higher than that of ipGTT curves from control mice ($p=0.0763$) (Fig. 16B). These findings were consistent with the increased levels of blood glucose, showing that SIRT2 plays an important role on glucose homeostasis under a stress condition of HFD feeding.

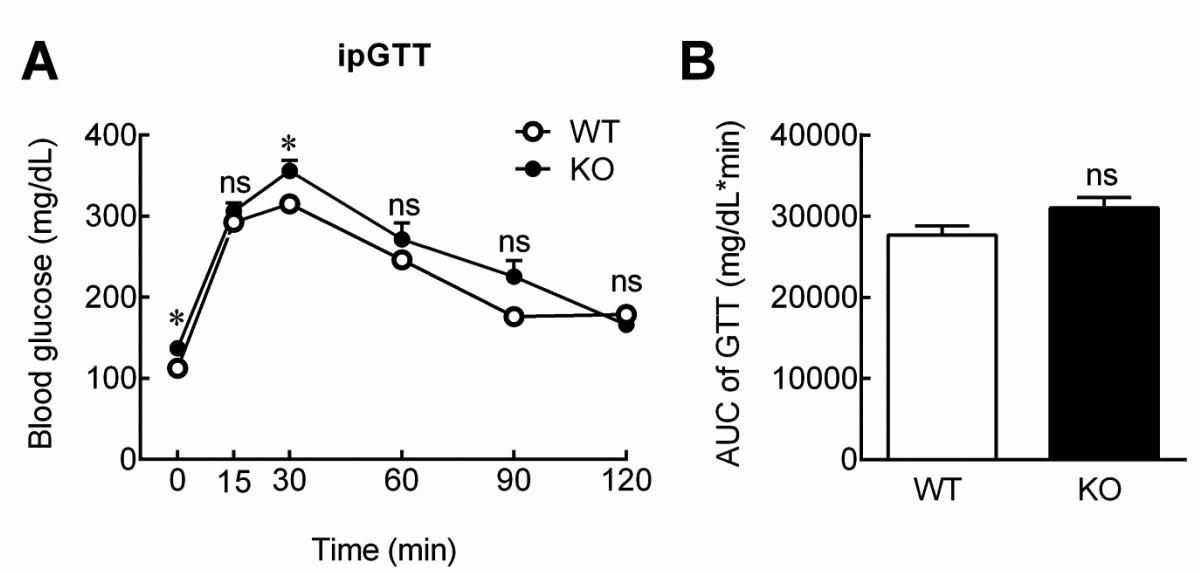

Figure 16. Intraperitoneal glucose tolerance test (ipGTT) was conducted in WT and KO mice after 24 days of HFD feeding. The area under the curve (AUC) of ipGTT curves was calculated. **(A)** Blood glucose levels of KO and WT mice at different time points after i.p injection of D-glucose (1.5 g glucose/kg body weight). **(B)** Average AUC of the ipGTT curves was calculated using the trapezoidal rule. Results are presented as mean ± SEM; n = 6-8/group. Statistical significance was determined by the unpaired Student's t-test (ns=non significant; * $p \leq 0.05$ compared to WT).

2.6 SIRT2-KO mice are markedly insulin resistant

To assess the effect of SIRT2 deficiency on insulin sensitivity, we performed an ipITT. The increased AUC of the ipITT curve of WT mice fed a HFD, compared with WT mice fed a CD diet, showed that HFD feeding for 28 days is able to induce insulin resistance in WT mice (Table 4). The rate of insulin-induced glucose excursion was markedly reduced in KO mice fed a HFD (Fig. 17A). Consistently, the AUC of the ipITT curves of KO mice was 42.1 ± 6.4 % higher than that of control mice (Fig. 17B), indicating that SIRT2 deficiency results in aggravated insulin resistance under HFD conditions.

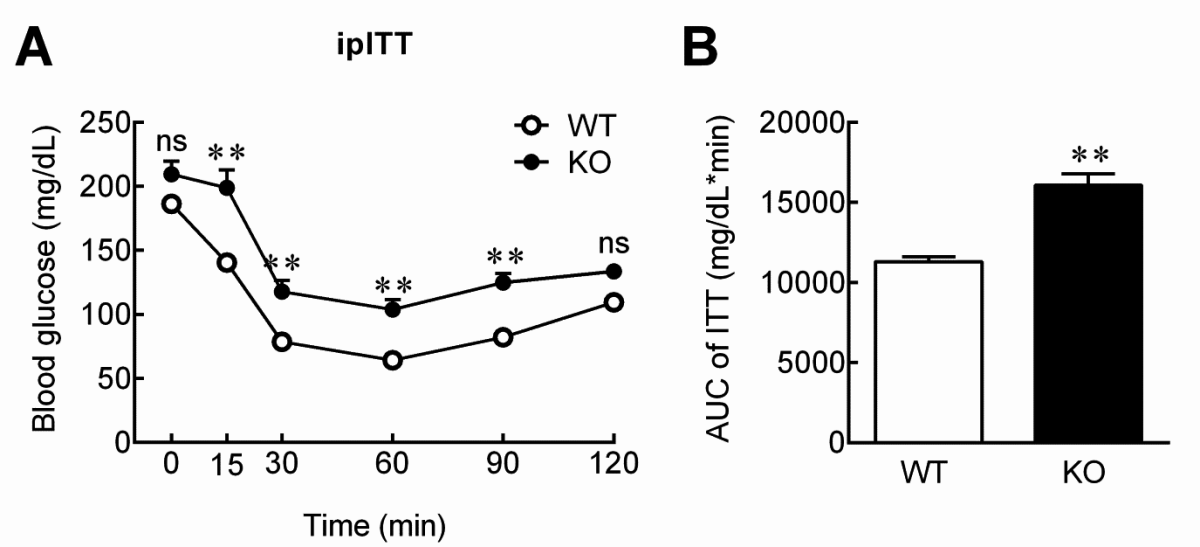

Figure 17. Intraperitoneal insulin tolerance test (ipITT) were performed in WT and KO mice fed a HFD for 28 days. Area under the curve (AUC) was calculated. **(A)** Blood glucose levels at different time points after i.p injection of human insulin (0.75 U / kg of body weight). **(B)** Average AUC of the ipITT curves. AUC was calculated using the trapezoidal rule. Results are presented as mean ± SEM; n = 7-8/group. Statistical significance was determined by the unpaired Student's t-test (ns=non significant; ** $p \leq 0.01$ compared to WT).

2.7 SIRT2-KO mice have cholesterol levels similar to WT mice

In order to evaluate the role of SIRT2 in lipid metabolism under a stress condition of HFD feeding, we measured serum cholesterol levels of mice fasted for 15 hours. The serum cholesterol levels of WT mice fed a HFD were 34.2 ± 13.4 % higher than that of WT mice fed a CD (Table 4), indicating that 31 days of HFD feeding leads to increased levels of circulating cholesterol in WT mice. SIRT2-KO mice fed a HFD had cholesterol levels similar to WT mice (Fig. 18), suggesting that SIRT2 does not play an important role in cholesterol metabolism.

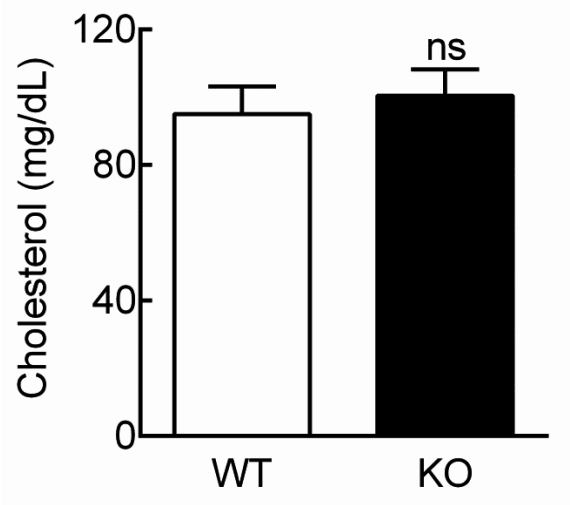

Figure 18. Serum cholesterol levels of WT and KO mice after 31 days of HFD feeding. Cholesterol levels were measured after 15 hours of fasting. Results are presented as mean ± SEM; n = 5-8/group. Statistical significance was determined by the unpaired Student's t-test (ns=non significant compared to WT).

2.8 SIRT2-KO mice have increased triglyceride levels

To further assess the impact of SIRT2 deficiency in lipid metabolism under a stress caused by HFD feeding, we measured serum triglyceride levels. No significant differences were observed in triglyceride levels in WT mice fed a HFD in comparison with WT mice fed a CD, suggesting that 31 days of HFD are not able to induce marked defects in triglyceride metabolism (Table 4). However, SIRT2-KO mice had 25.8 ± 7.1% higher levels of circulating triglycerides than that of WT mice (Fig. 19), suggesting that SIRT2 plays an important role in triglycerides metabolism under HFD-induced stress conditions.

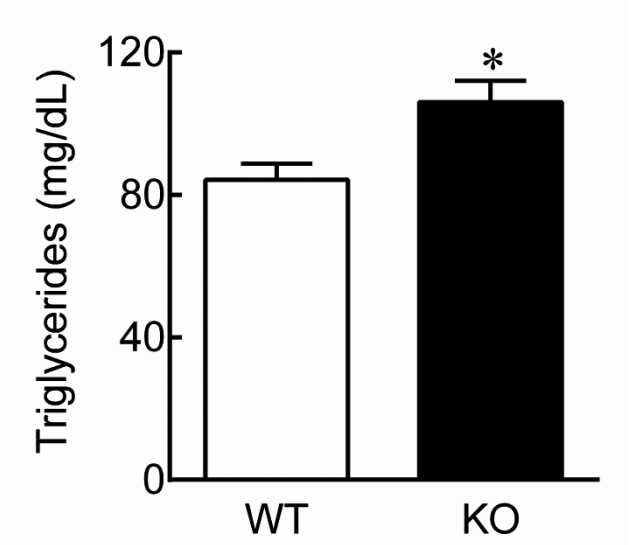

Figure 19. Serum triglyceride levels of WT and KO mice after 31 days of HFD feeding. Triglyceride levels were measured after 15 hours of fasting. Results are presented as mean ± SEM; n = 2-4/group. Statistical significance was determined by the unpaired Student's t-test (* $p \leq 0.05$ compared to WT)

Chapter IV

Discussion

The metabolic actions of SIRT2 have been studied primarily through *in vitro* models and few studies have reported the role of SIRT2 in metabolic homeostasis *in vivo*. The aim of the present study was to unravel the role of SIRT2 in whole-body metabolism. To this purpose, we performed a metabolic phenotyping of SIRT2-KO mice under a normal condition of chow diet (CD) feeding or under a stress condition induced by high fat diet (HFD) feeding. The current study demonstrates a role for SIRT2 in energy and glucose homeostasis and is the first study, to our knowledge, providing a detailed metabolic characterization of SIRT2-KO mice.

We show here that SIRT2-KO mice fed a CD diet lack an overt phenotype, when compared with gender- and age-matched WT mice. Metabolic parameters such as body/adipose tissue weight gain, food/energy intake, glucose tolerance and fasting blood glucose, cholesterol and triglyceride levels were similar between WT and SIRT2-KO mice. Consistent with the present study, previous body composition analyses of the same mouse model revealed no differences in the weight and lean/fat mass of mice fed a CD, when compared with WT mice (Bobrowska *et al.*, 2012; Belman *et al.*, 2015). We found that the liver weight of SIRT2-KO mice fed a CD is reduced compared with that of WT mice. Although we currently have no explanation for this finding, it is tempting to speculate that this could be related to accelerated aging of the liver of SIRT2-KO mice (Anantharaju *et al.*, 2002), since SIRT2 has been reported to play a crucial role in lifespan determination (North *et al.*, 2014). SIRT2 over-expression in an animal model of accelerated aging ($BubR1^{H/H}$) was reported to reverse the decreased heart weight associated with aging (North *et al.*, 2014). Overall, these findings suggest that SIRT2 does not play a crucial role in metabolic homeostasis under a normal metabolic condition of CD feeding.

Short-term HFD feeding has been reported to induce increased adiposity, hyperglycemia, glucose intolerance, insulin resistance and hyperphagia in WT mice (Ziotopoulou *et al.*, 2000; Wang *et al.*, 2001; Lee *et al.*, 2011; Turner *et al.*, 2013). Our data are consistent with these findings, since HFD feeding (60% energy from fat) for 31 days induced increased body weight gain and energy intake, hyperglycemia, glucose intolerance and insulin resistance in WT mice. The amount of ingested calories was clearly higher in WT

mice fed a HFD, due to the higher energy density of the HFD. However, the total amount of food ingested was lower in mice fed a HFD, what may be explained by a well-known compensatory response developed to resist to an increased energy intake (Ziotopoulou *et al.*, 2000; Chan *et al.*, 2008). We also observed that upon HFD feeding some mice were more prone to develop HFD-induced metabolic dysfunction than others, which is consistent with findings demonstrating the existence of obesity-prone and obesity-resistant mice (Choi *et al.*, 2016).

In contrast with a normal condition of CD feeding, when subjected to a metabolic stress condition induced by acute HFD feeding, SIRT2-KO mice developed evident metabolic abnormalities, compared with WT mice. Mice lacking SIRT2 gained 50.4% more body weight after 23 days of HFD feeding. Consistently, SIRT2-KO mice had a 13.3% higher food and energy intake, indicating that SIRT2 deficiency affects the regulation of energy homeostasis. One possible explanation is that SIRT2 is expressed in the hypothalamus to modulate the central regulation of energy metabolism, similar to SIRT1. Consistent with what has been reported for SIRT1 (Ramadori *et al.*, 2008; Satoh *et al.*, 2010), previous data from our group demonstrated that SIRT2 is expressed in the hypothalamus and that its expression is dependent on the energy availability, being downregulated upon HFD feeding (Santos, 2015; unpublished data). It is already known that SIRT1 is expressed in the hypothalamic neurons responsible for the regulation of food intake (i.e., orexigenic AgRP/NPY neurons and anorexigenic POMC/CART neurons), where it regulates the expression of AgRP and POMC through FOXO1. Hypothalamic FOXO1 directly binds the promoter of Agrp and Pomc genes, regulating their expression and promoting food intake (Cakir *et al.*, 2009; Sasaki *et al.*, 2010). SIRT2 was reported to deacetylate FOXO1 in adipocytes (Jing *et al.*, 2007; Wang and Tong, 2009); whether the same happens in hypothalamic neurons is still unknown, but plausible. Since hypothalamic overexpression of SIRT1 was reported to suppress hyperphagia and body weight gain induced by FOXO1-dependent expression of AgRP (Sasaki *et al.*, 2010), it would be interesting to test if SIRT2 overexpression has the same anorexigenic effect. Thus, the expression and action of SIRT2 in hypothalamic AgRP/NPY and POMC neurons, possibly through FOXO1, is a potential explanation for the impaired

energy homeostasis reported in SIRT2-KO mice, which resulted in increased body weight gain. A detailed evaluation of energy expenditure parameters, such as O_2 consumption, CO_2 production, heat production and respiratory quotient (RQ), is needed to further evaluate the impact of SIRT2 deficiency on the whole-body energy balance. Lack of hypothalamic SIRT1 was reported to suppress energy expenditure (Ramadori *et al.*, 2010; Satoh *et al.*, 2010). SIRT2 has been reported to be overexpressed in the BAT upon cold exposure, indicating a possible link between SIRT2 and thermogenesis (Wang and Tong, 2009). Further studies are required to delineate the role of SIRT2 in the regulation of food intake and energy expenditure.

SIRT2 has emerged as an important regulator of lipid metabolism, playing a role in adipogenesis, lipogenesis and fatty acid oxidation. SIRT2 was shown to suppress adipocyte differentiation and to stimulate lipolysis in mature adipocytes (Jing *et al.*, 2007; Wang and Tong, 2009). Interestingly, similar results were found for SIRT1 (Picard *et al.*, 2004). In line with the increased body weight gain, the adipose tissue weight of SIRT2-KO mice fed a HFD was 45% higher than that of WT mice. It should be pointed out that we considered the gonadal white adipose tissue (gWAT) as representative of the total body weight. The body weight gain would be better assessed using whole body composition analyzer, such as magnetic resonance imaging (MRI), which would allow an accurate evaluation of the body fat and lean mass contents. Previous data regarding the role of SIRT2 in cholesterol synthesis have been conflicting. SIRT2 inhibition was reported to decrease cholesterol synthesis (Luthi-Carter *et al.*, 2010; Taylor *et al.*, 2011). However, consistently with our results, Bobrowska *et al.* (2012) showed that the expression of enzymes involved in cholesterol biosynthesis is not altered in SIRT2-KO mice (Bobrowska et al., 2012). SIRT2 was reported to inhibit lipogenesis through ACLY deacetylation and consequent degradation (Lin *et al.*, 2013). ACLY deficiency was shown to result in decreased levels of circulating triglycerides (Wang *et al.*, 2010). These previous findings suggest that lack of SIRT2 leads to ACLY acetylation and stability, increasing the synthesis of triglycerides through lipogenesis. The present study supports this theory, since SIRT2-KO mice showed increased levels of circulating triglycerides.

Gluconeogenesis, the process that synthesizes glucose from non-carbohydrate carbon sources, such as pyruvate and lactate, is the main source of glucose during fasting (Bouche *et al.*, 2004). SIRT2 has been suggested as a critical sensor of glucose levels *in vitro*. It was demonstrated that SIRT2 deacetylates and stabilizes PEPCK1, a rate-limiting gluconeogenic enzyme (Jiang *et al.*, 2011). We have shown that SIRT2-KO mice fed a HFD for 24 days showed increased fasting blood glucose levels, which may be explained by the overexpression of gluconeogenic enzymes. We cannot exclude the possibility that SIRT2 may target other enzymes of the gluconeogenic pathway. In adipocytes, SIRT2 was reported to target PGC-1α (Krishnan *et al.*, 2012) which is known to be a key regulator of hepatic gluconeogenesis (Yoon *et al.*, 2001). To further evaluate the effect of SIRT2 deficiency in glucose production, the expression of gluconeogenic enzymes, such as PEPCK1 and G6Pase, should be assessed. Since deregulation of gluconeogenesis is an important marker of acute HFD-induced metabolic dysfunction (Lee *et al.*, 2011), the key role of SIRT2 in this pathway may explain the exacerbated phenotype in SIRT2-KO mice, only under HFD feeding.

Impaired glucose tolerance is developed early in the onset of HFD-induced metabolic disorders (Lee *et al.*, 2011; Turner *et al.*, 2013). After 24 days of HFD feeding, SIRT2-KO mice showed a tendency towards glucose intolerance. One previous study proposed that SIRT2 deficiency enhances glucose tolerance (Belman *et al.*, 2015). However, key experimental parameters, such as the fasting duration and the glucose load, were different from our study, hampering a direct comparison. Glucose tolerance is determined by the balance between insulin secretion and insulin-dependent and -independent glucose actions. Insulin levels should be quantified during the GTT to assess insulin secretion, since differences in glucose tolerance can be related to insulin levels rather than to glucose intolerance. Insulin-independent mechanisms triggered by glucose on the central nervous system may also explain differences in glucose clearance of SIRT2-KO mice (Fioramonti *et al.*, 2007; Parton *et al.*, 2007).

SIRT2-KO mice were markedly insulin resistant after 28 days of HFD feeding. Consistently, even when fed a CD, SIRT2 deficient mice exhibited a mild insulin resistant phenotype. SIRT2 was shown to play an important role in lipid metabolism, regulating lipid

synthesis (Lin *et al.*, 2013) and oxidation (Krishnan *et al.*, 2012). Acute lipid overload leads to the accumulation of lipid metabolites in peripheral tissues, which blocks the insulin signaling pathway leading to an insulin resistance state (Powell *et al.*, 2004; Magkos *et al.*, 2012). Taking this into account, the role of SIRT2 in lipid metabolism may explain the exacerbated insulin resistance of SIRT2-KO mice fed a HFD. It would be interesting to measure the hepatic content of lipids. It would be also important to perform a hyperinsulinemic-euglycemic clamp, since it is widely considered the standard procedure to assess whole-body insulin sensitivity *in vivo.* Previous findings suggest that SIRT2 binding is required for the optimal activation of Akt, a key downstream component of the insulin signaling pathway (Chen *et al.*, 2013; Ramakrishnan *et al.*, 2014). However, another study did not report any interaction between Akt and SIRT2 (Arora and Dey, 2014). The tissue-dependent interactions of SIRT2 may explain these contradictory results, since the same happens with SIRT1, whose ability to interact and deacetylate different proteins is cell- and tissue-specific (Sun *et al.*, 2007; Yoshizaki *et al.*, 2009; Wang *et al.*, 2011; Lu *et al.*, 2013). We have shown that SIRT2 deficiency has no impact on the insulin-dependent activation of Akt, at least in the liver of mice fed a CD. We should assess the insulin sensitivity of other metabolic relevant tissues, such as skeletal muscle and WAT.

Inflammatory response was reported to play a crucial role in the metabolic dysfunction induced by short-term HFD feeding, contributing to hepatic insulin resistance (Ji *et al.*, 2012; Van Beek *et al.*, 2012; Wiedemann *et al.*, 2013). SIRT2 has been shown to play an important inhibitory role in inflammation and oxidative stress-induced cell death (Rothgiesser *et al.*, 2010; Kim *et al.*, 2013; Pais *et al.*, 2013; Lo Sasso *et al.*, 2014). Therefore, we cannot exclude the possibility that SIRT2 deficiency may exacerbate the inflammatory response to HFD feeding, thereby explaining the severe metabolic dysfunction observed in SIRT2-KO mice fed a HFD. Thus, it would be interesting to evaluate inflammatory markers in several tissues of SIRT2-KO mice.

In conclusion, the present study is the first to show that SIRT2 deficiency leads to increased body weight gain and energy intake, hyperglycemia, glucose intolerance and insulin resistance under HFD feeding. These results underscore the importance of SIRT2 in

HFD-induced metabolic stress, which is of great importance as it is a major cause of obesity and type 2 diabetes. Since the prevalence of these metabolic diseases is increasing at an alarming rate, it is crucial to find novel therapeutic approaches. The present study strongly suggests that stimulation of SIRT2 expression and/or activity may represent a novel strategy to protect against the development of HFD-induced metabolic stress conditions. However, the regulation of metabolic homeostasis by SIRT2 is likely to be very complex, since tissue- and cell-specific processes are involved. Therefore, cell-specific manipulation of SIRT2 expression seems of great importance in future therapeutic approaches.

Chapter V

Conclusions

The present study was the first, to our knowledge, to report the effect of SIRT2 deficiency in the *in vivo* metabolic homeostasis. Metabolic phenotyping of SIRT2-KO mice revealed no overt phenotype under a normal metabolic condition of chow diet feeding. Interestingly, under a metabolic stress condition of short-term high fat diet (HFD) feeding, SIRT2 deficiency leads to increased body weight gain and energy intake, hyperglycemia, hypertriglyceridemia, glucose intolerance and insulin resistance. Further studies are required in order to evaluate the tissue- and cell-specific mechanism involved in the SIRT2 regulation of energy expenditure and expression of gluconeogenic enzymes. The effect of SIRT2 deficiency in specific metabolic relevant tissues, such as liver, skeletal muscle and adipose tissue, should be further assessed to evaluate the marked whole-body insulin resistance reported here.

Taking into account that SIRT2 deficiency leads to severe metabolic abnormalities under acute high fat diet feeding, the present study strongly suggests that stimulation of SIRT2 under metabolic stress conditions would result in improved energy and glucose metabolism. It is crucial to find novel therapeutic approaches to combat metabolic diseases such as obesity and type 2 diabetes, since their prevalence is increasing at an alarming rate. Our data suggest SIRT2 manipulation as a potential therapeutic target to ameliorate the metabolic dysfunction associated with the early onset of metabolic disorders.

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