REGULATION OF METABOLIC PROCESSES BY microRNAs AND CLASS I HISTONE DEACETYLASES

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by

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REGULATION OF METABOLIC PROCESSES BY microRNAs AND CLASS I HISTONE DEACETYLASES

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Obesity is a medical condition resulting from accumulation of excess body fat that affects more than 30% of the adult population in the U.S. Obesity-related pathological conditions include heart disease, stroke, type 2 diabetes and certain types of cancer. Despite the high incidence and the elevated social costs, the molecular basis of obesity and associated metabolic syndrome are still poorly understood. Yet, the need for novel therapeutic approaches for the treatment and prevention of obesity remains. In humans and animal model of disease, hallmarks of obesity include dysregulation of genes involved in mitochondrial function, lipid uptake and lipid storage. The dynamic and modifiable regulation of transcriptional pathways that control mitochondrial function and adipogenesis, as well as additional aspects of mammalian metabolism, will provide new approaches for pharmacological intervention in obesity. Thus, the modulation of epigenetic histone modifications and microRNA functions represents a potentially powerful approach for the treatment of metabolic disorders. We show that the *Ppargc1b*

gene, which encodes the PGC-1β protein, also co-transcribes two microRNAs, miR-378 and miR-378*. Mice lacking miR-378/378* are resistant to high fat diet-induced obesity and display enhanced mitochondrial fatty acid metabolism and elevated oxidative capacity of insulin-target tissues. Taken together, our findings reveal that miR-378 and miR-378* function as integral components of the regulatory circuit formed by PGC-1beta and nuclear hormone receptors to control the overall oxidative capacity and energy homeostasis of insulin-target tissues. MiR-378/378* mutant mice do not display overt phenotypes under normal laboratory conditions, whereas their phenotypes become apparent under conditions of stress, in this case in response to excessive caloric intake. Thus, pharmacological modulation of miR-378/378* function might represent an effective approach in the treatment of obesity. In obese humans and mice, the unused caloric energy resulting from excessive net caloric intake is converted to triglycerides and stored in adipocytes for further usage. Lipid accumulation within adipocytes is under the control of a cascade of transcription factors that interact with histone acetyltransferases and deacetylases. We show that histone deacetylase inhibitors efficiently block adipocyte differentiation *in vitro*. Furthermore, through a loss-of-function approach, we provide evidence that histone deacetylases 1 and 2 play redundant and requisite roles in adipogenesis. In conclusion, we unveiled previously unrecognized roles for miR-378/378* in the control of mitochondrial metabolism and energy homeostasis, and for histone deacetylases in the control of adipocyte differentiation.

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3. **Carrer M.**, Liu N., Grueter, C.E., Williams, A.H., Frisard, M.I., Hulver, M.W., Bassel-Duby, R., Olson, E.N. Control of mitochondrial metabolism and energy homeostasis by microRNAs 378 and 378*. *returned revised manuscript to Proc Natl Acad Sci.*

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LIST OF ABBREVIATIONS

ADIPOQ	adiponectin
AMPK	AMP-activated protein kinase
aP2	adipocyte lipid-binding protein 2
ASMs	acid soluble metabolites
ABCA1	ATP-binding cassette transporter subfamily A1
BAT	brown adipose tissue
BrdUrd	5-bromo-2-deoxyuridine
BSA	bovine serum albumin
C/EBP	CAAT/enhancer-binding protein
CaMK	Ca^{2+}/calmodulin-dependent protein kinase
CRAT	carnitine O-acetyltransferase
DMEM	Dulbecco's modified Eagle's medium
E	embryonic day
FBS	fetal bovine serum
FCS	fetal calf serum
FDA	Food and Drug Administration
FH1	fumarate hydratase 1
FOXO1	forkhead box O1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDACi	HDAC inhibitor
HFD	high fat diet
HNF4a	hepatocyte nuclear factor 4a
IBMX	isobutyl-1-methylxanthine
IFNγ	interferon γ
IL-1β	interleukin-1β
IRS	insulin receptor substrate
KO	knockout
LIPE	hormone-sensitive lipase
LNA	locked nucleic acid
MEF	mouse embryonic fibroblast
MEF2	myocyte enhancer factor 2
MiR	microRNA
MRI	magnetic resonance imaging
NAD	nicotinamide adenine dinucleotide
NEO	neomycin-resistance cassette
ORO	oil Red O staining
PEPCK	phosphoenol pyruvate carboxykinase

PGC-1	peroxisome proliferator activated receptor γ coactivator-1
PI3K	phosphatidylinositol 3 kinase
PKD	protein kinase D
PPAR	peroxisome proliferator activated receptor
RISC	RNA-induced silencing
RT-PCR	reverse transcription polymerase chain reaction
RUNX2	runt-related transcription factor 2
SAHA	suberoylanilide hydroxamic acid
SCFA	short-chain fatty acid
SDHD	succinate dehydrogenase complex subunit d
SREBP	sterol regulatory element-binding protein
STAT	signal transducer and activator of transcription
STZ	streptozotocin
T1D	type 1 diabetes
T2D	type 2 diabetes
TAC	thoracic aortic constriction
TCA	tricarboxylic acid
TNFα	tumor necrosis factor α
TSA	trichostatin A
UTR	untranslated region
WT	wild-type

Chapter I

MicroRNAs in Metabolism and Disease

Introduction

MicroRNAs are small non-coding RNAs that inhibit the expression of target mRNAs in a sequence-specific manner. The first microRNA to be discovered was *lin-4* in *C. elegans* in the early 1990s. Currently, microRNAs form one of the largest gene families, representing about 1% of the entire mammalian genome (Kim, 2005; Mendell and Olson, 2012). The number of biological pathways known to be modulated by microRNAs has also grown exponentially in recent years. The same is true for the complexity of the regulatory network formed by microRNAs and their target genes. In fact, over one third of the entire human gene array has been predicted to be targeted by microRNAs (Lewis et al., 2005). Additionally, a single microRNA can target multiple mRNAs, and a single mRNA can be targeted by multiple microRNAs. The common theme that emerges from studies on different microRNAs is their ability to modulate a variety of biological processes under conditions of physiological and pathological stress (Bartel, 2009). Taken together, these observations point to microRNAs both as promising targets for disease modulation and as potential diagnostic markers of pathological states.

Biogenesis and function of microRNAs in physiology and disease

MicroRNAs are single-stranded RNAs of about 22 nucleotides in length (Bartel, 2004; Kim, 2005). Most microRNAs are transcribed by RNA polymerase II within a longer hairpin-containing primary structure called pri-miRNA (Lee et al., 2004). These pri-miRNAs vary in length from hundreds to several thousands of nucleotides and

contain 5’ cap sequences, as well as polyadenylated tails (Cai et al., 2004). The mature microRNA is generated from the pri-miRNA by a multi-step process. Initially, the pri-miRNA is cleaved in the nucleus by a multi-protein complex containing the RNase III endonuclease Drosha to generate a precursor of about 70 nucleotides in length, called the pre-miRNA (Lee et al., 2003). The pre-miRNA stem-loop structure, which has a 5’ phosphate and a 2 nucleotide 3’ overhang, is actively transported from the nucleus to the cytoplasm by Exportin-5 via a Ran-GTP-dependent mechanism (Yi et al., 2003). In the cytoplasm, the pre-miRNA is processed by Dicer, a RNase III-type enzyme, to generate the miRNA:miRNA* duplex, an siRNA-like structure that contains the mature miRNA and a similar fragment, derived from the opposing arm of the pre-miRNA, called miRNA* (Hutvagner et al., 2001). Within the duplex, the miRNA strand is defined by both the absolute and relative stabilities of the base pairs at its 5' end. The miRNA* strand has higher stability of the base pairs and is typically degraded (Schwarz et al., 2003). The miRNA strand is incorporated in the RNA-induced silencing (RISC) complex, whose assembly is initiated by Dicer, together with Argonaute proteins (Gregory et al., 2005). The mature miRNA strand guides the RISC complex to target mRNAs via base pairing interactions between the miRNA seed region (nucleotides 2-8 of the mature miRNA) and complementary sequences in the 3’ untranslated region (UTR) of target mRNAs (Bartel, 2009). MicroRNAs repress gene expression at the post-transcriptional level via two mechanisms - either by blocking mRNA translation or by affecting mRNA stability (Bartel, 2009; Filipowicz et al., 2008). Mechanism of repression, either mRNA cleavage or blockade of translation, is determined by the identity and extent of base pairing between complementary sequences of the target

mRNA and the mature miRNA. If the target mRNA displays sufficient complementarity to the miRNA seed region, mRNA cleavage will be induced. Furthermore, the miRNA will repress translation if the target mRNA is not perfectly complementary to the miRNA seed sequence (Bartel, 2004). There is evidence that the miRNA-mediated block of translation occurs at the initiation step. However, a role of miRNA in the repression of the post-initiation step has not been excluded (Filipowicz et al., 2008). Often, multiple RISC complexes cooperate to mediate translational repression via binding to multiple miRNA-complementary sites present on the same target mRNA (Bartel, 2004).

Most of the known miRNAs recognize sequences in the 3' UTR of target mRNAs, probably to avoid ribosomal-mediated mRNA clearance. The residues of the miRNA responsible for the interaction are generally nucleotides 2-8 of the mature miRNA sequence. These residues form the seed region of the mature miRNA. However, many types of mRNA-miRNA interaction sequences differ from the canonical recognition site (Bartel, 2009). Despite the heterogeneity of the position and extent of base pairing between miRNA and target UTR sequences, the complementarity of the region starting at the second nucleotide of the 5' end of the mature miRNA seems to be required for proper miRNA function, probably due to structural reasons that involve the RISC ability to present only this core region for pairing with the target mRNA (Bartel, 2009). In keeping with this assumption, the residues 2-8 of different miRNAs are the most conserved among species (Lim et al., 2003). In recent years, the importance of base pairing of the seed region, as well as of flanking sequences, has been a topic of debate. It is not completely understood if the seed complementarity to the target UTR is the only determinant of specificity or if the local mRNA structure could limit the accessibility of

the RISC complex to the UTR. Similarly, the contribution of the base complementarity of regions flanking nucleotides 2-8 of the mature miRNA remains to be determined.

MicroRNAs can be classified depending on their genomic location. Most microRNAs are intergenic, as they reside in regions of the genome distant from annotated genes. These microRNAs are transcribed as independent units (Bartel, 2004). Other microRNAs reside in the introns of known genes. In general, these intronic microRNAs are in the same orientation of the host gene and are not transcribed from their own promoter (Kim, 2005). Other microRNAs are arranged in clusters and are transcribed as multi-cistronic units (Kim, 2005).

The high degree of conservation of different microRNAs in related species, including human and mouse, is indicative of their functional relevance. Some microRNAs are highly expressed in the cell, whereas the differential expression of other microRNAs in different stages of development and in different cell types allows for fine-tuning regulation of diverse cellular processes. Accordingly, the function of each microRNA is tightly linked to the array of cellular target mRNAs that are recognized by that microRNA. This observation is true for the first microRNA to be discovered in *C. elegans*, *lin-4*, which displays conserved regions of sequence complementarity to the *lin-14* UTR (Lee et al., 1993). The same is true for *let-7* and the *lin-41* UTR (Reinhart et al., 2000). Indeed, *lin-4* and *let-7* were identified by loss-of-function mutations that cause defects in developmental timing in the worm larvae, due to the deregulation of their target genes (Lee et al., 1993; Reinhart et al., 2000). Upon analysis of the functions of multiple microRNAs in different species, a common feature that becomes evident is the propensity of these genetic units to modulate their target gene expression under

conditions of stress (Leung and Sharp, 2010). In keeping with the role of microRNAs as key mediators of cellular stress responses, mutant animals lacking the expression of different microRNAs often do not display evident phenotypes under standard laboratory conditions. However, in some cases, abnormalities in development or viability become evident when these animals are subjected to changing environments, or more generally stress. This is true from flies, where genetic deletion of miR-7 causes developmental eye defects only when the flies are subjected to fluctuating temperatures (Li et al., 2009), to mice, where genetic deletion of miR-208 compromises the response to cardiac pressure overload (van Rooij et al., 2007). Other examples in mice include genetic deletion of the miR-143/145 cluster, which blocks neointima formation only in response to vascular injury (Xin et al., 2009), and loss of miR-375 expression, which results in proliferative defects of pancreatic β-cells upon an obesity-induced insulin resistance state (Poy et al., 2009). A possible explanation for the absence of severe developmental defects in mice and other organisms lacking the expression of different miRNAs is redundancy among related miRNAs, as these genes would share common seed regions and target common mRNAs. Indeed, this premise has been demonstrated for the miR-17-92 and miR-106b-25 clusters (Ventura et al., 2008), the miR-133 family members a-1 and a-2 (Liu et al., 2008), and miR-208b/miR-499 (van Rooij et al., 2009). While single genetic deletions of either miR-106b-25 or miR-106a are viable, compound knockout embryos for both miR-106b-25 and miR-17-92 die at mid-gestation (Ventura et al., 2008). Similarly, while mice lacking either miR-133a-1 or miR-133a-2 are normal, a large fraction of double knockout embryos die due to ventricular-septal defects (Liu et al., 2008). Furthermore, miR-208b and miR-499 has been shown to play redundant roles in the specification of

muscle fiber identity (van Rooij et al., 2009). The complexity of the regulation of microRNA functions under conditions of stress is increased by the modulation of the miRNA expression, of the amount of target mRNA, and of the degree of activity of the RISC complex (Leung and Sharp, 2010).

Given their abundance and diffuse expression, microRNAs modulate a wide variety of biological processes, both under physiological and pathological conditions. One of the most extensively studied aspects of microRNA functions is their contribution in the regulation of cell proliferation, cell differentiation, and apoptosis. Consequently, microRNAs have been shown to play a fundamental role in tumor formation and progression. Yet, the first microRNAs to be discovered, *lin-4* and *let-7*, have been shown to allow the timely division and differentiation of stem cell lineages in *C. elegans*. Through the years, the identification of the functions of different microRNAs in multiple tumor cell lines and cancer types allowed the generation of microRNA signature patterns, which represent a powerful tool in tumor classification and prognosis (Calin and Croce, 2006; Mendell and Olson, 2012). Among the examples of microRNAs involved in tumorigenesis, miR-378 has been shown to promote cell survival, tumor growth and angiogenesis by targeting of two oncosuppressor genes, *SuFu* and *Fus-1* (Lee et al., 2007). Similarly, miR-378* has been shown to promote a metabolic shift in breast cancer cells towards glycolysis by targeting the PGC-1β/ERRγ transcriptional pathway, thus reducing tricarboxylic acid cycle gene expression and oxygen consumption, while increasing lactate production and cell proliferation (Eichner et al., 2010). Other examples of microRNAs that function as oncogenes include the miR-15a/16-1 cluster, which is frequently deleted in chronic lymphocytic leukemia (Calin et al., 2002), the miR-17-92

cluster, which mediates signaling through the Myc pathway, miR-155, whose overexpression is sufficient to initiate lymphomagenesis in mice, and miR-21, which is considered an anti-apoptotic factor overexpressed in different human cancers (Mendell and Olson, 2012). In contrast, the miR-15a/miR-16-1 cluster targets proliferative factors, miR-34a mediates signaling through the p53 pathway, and miR-143/145, which is repressed by oncogenic Ras, functions as tumor suppressors *in vivo* (Mendell and Olson, 2012).

Together with their well documented role in cancer biology, microRNAs have been shown to modulate different aspects of the cardiac response to stress. In this setting, miR-29 and miR-15 family members regulate fibrosis, cardiomyocyte proliferation and apoptosis, miR-208a, miR-126, miR-21, miR-199a, and the miR-23a/27a/24-2 cluster regulate cardiac remodeling and angiogenesis, miR-133a function as a cardio-protective factor against heart failure, and the miR-143/145 cluster is required for smooth muscle cell plasticity and proliferation in response to injury (Mendell and Olson, 2012).

A role for different microRNAs in complex genetic diseases, including obesity and related metabolic syndrome, is beginning to emerge in recent years, expanding the range of possible therapeutic applications of specific miRNA inhibitors to multi-factorial pathologic conditions.

Role and regulation of microRNAs in metabolism and metabolic syndrome

MicroRNAs have recently been recognized as important modulators of physiological and pathological metabolic states. A role for different microRNAs has been described in the control of glucose uptake, glucose utilization, cholesterol and lipid

metabolism, and global energy homeostasis (Naar, 2011; Rottiers and Naar, 2012; Rottiers et al., 2011). As a consequence, microRNAs represent a potential therapeutic target for the prevention and treatment of obesity, type II diabetes, and related metabolic syndrome.

The first microRNA to be identified as a regulator of metabolic homeostasis was miR-122. Using pharmacological inhibition of miR-122 in mice, two separate studies revealed a role for miR-122 in the control of hepatic cholesterol and lipid metabolism (Esau et al., 2006; Krutzfeldt et al., 2005). Administration of antisense oligonucleotides against miR-122, in mice and in non-human primates, results in the reduction of plasma cholesterol levels via decreased hepatic biosynthesis of cholesterol and fatty acids (Esau et al., 2006; Krutzfeldt et al., 2005). Hepatic steastosis and circulating total cholesterol and triglycerides are also reduced in antimiR-122-treated mice fed a high fat diet (Esau et al., 2006).

Probably the best characterized metabolic microRNAs are the members of the miR-33 family. MiR-33a and miR-33b are two intronic microRNAs located in the human *SREBF2* and *SREBF1* genes, respectively. MiR-33a in rodents, and both miR-33a and miR-33b in non-human primates, have been shown to collaborate with the host gene in a regulatory circuit that controls intracellular cholesterol levels and lipid homeostasis (Rayner et al., 2010). MiR-33a and miR-33b repress the ATP-binding cassette transporter subfamily A1 (ABCA1), which mediates the transport of free cholesterol from the cell to plasma HDL lipoprotein complexes (Najafi-Shoushtari et al., 2010). Genetic deletion and pharmacological inhibition of miR-33a in mice increases plasma HDL levels and promotes cholesterol efflux from mouse macrophages (Rottiers and Naar, 2012).

Consistently, administration of anti-miR against miR-33a in LDLR-knockout mice fed a high fat diet increases circulating HDL levels and enhances cholesterol efflux, resulting in the reduction of atherosclerotic plaque size and lipid content (Rayner et al., 2011). These findings confirm the therapeutic potential of miR-33 inhibition for the treatment of hypercholesterolemia and cardiovascular disease. The importance and complexity of maintaining appropriate cholesterol levels is exemplified by the observation that two other microRNAs, miR-758 and miR-106b, also repress cellular cholesterol efflux via targeting of *ABCA1* (Kim et al., 2012; Ramirez et al., 2011). In keeping with the hypothesis that miR-33a and miR-33b collaborate with their host genes to form a complex regulatory network required for the maintenance of cholesterol and lipid homeostasis, many other genes involved in metabolic processes controlled by SREBP have been identified as targets of miR-33 family members. For example, miR-33a and miR-33b regulate the expression of proteins involved in fatty acid β-oxidation. Similarly, miR-33a and miR-33b modulate the expression and functions of upstream regulators of glucose, fatty acid and lipid homeostasis, including SIRT6, AMPKα1, and IRS2 (Rottiers and Naar, 2012).

Other microRNAs are involved in the proliferation and differentiation of pancreatic α and β cells, as well as in the maintenance of pancreatic insulin-secreting functions. Examples include miR-375 and miR-124, two co-expressed microRNAs that are required *in vivo* for proper islet formation and development, and insulin exocytosis and secretion (Baroukh and Van Obberghen, 2009). In contrast, the miR-29 family members, miR-29a and miR-29b, repress insulin secretion via targeting of the transporter MCT1 (Pullen et al., 2011). The expression profile of miR-29a and miR-29b is

indicative of their important role not only in insulin secretion but also in glucose uptake and utilization in insulin-target tissues. Indeed, miR-29a and miR-29b are expressed in skeletal muscle, white adipose, and liver, where they function as repressors of insulin signaling via targeting of caveolin 2. Consistently, miR-29a and miR-29b are markedly up-regulated in animal models of diabetes and their overexpression causes insulin resistance (He et al., 2007). Other examples of microRNAs involved in the regulation of downstream insulin signaling are miR-126, which targets IRS1, miR-223, which targets GLUT4, miR-33a and miR-33b, which target IRS2, SIRT6 and AMPKα (Rottiers and Naar, 2012). Through sequence-specific repression of their target genes, these microRNAs inhibit glucose uptake and induce the development of insulin resistance in mice (Rottiers and Naar, 2012). Multiple animal models of obesity and insulin resistance show the differential regulation of microRNA patterns in liver and adipose tissue. MiR-143, miR-103, miR-107, and members of the let-7 family, all negatively regulate insulin signaling and are up-regulated in liver of leptin-deficient mice and in diet-induced obesity models (Frost and Olson, 2011; Rottiers and Naar, 2012; Zhu et al., 2011).

Among the metabolic dysfunctions that occur in insulin-target tissues during obesity and related diseases, the ones affecting adipocyte proliferation, hypertrophy, hyperplasia and endocrine functions are some of the most profound *in vivo*. Recently, it has been shown that the heart-specific miR-208a regulates systemic energy homeostasis via targeting of MED13, a subunit of the Mediator complex (Grueter et al., 2012). Pharmacological inhibition of miR-208a in mice confers resistance to high-fat diet-induced obesity and improves systemic insulin sensitivity and glucose tolerance (Grueter

et al., 2012). These findings identify miR-208a as an important component of the transcriptional circuit formed by thyroid hormone and nuclear hormone receptors.

Mir-143, miR-204, miR-141, miR-429, and the miR-200 family members are all involved in adipocyte differentiation (Rottiers and Naar, 2012). Similarly, mir-17, miR-92, miR-130, miR-27a, miR-27b, miR-335 and the miR-378/378* cluster are all involved in lipid metabolism and adipogenesis (Rottiers and Naar, 2012). In particular, overexpression of miR-378/378* in ST2 adipocytes increases triacylglycerol accumulation, and up-regulation of genes involved in fatty acid metabolism, including FABP4, FAS, and SCD1 (Gerin et al., 2010a). In addition, overexpression of miR-378* in breast cancer cells induces a metabolic shift away from fatty acid utilization towards glycolysis (Eichner et al., 2010).

Another microRNA highly expressed in metabolically active tissues is miR-34a. This microRNA is upregulated in obese human patients, as well as in rodent models of obesity, type 2 diabetes and non-alcoholic fatty liver disease (Rottiers and Naar, 2012). MiR-34a targets SIRT1 and, in so doing, it reduces the activation of PPARs and LXR nuclear hormone receptors on one side, and SREBPs and NF-kB on the other side (Lee and Kemper, 2010). In turn, miR-34a is inhibited at multiple levels by SIRT1 in a regulatory loop that includes also FXR and p53. The repressive functions of miR-34a result in the negative regulation of lipogenesis, cholesterogenesis, energy homeostasis and inflammation (Lee and Kemper, 2010).

In summary, multiple microRNAs play a fundamental role in the regulation of different aspects of metabolism in various tissues, from the upstream modulation of the insulin signaling cascade, to the repression of downstream mediators of glucose uptake

and utilization. Some microRNAs regulate pancreatic cell development and maintenance, while many other microRNAs control cholesterol homeostasis, adipocyte differentiation and lipid uptake. This complex and diffuse regulatory network, even if challenging to dissect, offers multiple possibilities for therapeutic intervention in the modulation of metabolic disorders.

MicroRNAs as therapeutic targets

Based on the genetic evidence that perturbations in the levels of single miRNAs are either a cause or a marker of pathological states, the pharmacological modulation of microRNA abundance and function represents a promising therapeutic approach for the treatment of a wide variety of human diseases.

MicroRNAs can be inhibited by chemically modified antisense oligonucleotides containing sequences of complementarity to the mature microRNA. The development of microRNA-based antisense oligonucleotides called anti-miRs aims to inhibit the function of specific disease-associated microRNAs. Through the years, microRNA-based therapeutics have evolved in the antisense chemistries and delivery strategies to enhance their specificity, safety, potency, and bioavailability. The assessment of microRNA inhibition and potential off-target effects are important issues that have been addressed in the development of RNA-based therapeutic approaches. In particular, methods for controlled tissue-specific delivery of microRNA inhibitors or microRNA mimics, as well as novel modification, conjugation and formulation strategies, are required to reduce the toxicity and off-target effects of these molecules. Nonetheless, the delivery of oligonucleotide-based inhibitors of a specific microRNA remains difficult for some

anatomical compartments, such as brain and muscle (Broderick and Zamore, 2011; Stenvang et al., 2012). However, as demonstrated by loss-of-function studies, microRNAs function in cellular stress responses, whereas their expression is often dispensable under normal laboratory conditions. This characteristic suggests that the pharmacological inhibition of microRNAs is a safe means of disease modulation.

The first-generation microRNA inhibitors consisted of 2'-O-methyl ribose-modified RNA. They were administered in formulations that allowed lipid-mediated transfection into cultured human cells or injection in *C. elegans* (Broderick and Zamore, 2011). The first RNA-based microRNA inhibitors able to effectively reduce the amount of mature microRNA in mammals were named antagomirs. These molecules contain a cholesterol group to improve their intracellular delivery (Broderick and Zamore, 2011). However, antagomirs present numerous limitations related to the affinity of target binding and especially to their poor distribution, bioavailability and stability properties, as they are readily degraded *in vivo* by nucleases. These limitations prompted the development of alternative RNA chemistries, such as 2'-O-methoxyethyl, 2'-fluoro and 2',4'-methylene locked nucleic acids (LNAs), which have greater affinity for the target microRNAs and are more stable. The chemistry of LNAs allows the formation of a very stable anti-miR structure, as the ribose is constrained in the C3' endo conformation. As a consequence, the LNA modification reduces anti-miR degradation by endonucleases and increases the melting temperature of RNA duplexes, conferring high specificity for target sequences. Antagomirs function by blocking the association of target microRNAs with the RISC complex, thus promoting degradation of the microRNA. Furthermore, LNA-based anti-miRs are thought to bind to microRNAs but not to promote their degradation.

The high target binding affinity of these locked oligonucleotide structures allowed their further development in recent years to generate tiny LNAs, 8-mer LNA oligonucleotides complementary to the seed region of the target microRNAs (Broderick and Zamore, 2011; Mendell and Olson, 2012; Obad et al., 2011; Stenvang et al., 2012). The use of tiny LNAs has been shown to be particularly effective in the simultaneous inhibition of multiple members of microRNA families, which share the same seed sequence (Obad et al., 2011).

Multiple studies have proven the efficacies of anti-miR molecules in reducing the amount of specific target microRNAs both *in vitro* and *in vivo*. For example, inhibition of miRNA-132 prevents angiogenesis in a carcinoma mouse model. More strikingly, an LNA-modified anti-miR against miRNA-122 is now being tested in clinical trials for the treatment of hepatitis C virus infection (Lanford et al., 2010). In another study, subcutaneous delivery of an anti-miR against miR-208a in hypertensive rats prevented pathological cardiac remodeling, improved cardiac function, and survival (Montgomery et al., 2011). Despite the successful application of multiple anti-miRs, some improvements are still required, including the reduction of off-target effects, the effective delivery to tissues such as brain and skeletal muscle, the evaluation of long-term administration regimens, and the development of efficient methods to determine microRNA activity on downstream target genes. Similarly to microRNA inhibitors, injectable microRNA mimics has been developed to enhance the function of specific microRNAs. However, their application has proven to be more problematic and less effective *in vivo* compared to anti-miRs (Mendell and Olson, 2012). Nevertheless, the potential applications of both microRNA mimics and LNA-modified anti-miRs are

numerous. In particular, LNA-modified anti-miRs seem to be specific, stable and non-toxic when administered *in vivo*. They mediate efficient microRNA silencing and are powerful tools to validate the role of specific microRNAs or microRNA families in multiple disease settings. In the same way, anti-miR-based treatments represent a new therapeutic approach for disease modulation.

Concluding remarks

Mouse models lacking expression of different microRNAs have proven to be important tools for the analysis of microRNA function in development, physiology and disease. Together with the new understanding of the role of microRNAs in regulating multiple steps of the cellular stress response, the intense research effort that involved microRNA biology in recent years has provided new opportunities for therapeutic intervention in disease treatment. Identification and validation of mRNA targets remains a challenge on multiple fronts, both at the experimental and computational level. However, it will undoubtedly reveal novel mediators of cellular responses to a variety of pathophysiological signals in different cellular contexts. Another issue that has to be addressed is the functional redundancy among microRNAs, especially within microRNA families. The development of both genetic and pharmacological tools for the inhibition of specific microRNAs will provide a powerful base for functional studies, as well as fine modulation of complex genetic regulatory networks. To this end, the systemic administration of anti-miR molecules and microRNA mimics represents a method to alter the level of specific microRNAs and their target genes, providing a new approach for

therapeutic intervention, potentially void of toxicity, in the setting of multiple pathologic states from cancer to obesity.

Chapter II

Histone Deacetylases in Metabolism and Disease

Introduction

In all eukaryotic cells, the transcriptionally inactive DNA is compacted around histone proteins within the nucleosome. The nucleosome is the fundamental unit of chromatin and consists of four core histones - an H3/H4 tetramer and two H2A/H2B dimers (Kornberg and Lorch, 1999). Transcriptional regulation of eukaryotic gene expression is tightly linked to the dynamic post-translational modification of the amino group of lysine residues located on the tail of histone proteins. Indeed, these covalent modifications influence local chromatin structure, ultimately determining the accessibility of DNA to multiple factors, including the core transcriptional machinery (Luger and Richmond, 1998; Strahl and Allis, 2000). The best characterized covalent modification of histone tails is acetylation. Two opposing classes of enzymes act in an antagonizing manner to control the acetylation state of all core histones - the histone acetyltransferases (HATs) and the histone deacetylases (HDACs), respectively, add and remove acetyl groups from lysine residues on the histone tails (Grunstein, 1997; Struhl, 1998). In general, decreased acetylation levels of histone tails are associated with a compact chromatin architecture, where the DNA is inaccessible to transcription factors. In contrast, acetylation of histone tails is associated with increased transcriptional activity (Strahl and Allis, 2000; Struhl, 1998). Thus, the balance between the actions of HATs and HDACs represents the key mechanism in the regulation of physiological transcriptional states in mammals. As predicted, aberrant gene expression that characterizes different pathological states is often associated with alterations in the expression and function of HDACs (Lee et al., 2007).

Classification and biological function of histone deacetylases

The first HDAC was discovered in the second half of the 1990s (Brownell and Allis, 1995). HDACs catalyze the reversible addition of an acetyl moiety to the ε-amino group of lysine residues. It soon became evident that the substrates of HDAC enzymes are not limited to core histone proteins but include other factors, such as p53, E2F, α-tubulin, and MyoD (Choudhary et al., 2009). This substrate plasticity, together with a well-defined role of HDACs in the control of basic transcriptional activity, demonstrates how HDACs function at multiple levels to govern specific patterns of gene expression in different cell types, during different times of the cell cycle, and in response to different physiological and pathological stimuli. Since HDACs do not bind DNA directly, they depend on designated sets of transcription factors for recruitment to specific target genes, often within large multi-protein complexes (Yang and Seto, 2003). In keeping with the notion that acetylation of histones is associated with increased gene expression, HDACs are generally considered transcriptional repressors (Clayton et al., 2006; Shahbazian and Grunstein, 2007). Indeed, the versatility of HDACs is exemplified by their ability to activate the expression of some genes (Gallinari et al., 2007). Of note, genetic deletion of different HDAC isoforms causes the up-regulation and down-regulation of approximately the same number of genes (Glaser et al., 2003; Montgomery et al., 2007).

The mammalian genome encodes 11 classical HDAC proteins, which are grouped in four classes according to sequence similarity, subcellular localization, expression pattern, and cofactor dependency (Figure 2.1) (Gregoretti et al., 2004). Class I HDACs display sequence similarity to the yeast RPD3 transcription factor and include HDAC1, -2, -3, and -8 (Taunton et al., 1996). Class IIa HDACs are closely related to the

yeast HDA1 deacetylase and consist of HDAC4, -5, -7, and -9. HDACs -6 and -10 belong to the class IIb. HDAC11 forms the class IV. In addition, mammalian deacetylases include seven sirtuin enzymes, also referred to as class III HDACs, which requires NAD^{+} instead of Zn^{2+} as cofactor (Yang and Seto, 2007).

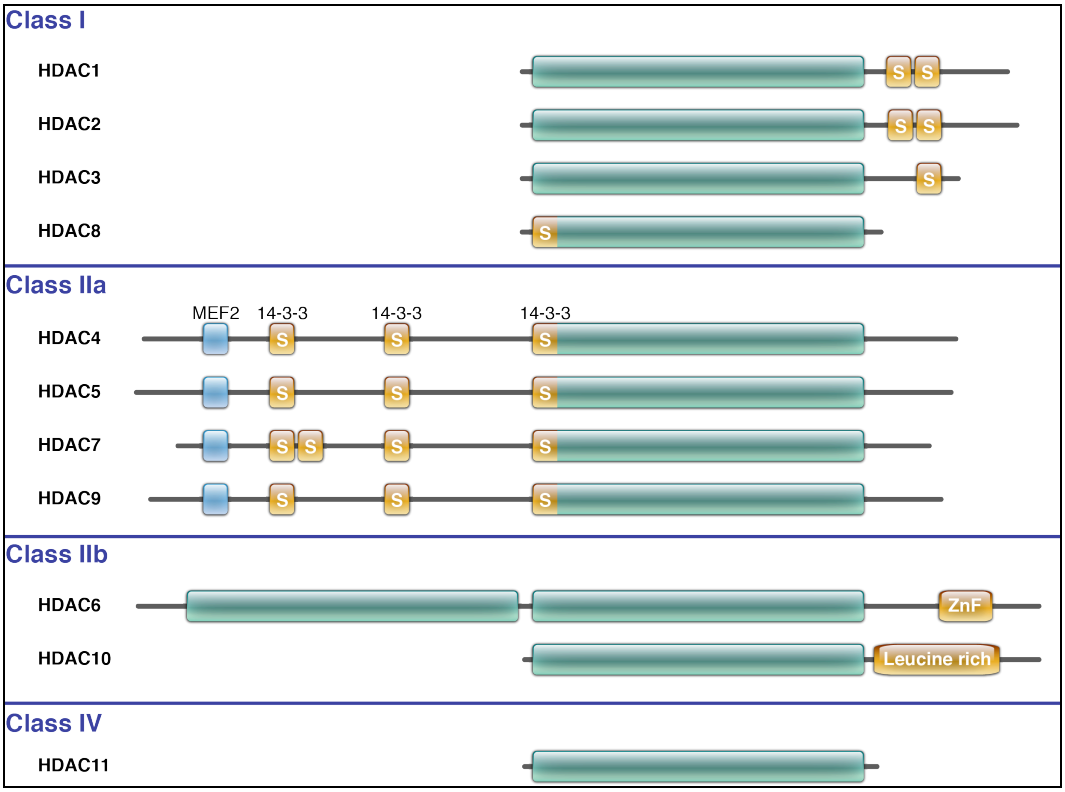


Figure 2.1. HDAC isoforms. Schematic representation of the various members of the HDAC superfamily showing their different protein domains (adapted from Haberland et al., 2009c). The conserved HDAC domains are shown in green. The MEF2 and 14-3-3 binding sites are indicated. S, serine phosphorylation sites; ZnF, zinc finger domain.

After the first HDAC was identified there was a surge in research interest aimed at understanding the biological functions of the various HDACs in development, physiology and pathogenesis. Initially, *in vitro* investigation offered the ability to identify numerous target genes of different HDACs, confirming the role of HDACs as

transcriptional repressors in multiple cell types and conditions. HDAC inhibitors (HDACi) also represented an important tool to identify target genes and to study the biochemical functions of HDACs, despite several issues related to the limited isoform-specificity of the first-generation of HDACi (Dokmanovic, 2007; Wagner et al., 2010). However, *in vivo* analysis, and the generation of isoform-specific knockout mice, uncovered the mechanism of action of HDACs, in some cases highlighting the redundancy of function among different isoforms (Haberland et al., 2009c).

Class I HDACs The class I HDACs include HDAC1, -2, -3, and -8. These HDACs share a high degree of sequence homology, common substrates and a ubiquitous expression pattern. Class I HDACs display a conserved deacetylase domain and short amino- and carboxy-terminal regions (Yang and Seto, 2003, 2008). HDAC1, -2, and -8 localize exclusively in the nucleus, while HDAC3 can be found in the cytoplasm, as it has both a nuclear export signal and an import signal (de Ruijter et al., 2003). The subcellular localization of HDAC3 is often determined by the interaction with other nuclear cofactors, such as NCoR, SMRT, as well as HDAC4, -5, and -7 (Fischle et al., 2002). Four repressive protein complexes that contain both HDAC1 and HDAC2: Sin3, NuRD, Co-REST and PRC2 have been characterized (Yang and Seto, 2003). Indeed, to be functionally active, HDAC1 and -2 require cofactors that mediate their binding to DNA and direct them to specific target gene promoters. Phosphorylation of serine residues of HDAC1 and -2 is responsible for increased deacetylase activity (Grunstein, 1997; Shahbazian and Grunstein, 2007). While global genetic deletion of HDAC1 causes lethality in mice at embryonic day 10.5, mice with tissue-specific knockout of HDAC1 in heart, skeletal muscle and smooth muscle are viable, likely due to redundancy with

HDAC2 functions (Haberland et al., 2009c; Montgomery et al., 2007). HDAC1-null mice display proliferation defects and growth delay associated with increased expression of p21 and p27 cyclin-dependent kinase inhibitors (Zupkovitz et al., 2006). Genetic deletion of HDAC2 in mice causes lethality around post-natal day 1, due to uncontrolled proliferation of cardiomyocytes and consequent severe cardiac defects (Montgomery et al., 2007). Skeletal muscle deletion of both HDAC1 and HDAC2 results in perinatal lethality of a subset of mice due to progressive myopathy, mitochondrial abnormalities, sarcomere degeneration and abnormal metabolism resulting from a blockade of the autophagy flux (Moresi et al., 2012).

The poorly conserved C-terminal region of HDAC3 is responsible for its activity (de Ruijter et al., 2003). HDAC3 functions in corepressor complexes that include SMRT and NCoR (Fischle et al., 2002). Mice lacking the expression of HDAC3 die at embryonic day 9.5 due to gastrulation abnormalities (Knutson et al., 2008; Montgomery, 2008). Liver and heart-specific knockout of HDAC3 revealed its fundamental role within the nuclear hormone receptor transcriptional circuit, which is responsible for the maintenance of energy homeostasis, fatty acid uptake, and lipid metabolism in mice (Knutson et al., 2008; Montgomery, 2008). In fact, loss of HDAC3 in both hepatic and cardiac tissue results in lipid accumulation and reduced glycogen pools as a consequence of the deregulated expression of genes downstream of peroxisome proliferator-activated receptors (Knutson et al., 2008; Montgomery, 2008). Mice with cardiac-specific HDAC3 deletion die at about 4 months of age with cardiac hypertrophy, fibrosis and ectopic lipid accumulation (Montgomery, 2008).

HDAC8 shares 54% sequence similarity with HDAC1 and HDAC2. It is ubiquitously expressed, with strongest expression in brain (de Ruijter et al., 2003). Genetic deletion of HDAC8 in mice results in the derepression of homeobox transcription factors specifically in cranial neural crest cells, which causes the loss of neural crest cells and cranial skeletal elements (Haberland et al., 2009b). Also, knockdown of HDAC8 by RNA interference showed an anti-proliferative effect in human lung, colon, and cervical cancer cell lines (Vannini et al., 2004).

Class II HDACs HDAC4, -5, -6, -7, -9, and -10 form the class II family. In detail, HDAC4, -5, -7, and -9 are class IIa HDACs, whereas HDAC6 and -10 are class IIb HDACs. The class II HDACs can generally shuttle between the nucleus and the cytoplasm in response to different stimuli. In particular, the subcellular localization of HDAC4 and -5 is tightly regulated during muscle cell differentiation. During proliferation of muscle cells, HDAC4 is phosphorylated by the Ca^{2+}/calmodulin-dependent protein kinase (CaMK) and exported in the cytoplasm, where it is sequestered by 14-3-3 chaperone protein. In the same stage, HDAC5 is present in the nucleus. During terminal differentiation of muscle cells, HDAC5 localizes to the cytoplasm while HDAC4 returns to the nucleus following dephosphorylation and release from 14-3-3 (Backs et al., 2006; Bassel-Duby and Olson, 2006). Mutant mice that lack the expression of each member of the class II HDACs have been generated, revealing a high degree of redundancy and a common repressive activity on the myogenic transcription factor myocyte enhancer factor 2 (MEF2) (Haberland et al., 2009c). In particular, HDAC4, -5, and -7 bind to and repress MEF2 via their N-terminus domain. Interaction of MEF2 with the class IIa HDACs is regulated by CaMK and protein kinase D (PKD)-mediated

phosphorylation of these HDACs (Backs et al., 2006; Kim et al., 2008). Dissociation of HDACs from MEF2 allows the conversion of MEF2 to a transcriptional activator via acetylation by p300 (Ma et al., 2005). The repressive function of class IIa HDACs on MEF2 is particularly relevant in skeletal muscle, where the expression of slow myofiber genes is under the control of MEF2. Indeed, genetic deletion of class IIa HDACs in skeletal muscle results in the conversion of fast myofibers to slow myofibers (Potthoff et al., 2007). Thus, HDAC4, -5, and -7 function in multi-protein complexes to link extracellular signals and transcriptional responses. They provide a scaffold for the interaction of DNA-binding corepressor molecules and class I HDACs, including HDAC3 (Grozinger and Schreiber, 2000). In fact, it has been concluded that the intrinsic catalytic activity of class IIa HDACs is not necessarily required for transcriptional repression (Lahm et al., 2007).

HDAC4 is highly expressed in brain and chondrocytes. Knockout mouse models revealed a fundamental role of HDAC4 in the regulation of the conversion of cartilaginous skeletal elements to ossified bone (Vega et al., 2004). In addition, it has been shown that mice lacking HDAC4 and -5 in skeletal muscle fail to preserve muscle mass following denervation due to the inability to up-regulate the transcription factor myogenin (Moresi et al., 2010).

HDAC5 and HDAC9 are highly enriched in muscles, heart and brain. Double knockout mice for HDAC5 and HDAC9 display abnormalities in cardiomyocytes growth, increased susceptibility to cardiac mechanical stress, and propensity to develop myocardial defects (Chang et al., 2004). HDAC9 knockout mice are sensitized to other types of stress, such as muscle denervation. Conversely, overexpression of HDAC9 in

skeletal muscle confers resistance to the effects of denervation. The importance of HDAC9 in muscle differentiation is further confirmed by its MEF2-dependent regulation. In fact, MEF2 can bind to the HDAC9 promoter to induce the expression of its own repressor in a negative forward loop (Haberland et al., 2007; Mejat et al., 2005).

HDAC7 is enriched in T-cell precursors and endothelial cells. This pattern of expression explains the embryonic lethality associated with the lack of HDAC7 expression in mice, where the absence of HDAC7 results in the loss of integrity of the blood vessel walls (Miano and Berk, 2006).

HDAC6 displays two catalytic domains, a zinc finger domain and an ubiquitination site. It can be found in the nucleus in complex with HDAC11. However, HDAC6 mainly resides in the cytoplasm where it regulates cell motility by controlling acetylation of tubulin (Zhang et al., 2008).

Two splice variants exist for HDAC10. Mutagenesis analysis revealed that both the catalytic and the C-terminal domain are required for HDAC10 activity. Furthermore, HDAC10 interacts with HDAC1, -2, -3, and -4 via its N- and C-terminal domains (Kao et al., 2002).

Class IV HDACs The only member of the class IV is HDAC11. The catalytic domain of HDAC11 is located at the N-terminus (Gao et al., 2002). The activity of HDAC11, which is not incorporated in corepressor complexes, is inhibited by trapoxin (Gao et al., 2002). HDAC11 has been shown to repress the production of IL-10 (Villagra et al., 2009). In addition, its expression is up-regulated in various cancer cell lines (Gao et al., 2002).

Role of histone deacetylases in metabolism and metabolic disorders

In recent years, substantial evidence has accumulated for an important contribution of HDACs in the maintenance of energy homeostasis, and in the pathogenesis of metabolic conditions, such as insulin resistance, diabetes and obesity. Consequently, HDACi could be used for the design of new therapeutic approaches in the treatment and prevention of metabolic syndrome.

One of the first indications that HDACs may play a role in the etiology of diabetes came from genome-wide association scans of both type 1 (T1D) and type 2 (T2D) diabetes loci. A significant linkage was found between the chromosomal region 6q21, where HDAC2 is located, and both T1D and T2D (Nerup and Pociot, 2001; Xiang et al., 2004).

In addition, several studies demonstrated that class IIa HDACs, especially HDAC4 and -5, cooperatively regulate the expression of metabolic genes in heart and skeletal muscle, where oxidative function is compromised upon obesity and T2D (McGee and Hargreaves, 2010). The phosphorylation-dependent nuclear export of class IIa HDACs is controlled by kinases, including the AMP-activated protein kinase (AMPK) and PKD, two pivotal energy sensors in the mammalian cell (McGee and Hargreaves, 2010; McGee et al., 2008).

The role of HDACs in the regulation of metabolic pathways in heart and skeletal muscle reflects the intimate connection between these enzymes and MEF2. Indeed, class II HDACs represses MEF2 activity. This repression is released in response to calcium signaling (Ha et al., 2010; McGee and Hargreaves, 2010; McGee et al., 2008; Potthoff et al., 2007). Inducible cardiac overexpression of a signal-insensitive form of HDAC5

resulted in sudden death in male mice accompanied by defects in the function and morphology of cardiac mitochondria, due in part to down-regulation of the transcriptional coactivator PGC-1α (Czubryt et al., 2003). Two MEF2 binding sites in the PGC-1α upstream region mediate transcriptional activation by MEF2 and repression by HDAC5 (Czubryt et al., 2003). Consistently, administration of trichostatin A (TSA), an HDACi, in mice induces up-regulation of PGC-1α expression in skeletal muscle (Minetti et al., 2006). Activation of the PGC-1 transcriptional cascade in myocytes could be beneficial in the context of T2D.

Thus, the HDAC/MEF2 axis plays an important role in the regulation of oxidative metabolism in heart and in skeletal muscle. Indeed, in oxidative myofibers, the proteasome-mediated degradation of class II HDACs allows derepression of MEF2 transcription, and the consequent activation of the slow fiber program (Potthoff et al., 2007). In the heart, loss of class II HDACs has been shown to confer cardioprotection mediated by estrogen receptor and its regulation by MEF2 (van Rooij et al., 2010).

In addition, MEF2 family members can induce the transcription of *Glut4* in response to insulin signaling (Karnieli and Armoni, 2008). Diabetic rodents have reduced expression of MEF2A and MEF2D in skeletal muscle and adipose tissue, respectively (Mora et al., 2001). As a regulator of peripheral glucose uptake, GLUT4 plays a fundamental role in the insulin pathway, both in striated muscle and in adipose. In fact, insulin signaling is initiated by binding to the insulin receptor and phosphorylation of members of the insulin receptor substrate (IRS) family. Upon phosphorylation, IRSs activate phosphatidylinositol 3 kinase (PI3K) that in turn phosphorylates Akt . Activation of this protein kinase induces translocation of GLUT4

from intracellular vesicles to the plasma membrane, promoting glucose uptake (Huang and Czech, 2007). *In vitro* studies using the 3T3-L1 cell line and human primary myotubes demonstrated that *Glut4* expression is dependent on nuclear HDAC5, confirming on one side the importance of AMPK-mediated phosphorylation/nuclear export of HDAC5 and on the other side the role of the MEF2/GLUT4/HDACs circuit in insulin-target tissues (Weems and Olson, 2011). HDAC1 and HDAC4 are also able to repress *Glut4* expression in adult muscle tissue and adipocytes in response to adrenergic activation (Weems et al., 2012).

In addition to the regulation of GLUT4 expression and translocation, HDACs control other aspects of muscle metabolism. For example, HDAC1 and -2 control muscle homeostasis and autophagy flux in skeletal muscle (Moresi et al., 2012). Mice lacking both HDAC1 and -2 in skeletal muscle develop a progressive myopathy due to impaired autophagy flux. Furthermore, myocytes from HDAC1 and -2 skeletal muscle knockout mice display a shift toward oxidative metabolism (Moresi et al., 2012).

Using different animal systems, several studies have proposed a beneficial effect of HDACi treatment in ameliorating global insulin sensitivity through increased oxidative metabolism in skeletal muscle (Dokmanovic, 2007). For example, prolonged treatment of myocytes with the inhibitor scriptide induces glucose uptake (Takigawa-Imamura et al., 2003). Other HDACi, such as sodium butyrate, improve metabolic dysfunction in mice fed a high fat diet (Gao et al., 2009). Indeed, supplementation with sodium butyrate prevents development of diet-induced insulin resistance and obesity. The metabolic changes observed upon administration of sodium butyrate reflect increased adaptive thermogenesis in brown adipose tissue. Energy expenditure and mitochondria function

are also enhanced in skeletal muscle, together with fatty acid oxidation and the number of slow myofibers (Gao et al., 2009).

HDACs play an important role in the modulation of metabolic gene expression not only in heart and skeletal muscle but also in the liver. In hepatocytes from ob/ob mice, a model of insulin resistance, IRS-1 signaling is repressed by HDAC2, whereas RNAi-mediated knockdown of HDACs partially restores insulin sensitivity (Kaiser and James, 2004).

In the liver, chromatin dynamics are sensitive to a regulatory cascade initiated by bile acids (De Fabiani et al., 2010). The atypical orphan nuclear receptor SHP, an important component of bile acid-mediated cholesterol metabolism, has been shown to interact with HDAC1 and -2 (De Fabiani et al., 2010). Furthermore, bile acids induce the recruitment of multiple HDACs, including HDAC1, -3, and -7, to a repressor complex that silences the *Cyp7a1* gene, and consequently reduces hepatic gluconeogenesis and bile acid synthesis (De Fabiani et al., 2010). Treatment of cultured hepatocytes with HDACi causes the release of *Cyp7a1* expression (De Fabiani et al., 2010). Treatment of mice on high fat diet with a chlamydocin-hydroxamic acid analog that is able to inhibit class I HDACs activity reduces gluconeogenesis in the liver via down-regulation of phosphoenol pyruvate carboxykinase (*Pepck*), hepatocyte nuclear factor 4a (*Hnf4a*) and suppression of forkhead box O 1 (FOXO1) activity (Oiso et al., 2011).

In the liver, genetic deletion of HDAC6 results in impaired dexamethasone-induced glucocorticoid receptor translocation and gluconeogenesis (Winkler et al., 2012). Consequently, liver-specific HDAC6 knockout ameliorates glucocorticoid-induced hyperglycemia, glucose intolerance, and insulin resistance (Winkler et al., 2012). Thus,

selective inhibition of HDAC6 could provide a novel approach to control hepatic gluconeogenesis and glucose metabolism through modification of glucocorticoid receptor nuclear translocation.

The pancreas, and in particular pancreatic β-cells, play a central role in the regulation of serum glucose and insulin levels. HDACs have been shown to regulate β-cells replication and apoptosis via transcriptional regulation of different factors implying that alterations of acetylation influence diabetes pathogenesis. During low glucose conditions, HDAC1 and -2 interact with PDX-1 to repress transcription of insulin in insulin-producing pancreatic cells (Christensen et al., 2011). In the same cells, expression of Pax4, a critical regulator of pancreatic cell fate, is repressed by the NRSF/REST complex via recruitment of HDACs (Gray and De Meyts, 2005).

Endocrine β-cells of the pancreas express class IIa HDAC4, -5, -7, and -9 (Lenoir et al., 2011). Mice lacking the expression of either HDAC5 or HDAC9 in the pancreas have an increased pool of insulin-producing β-cells (Lenoir et al., 2011). Treatment of pancreatic explants with the selective class IIa HDACi MC1568 results in up-regulation of Pax4 and an increased number of endocrine β-cells (Lenoir et al., 2011). Similarly, sodium butyrate increases both glucagon and insulin gene expression in rat islet cell lines, and induces pancreatic β-cell differentiation (Philippe et al., 1987).

Immunological and inflammatory responses are essential components of the metabolic syndrome that is associated with obesity and diabetes. Generally, proinflammatory cytokines produced by immunocompetent cells have deleterious effects on pancreatic β-cells (Christensen et al., 2011). In T1D, for example, interleukin-1β (IL-

1β), IL-12, tumor necrosis factor α (TNFα) and interferon γ (IFNγ) contribute to β-cell destruction, mediated in part by nitric oxide (Christensen et al., 2011).

Several HDACi, including ITF2357, possess anti-inflammatory and cytokine-suppressing properties in streptozotocin (STZ)-treated mice, an animal model of diabetes (Lewis et al., 2011). *In vitro* experiments have shown that ITF2357 increases islet cell viability, insulin secretion, and reduces nitric oxide production and β-cells apoptosis (Lewis et al., 2011). Thus, HDACi promote β-cell development, proliferation, differentiation and function. In addition, HDACi have been shown to positively affect late diabetic micro-vascular complications (Advani et al., 2011; Gilbert et al., 2011; Kadiyala et al., 2012).

Adipose tissue is a key metabolically relevant tissue with important endocrine functions. In humans, as well as in rodents, fat cells play a fundamental role in storing physiological levels of triglyceride and free fatty acid, as well as in maintaining systemic insulin sensitivity (Kershaw and Flier, 2004). Alteration of these functional properties of adipose tissue often causes adverse metabolic effects. Thus, regulation of the endocrine functions of the adipose compartment, as well as the modulation of adipogenesis and adipocyte proliferation are important aspects in the design of therapeutic approaches for the treatment of increasingly prevalent disorders such as obesity, diabetes and related metabolic syndrome.

Both *in vitro* and *in vivo* experiments have demonstrated a fundamental role of HDACs in fat cell hypertrophy and hyperplasia. HDACs are also involved in lipid uptake and ectopic lipid deposition (Chatterjee et al., 2011; Miard and Fajas, 2005; Nebbioso et al., 2010; Weems and Olson, 2011). In addition, changes in the expression

profile of HDAC3, -4, -5, -10, and -11 in the medial hypothalamus of mice in response to either fasting or high fat diet suggest an important role of HDACs in the regulation of gene expression in the hypothalamus under different metabolic states (Funato et al., 2011). In the preadipocyte cell line 3T3-L1, the class II-selective inhibitor MC1568 attenuates adipogenesis while the class I-selective MS275 blocks adipocyte differentiation completely (Nebbioso et al., 2010).

HDAC9 overexpression in 3T3-L1 preadipocytes suppressed adipogenic differentiation (Chatterjee et al., 2011). The role of HDAC9 as negative regulator of adipogenesis is supported by the down-regulation of this HDAC preceding adipogenic differentiation (Chatterjee et al., 2011). Furthermore, primary adipocytes from HDAC9 knockout mice exhibit enhanced expression of the master regulator of adipogenic differentiation, C/EBPβ. Indeed, inhibition of adipogenesis by HDAC9 is independent of its deacetylase activity and is associated with the recruitment of HDAC9 at the promoter region of C/EBPβ (Chatterjee et al., 2011).

Other important factors involved in the modulation of the adipogenic gene program are PPARγ and the signal transducer and activator of transcription (STAT) proteins. *In vivo*, the class II-selective inhibitor MC1568 attenuates PPARγ signaling mostly in the heart and adipose tissues (Kimura et al., 2012; Miard and Fajas, 2005; Nebbioso et al., 2010). STAT1 and -2 have been shown to interact with HDAC1, while STAT5 associates with the HDAC3/NCoR/SMRT repressor complex (Gray and De Meyts, 2005). Similarly, the ability of PPARγ to activate the adipogenic gene program is repressed by a multi-protein complex containing HDAC3 (Fajas et al., 2002).

Class I HDAC3 is a component of the transcriptional regulatory circuit involving nuclear hormone receptors. Inactivation of HDAC3 in cancer cell lines induces apoptosis, while global *Hdac3* genetic deletion causes embryonic lethality in mice (Knutson et al., 2008; Montgomery, 2008; Sun et al., 2011). Tissue-specific knockout of HDAC3 revealed a fundamental role of this HDAC isoform in the maintenance of lipid metabolism and energy homeostasis in the heart and liver (Knutson et al., 2008; Montgomery, 2008; Sun et al., 2011). At around 4 months of age, mice lacking HDAC3 expression in the heart show massive cardiac hypertrophy and myocardial up-regulation of genes associated with fatty acid uptake, fatty acid oxidation, and oxidative phosphorylation (Montgomery, 2008). These metabolic changes derive from excessive activity of the nuclear receptor PPARα and are accompanied by myocardial lipid accumulation and elevated triglyceride levels (Montgomery, 2008). When fed a high fat diet, mutant mice lacking *Hdac3* postnatally in heart and skeletal muscle, die within weeks due to the severe hypertrophic cardiomyopathy and heart failure (Sun et al., 2011). These morphological changes in the heart are caused by dysregulation of myocardial mitochondrial genes involved in lipid metabolism (Sun et al., 2011).

Additional evidence showing the role of HDAC3 in the regulation of cell proliferation and lipid metabolism comes from genetic deletion of *Hdac3* in the postnatal mouse liver. Mice lacking the expression of HDAC3 in the liver display aberrant hepatocyte hypertrophy and consequent hepatomegaly accompanied by changes in the expression of genes controlled by nuclear hormone receptors, which utilize the NCoR/SMRT/HDAC3 complex to repress transcription (Knutson et al., 2008). Genes that regulate lipid and cholesterol biosynthesis are up-regulated in the hepatic tissue from

mutant mice, leading to increased liver triglycerides and cholesterol levels (Knutson et al., 2008).

In summary, different HDAC proteins play a central role in the regulation of several biological pathways relevant for the etiology and pathogenesis of obesity and diabetes. There is evidence that points to HDACs as important transcriptional regulators that link the metabolic state of the cell to gene transcription in both physiological and pathological conditions. Thus, the design of isoform-specific HDACi to modulate HDACs function represents a promising therapeutic approach in the treatment of obesity, diabetes and related metabolic syndrome.

Histone deacetylase inhibitors

The first compound to be recognized as an inhibitor of HDACs was sodium butyrate in the late 1970s (Candido et al., 1978). About ten years later, TSA was discovered to have potent inhibitory properties against HDACs (Yoshida et al., 1990). Suberoylanilide hydroxamic acid (SAHA) was identified shortly after and, in 2006, it became the first HDACi to be approved by the Food and Drug Administration (FDA) with the name of Vorinostat (Zolinza) for treatment of refractory cutaneous T-cell lymphoma (Dokmanovic, 2007; Richon et al., 1998; Wagner et al., 2010).

HDACi alter the acetylation state of both histone and non-histone proteins, and in so doing they modify the cellular gene expression profile. The most common effects include induction of apoptosis, cell cycle arrest, and inhibition of angiogenesis and metastasis (Dokmanovic, 2007; Wagner et al., 2010). Many HDACi also have immuno-modulatory and anti-inflammatory effects (Dokmanovic, 2007; Wagner et al., 2010).

Despite the ubiquitous expression of HDACs, HDACi selectively affect a small fraction of the cellular transcriptome (2-10%). Transformed cells generally show a higher sensitivity to HDACi compared to normal cells (Christensen et al., 2011; Dokmanovic, 2007; Wagner et al., 2010; Yang and Seto, 2007). The high tolerability and efficacy of the first HDACi used in *in vivo* treatment of cancer prompted the pharmaceutical industry to develop new HDACi with an increasing degree of isoform-specificity.

Four structurally distinct classes of inhibitors - hydroxamic acids, short-chain fatty acids, cyclic peptides, and benzamides - are now in clinical development (Figure 2.2) (Dokmanovic, 2007; Wagner et al., 2010).

The group of hydroxamic acids includes TSA, Scriptaid and the FDA-approved Vorinostat (SAHA). Seven compounds in this class are in clinical trials, including Belinostat. In general, these compounds display pan-HDAC inhibitory activity, high potency (nanomolar range), and anti-proliferative activities in different tumor cell lines. Often, preclinical efficacy has also been demonstrated in tumor xenograft models (Wagner et al., 2010).

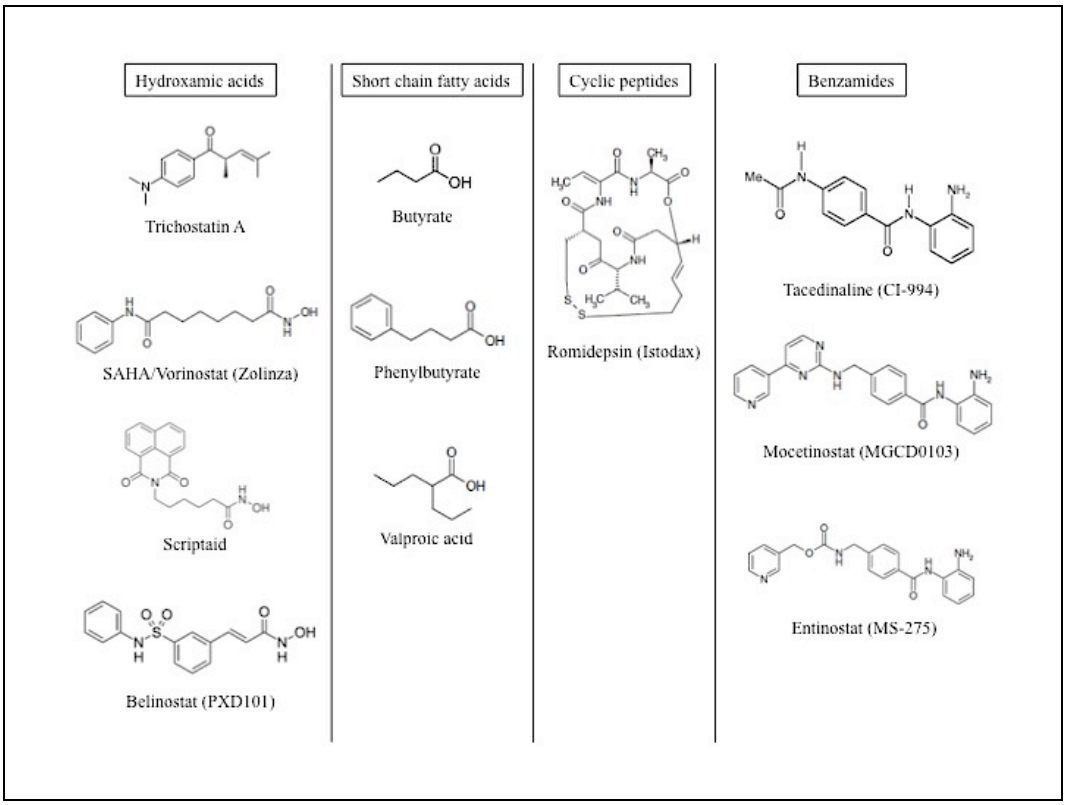


Figure 2.2. Chemical structure of HDACi. The structure of the HDACi described in the text is shown. Compounds are divided into the four different classes that are currently in clinical trials.

The group of short-chain fatty acids HDACi includes butyrate, phenylbutyrate and valproic acid. These compounds are less potent than TSA and other hydroxamic acid-based HDACi (micromolar to millimolar range). Compounds from this class have been used in the clinic. For example, valproic acid has been used as an antiepileptic drug for many years (Dokmanovic, 2007; Wagner et al., 2010).

Cyclic tetrapeptide antibiotics form the third group of inhibitors. These compounds are structurally complex and display high potency (nanomolar range). This

class includes the second FDA-approved HDACi, Romidepsin (Istodax) (Dokmanovic, 2007; Wagner et al., 2010).

The fourth class of HDACi in clinical trials is formed by the benzamides or amino anilides, which include Tacedinaline (CI-994), Mocetinostat and the class I-selective MS-275 (Entinostat). These compounds show anti-proliferative activity and anti-tumor activity in several tumor cell lines and in different xenograft models when used in the micromolar range (Dokmanovic, 2007; Wagner et al., 2010).

The mechanism of action of HDACi is not completely elucidated. However, most of these compounds bind to the zinc-containing catalytic site of HDACs in a reversible manner. Common downstream effects of HDAC inhibition include increased acetylation of histones, induction of p21, cell cycle arrest, inhibition of angiogenesis and metastasis formation, but also downregulation of IGF-1 receptor, inflammatory interleukins, and antiapoptotic genes (Dokmanovic, 2007; Wagner et al., 2010). These observations point to HDACi as potential therapeutics in nonmalignant diseases, such as inflammatory conditions. HDACi have been reported to delay the progression of neurodegenerative disorders in mice (Fischer et al., 2007). HDACi treatment has been shown to regulate cardiac plasticity in response to stress, and in particular to attenuate cardiac hypertrophy (Cao et al., 2011). Furthermore, TSA causes functional recovery of dystrophic muscles in mice, while other HDACi are potent activators of globin genes, which has beneficial implications for treatment of sickle-cell anemia (Cao et al., 2005; Minetti et al., 2006).

Concluding remarks

HDACs are enzymes that control a vast number of biological processes, both in physiological and pathological conditions. Their classical substrates are histones but evidence for unconventional targets is emerging. Non-histone substrates of HDACs include several transcription factors and signaling molecules, which could account for the specificity of phenotypes observed in mice lacking different HDAC isoforms. Debates on the possible redundancy of function of different HDACs, especially within the same class, and on whether HDACs control specific gene programs are still on-going. Genetic mouse models lacking expression of specific HDAC isoforms or expressing catalytically inactive forms of HDACs will provide insights on target specificity of different HDAC isoforms and will allow the design of more selective therapeutic approaches for HDACi. Currently, several clinical trials are being conducted to investigate the possible applications of HDACi in the treatment of a variety of disorders, ranging from cancer, immunological and inflammatory diseases, and neurodegenerative diseases. Reversibility of the inhibitory effects of HDACi and maintenance of the structural integrity of HDAC-containing multiprotein complexes following the administration of HDACi foster administration regimes that are well tolerated in humans. It remains to be determined whether selective inhibition of HDACs will be advantageous in the setting of cancer treatment. In fact, the plurality of target proteins of HDACi may be beneficial in the treatment of a broad spectrum of hematologic and solid tumors. However, in the setting of other pathological states, such as metabolic disorders, the development of isoform-selective inhibitors should lead to improved drug efficacy and safety.

Chapter III

Control of Mitochondrial Metabolism and Systemic Energy Homeostasis by MicroRNAs 378 and 378*

ABSTRACT

Obesity and related metabolic disorders are associated with aberrant energy homeostasis and are major risk factors for cardiovascular disease and diabetes. Multiple microRNAs (miRNAs) have recently emerged as important regulators of metabolic processes, however the contribution of individual miRNAs in the control of cellular energy homeostasis remains elusive. We showed that miR-378 and 378*, which are embedded in the *Ppargc1b* gene, are co-regulated with the transcriptional coactivator PGC-1β. Using a loss-of-function approach we demonstrated that miR-378/378* knockout mice are protected against high fat diet-induced obesity and are resistant to the development of metabolic syndrome. Further analysis showed enhanced mitochondrial fatty acid metabolism, up-regulation of tricarboxylic acid (TCA) cycle genes, and improvement of oxidative capacity of insulin-target tissues in the mutant mice lacking miR-378/378* expression. We identified *Crat* (carnitine O-acetyltransferase), which encodes a mitochondrial enzyme involved in fatty acid metabolism, as a target of miR-378. In addition, we showed that miR-378* targets a regulatory component of the Mediator complex, Med13. These observations indicate that miR-378 and miR-378* participate in the regulatory circuit centered on PGC-1β and the nuclear hormone receptors to govern the oxidative capacity and energy homeostasis of insulin target tissue under conditions of metabolic stress. Our findings point to miR-378 and miR-378* as potential targets for pharmacological intervention in the treatment of obesity and metabolic syndrome.

INTRODUCTION

Metabolic syndrome refers to a group of medical disorders that when clustered together increase the risk of obesity, diabetes and cardiovascular diseases (Alberti et al., 2009; Ford et al., 2010; Grundy et al., 2004). In industrialized countries metabolic syndrome affects more than 30% of the adult population (Ford et al., 2010). Recent studies show that altered mitochondrial function plays a critical role in the pathogenesis and progression of metabolic syndrome (Morino et al., 2006; Nisoli et al., 2007). Defects in mitochondrial oxidative metabolism of different energy sources, in particular fatty acids, have been linked to diet-induced obesity and the development of insulin resistance in adipose tissue and skeletal muscle (Bonnard et al., 2008; Gianotti et al., 2008; Kusunoki et al., 2006; Maassen et al., 2007; Zhang et al., 2012). Consistent with the observation that mitochondrial dysfunction is a risk factor for the development of metabolic syndrome, obese individuals have been reported to have mitochondria with compromised bioenergetic capacity (Anderson et al., 2009; Bonnard et al., 2008; Gianotti et al., 2008; Li et al., 2010).

PGC-1 proteins are co-activators that interact with a broad range of transcription factors to regulate mitochondrial biogenesis and function, thermogenesis, glucose metabolism and fatty acid oxidation (Finck and Kelly, 2006; Handschin and Spiegelman, 2006; Lin et al., 2005). The *Ppargc1b* gene, encodes PGC-1β, one of the members of the PGC-1 family, and is preferentially expressed in tissues with elevated mitochondrial content, such as heart, slow skeletal muscle and brown adipose tissue (Esterbauer et al., 1999). PGC-1β functions as a master regulator of metabolism in mammals (Lelliott et

al., 2006; Meirhaeghe et al., 2003; Sonoda et al., 2007; Vianna et al., 2006). Embedded in the first intron of the *Ppargc1b* gene are two microRNAs, miR-378 and miR-378*, which originate from a common hairpin RNA precursor (http://www.mirbase.org/).

MiRNAs are ~22 nucleotide single-stranded RNAs that mediate the degradation or inhibition of specific target mRNAs (Bartel, 2004, 2009; Djuranovic et al., 2011; Filipowicz et al., 2008). Approximately one-third of miRNAs are encoded by introns of protein-coding genes. Frequently, intronic miRNAs have been found to modulate the same biological processes as the protein encoded by the host gene (Baskerville and Bartel, 2005; Dill et al., 2012; Najafi-Shoushtari et al., 2010; Rodriguez et al., 2004). Recently, miRNAs have been linked to the post-transcriptional silencing of components of metabolic gene networks (Eichner et al., 2010; Frost and Olson, 2011; Gerin et al., 2010a; Gerin et al., 2010b; Grueter et al., 2012; Jordan et al., 2011; Naar, 2011; Najafi-Shoushtari et al., 2010; Rottiers and Naar, 2012; Trajkovski et al., 2011; Varghese et al., 2010; Zhu et al., 2011). Since *Ppargc1b*, the host gene of miR-378 and miR-378*, plays a critical role in the control of cellular energy homeostasis in conjunction with the nuclear hormone receptors, we investigated the role of miR-378 and miR-378* by deleting these miRNAs, while leaving the host gene intact. We showed that genetic deletion of miR-378 and -378* in mice is sufficient to protect against diet-induced obesity and insulin resistance *in vivo*. We identified *Crat* (carnitine O-acetyltransferase), which encodes a mitochondrial enzyme involved in fatty acid metabolism (Cordente et al., 2004; Noland et al., 2009; O'Donnell et al., 2002), as a target of miR-378. Furthermore, we demonstrated that Med13, a component of the Mediator complex involved in the regulation of nuclear hormone signaling (Ge et al., 2002; Grueter et al., 2012; Ito and

Roeder, 2001; Malik et al., 2004; Malik and Roeder, 2010; Pospisilik et al., 2010; Wang et al., 2002), is a target of miR-378*. Taken together, these results suggest that miR-378 and miR-378* regulate mitochondrial metabolism by counteracting the functions of the host gene *Ppargc1b*.

RESULTS

MiR-378 and miR-378* are co-regulated and co-expressed with PGC-1β

Both miR-378 and miR-378* are embedded in the first intron of the *Ppargc1b* gene (Figure 3.1A) and, similar to PGC-1β, are highly enriched in tissues with a high oxidative capacity, such as brown adipose tissue (BAT), heart and skeletal muscle (Figure 3.1B). Although it is common that the miRNA* strand is degraded, we and others detected miR-378* in various tissues (Figure 3.1B) (Eichner et al., 2010). To examine the response to metabolic stress we subjected mice to a high fat diet (HFD) and observed that *Ppargc1b*, miR-378, and miR-378* are up-regulated in the murine liver (Figure 3.1C). These findings show co-regulation of miR-378 and miR-378* with their host gene, *Ppargc1b*.

To further explore the regulation of the miR-378/378* cluster and its host gene, *Ppargc1b*, in different stress conditions, we examined their expression in the heart during pathological cardiac hypertrophy. Under normal conditions, the heart derives the majority of its energy from mitochondrial oxidation of fatty acids (Doenst et al., 2001; Goodwin et al., 1998; Taegtmeyer et al., 2004). However, during heart failure mitochondrial oxidative capacity is diminished and the metabolism of the heart shifts away from fatty acids toward glycolytic metabolism (Goodwin et al., 1998).

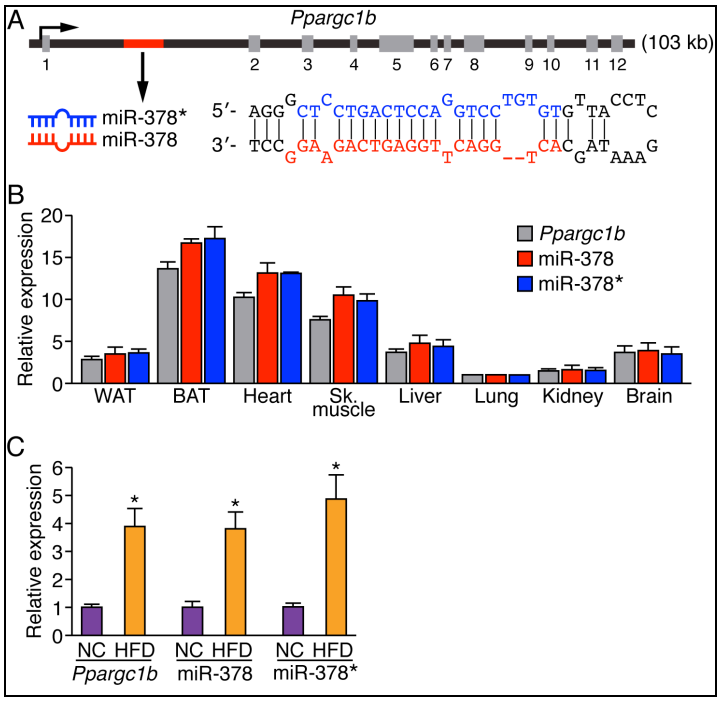
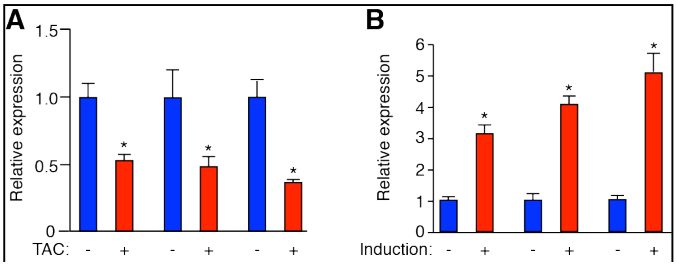


Figure 3.1. MiR-378/378* genetic locus and coexpression of miR-378/378* with the host gene, *Ppargc1b*. (A) Schematic representation of the genomic locus encoding *Ppargc1b* and the two conserved intronic microRNAs, miR-378 and 378*. (B) Quantitative real time RT-PCR shows that the host gene, *Ppargc1b*, as well as miR-378 and miR-378* are highly expressed in tissues rich in mitochondria. WAT, white adipose tissue; BAT, brown adipose tissue; Sk.muscle, skeletal muscle. (C) Quantitative real time RT-PCR shows that miR-378, miR-378* and *Ppargc1b* are up-regulated in the murine liver in response to high fat diet (HFD) compared to normal chow (NC). *P < 0.05.

We observed that miR-378 and miR-378* are both down-regulated in the heart following thoracic aortic constriction (TAC), a well characterized mouse model of pressure overload-induced cardiac hypertrophy (Figure 3.2A). Consistent with the co-regulation of miR-378, miR-378* and their host gene, we observed concomitant down-regulation of *Ppargc1b* following TAC (Figure 3.2A).

Additional evidence of the co-expression of miR-378, miR-378* and *Ppargc1b* comes from examining the



regulation of the miR-378/378* cluster during adipocyte differentiation. We observed a dramatic up-regulation of the expression of miR-378 and miR-378* during the differentiation of primary fat cells into mature adipocytes (Figure 3.2B). Consistently, the adipogenic program is characterized by the up-regulation of PGC-1β.

Figure 3.2. MiR-378, miR-378* and *Ppargc1b* are co-regulated. (A) Up-regulation of miR-378, miR-378* and *Ppargc1b* expression analyzed by quantitative real time RT-PCR in the murine heart following thoracic aortic constriction (TAC). (B) Up-regulation of miR-378, miR-378* and *Ppargc1b* upon differentiation of primary pre-adipocytes into fully mature adipocytes. Differentiation of the cultured cells was achieved using a hormonal cocktail (induction). *$P < 0.05$.

Genetic deletion of miR-378/378* confers resistance to high fat diet-induced obesity

Given the importance of PGC-1β in the regulation of mitochondrial biogenesis and metabolism, we sought to determine the role of miR-378 and miR-378* under metabolic stress. To explore the functions of miR-378/378* *in vivo*, we used homologous recombination to delete the region of intron 1 of the *Ppargc1b* gene encoding miR-378/378* and insert a neomycin-resistance cassette (neo) flanked by loxP sites (Figure 3.3A). Removal of the neomycin cassette from the *Ppargc1b* intron was accomplished by breeding miR-378/378*$^{neo/+}$ mice to mice expressing Cre recombinase under control of the CAG promoter. MiR-378/378*$^{-/+}$ mice were intercrossed to obtain miR-378/378*$^{-/-}$ knockout (KO) mice. Deletion of miR-378/378* KO was confirmed by Northern blot

analysis (Figure 3.3B). The targeted mutation did not alter the expression of PGC-1β mRNA or protein in tissues from homozygous mutant mice, as determined by quantitative real time RT-PCR (Figure 3.3C) and Western blot analysis (Figure 3.3D). MiR-378/378* KO mice were born at normal Mendelian ratios and had no overt abnormalities under basal conditions.

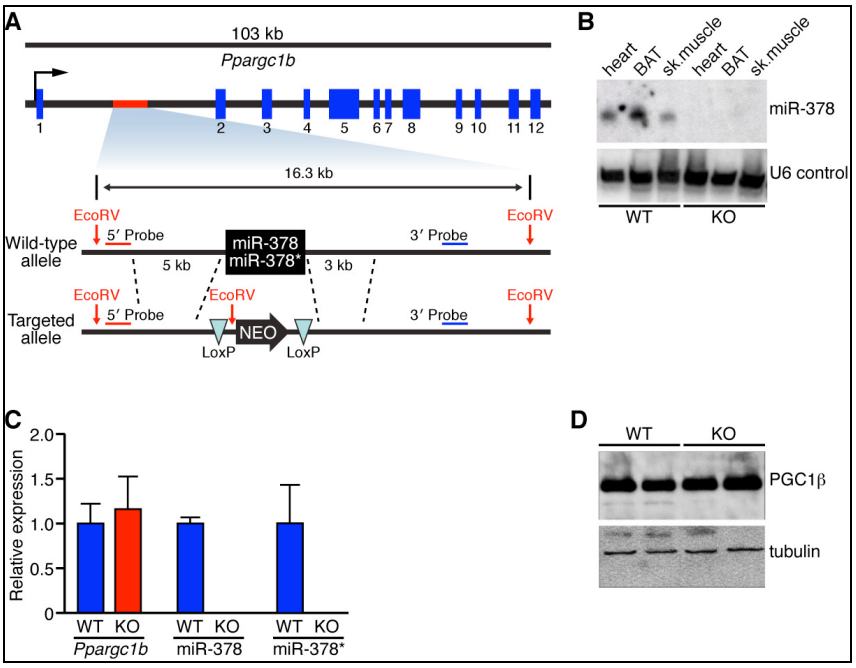


Figure 3.3. Targeting of the miR-378/378* locus and generation of knockout mice. (A) Targeting strategy used to generate miR-378/378* knockout mice. (B) Northern blot analysis shows the expression of mature miR-378 in different tissues from wild-type and knockout mice. A probe against U6 small nuclear RNA was used as control. BAT, brown adipose tissue. sk.muscle, skeletal muscle. (C-D) Analysis of the expression of the host gene *Ppargc1b*, by quantitative real time RT-PCR (C) and Western blot for PGC1β (D) in miR-378/378* knockout (KO) compared to wild-type (WT) mice. Tubulin was used as loading control in the Western blot analysis.

Often, the functions of miRNA, especially in the context of a loss-of-function analysis, become evident under conditions of stress (Leung and Sharp, 2010; Mendell and Olson, 2012). Thus, we subjected miR-378/378* KO mice to metabolic stress by feeding a high

fat diet. Indeed, we observed that miR-378/378* KO mice were resistant to high fat diet-induced obesity (Figure 3.4A). Body composition analysis by magnetic resonance imaging (MRI) revealed that the weight difference observed between miR-378/378* KO and wild-type (WT) mice after 8 weeks on high fat diet is due to reduced mass of fat depots while the lean mass is unchanged (Figure 3.4B). In particular, the subcutaneous and visceral white adipose fat pads were significantly smaller in the miR-378/378* KO mice compared to WT littermates (Figure 3.4C, D). Histological analysis showed that white adipocytes of miR-378/378* KO mice were smaller compared to adipocytes from WT mice (Figure 3.4D, E). Gross anatomy of WT liver showed ectopic deposition of fat after 8 weeks of high fat diet (Figure 3.4F). The red color of the liver from KO mice indicated the absence of lipids within the tissue. Further evidence of reduced hepatic steatosis in the mutant compared to WT mice came from oil red O staining of liver sections after 8 weeks on high fat diet (Figure 3.4F).

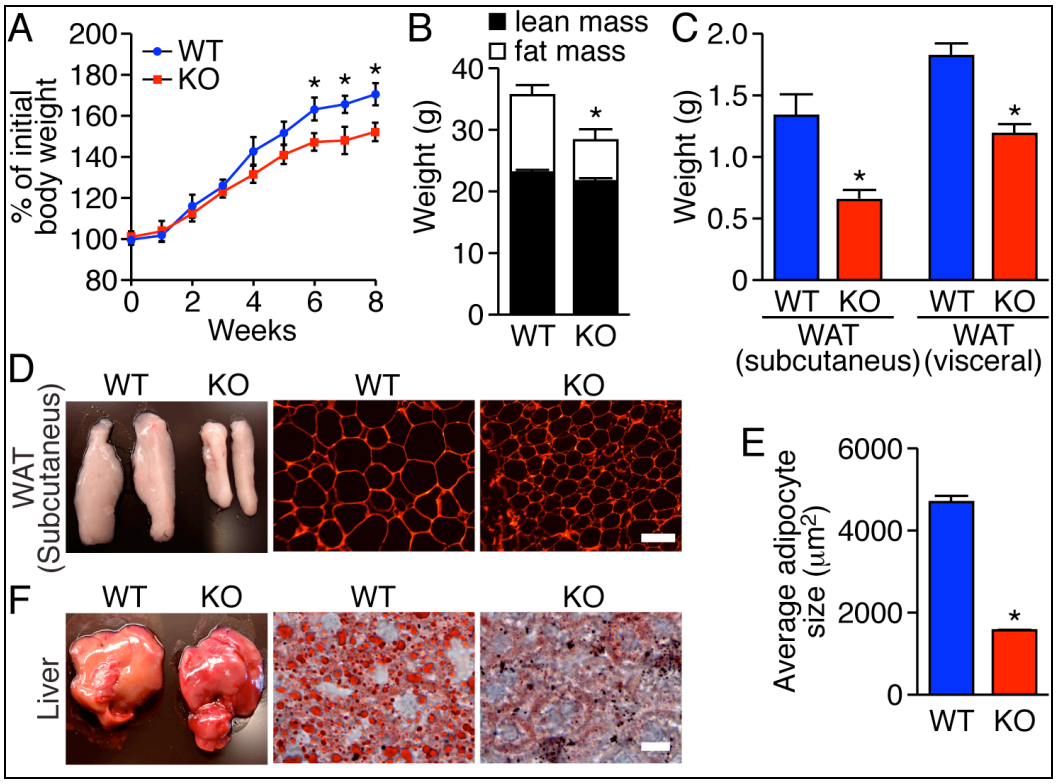


Figure 3.4. MiR-378/378* KO mice are resistant to high fat diet-induced obesity. (A) MiR-378/378* knockout mice (KO, red) gain less weight compared to wild-type mice (WT, blue) when fed with high fat diet. The increase in body weight is expressed as percentage of the body weight at the beginning of the high fat diet. (B) Analysis of body composition by MRI in miR-378/378* knockout (KO) and wild-type mice (WT) after 8 weeks of high fat diet. (C) Quantification of the weight difference of subcutaneous and visceral fat pads isolated from miR-378/378* knockout (KO) and wild-type mice (WT) after 8 weeks of high fat diet. (D) On the left panel, representative picture of the subcutaneous fat pads from KO and WT mice. WAT, white adipose tissue. On the middle and right panel, histological analysis of white adipocytes in miR-378/378* knockout (KO) and wild-type mice (WT) after 8 weeks of high fat diet. (F) On the left panel, representative picture of the liver from miR-378/378* knockout (KO) and wild-type mice (WT) after 8 weeks of high fat diet. On the middle and right panel, oil red O staining of histological section of the liver from WT and KO mice after 8 weeks of high fat diet. *P < 0.05.

In humans, as well as in rodents, obesity is characterized by reduced insulin sensitivity in target tissues such as skeletal muscle, liver and adipose. We tested whether genetic

deletion of miR-378/378* in mice resulted in improvement of the pre-diabetic state of insulin resistance associated with diet-induced obesity. Indeed, absence of miR-378/378* ameliorated the response to acute intraperitoneal administration of glucose during a glucose tolerance test performed after 7 weeks of high fat diet (Figure 3.5A). In addition, glucose measurements after 8 weeks of high fat diet confirmed reduced blood glucose levels in the KO mice compared to WT controls (Figure 3.5B). Serum triglycerides were also lower in the miR-378/378* KO mice, suggesting a global amelioration of the metabolic syndrome that accompanies the obese phenotype (Figure 3.5B).

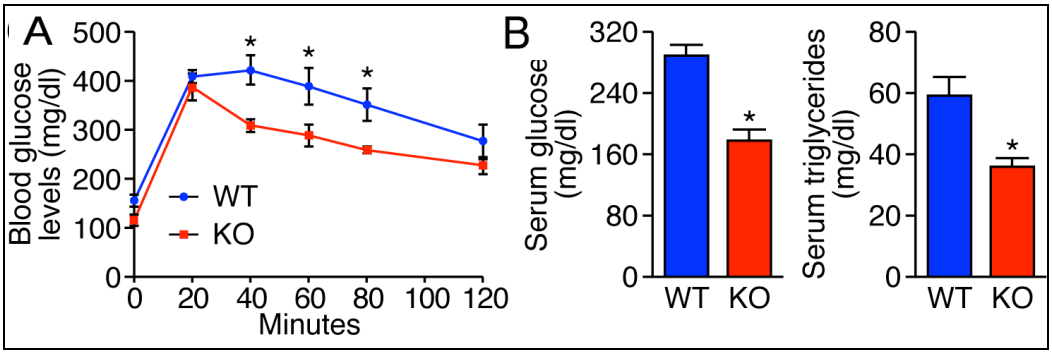


Figure 3.5. Genetic deletion of miR-378/378* leads to a global amelioration of obesity-associated metabolic syndrome. (A) Profile of blood glucose levels in miR-378/378* knockout (KO) and wild-type mice (WT) during a glucose tolerance test after 7 weeks of high fat diet. (B) Random measurements of serum glucose and serum triglycerides in miR-378/378* KO and WT mice after 8 weeks of high fat diet. *P < 0.05.

MiR-378/378* mutant mice display increased energy expenditure and mitochondrial oxidative capacity

To determine if the obesity-resistant phenotype observed in the miR-378/378* KO mice was due to enhanced activation of the PGC-1β-controlled regulatory pathways in mitochondria, we measured body energy consumption, mitochondrial number and function in mutant mice. Metabolic phenotyping of the miR-378/378* KO mice revealed

a significant increase in energy expenditure, as measured by body oxygen consumption and CO_2 production, in the mutant mice compared to WT controls on high fat diet (Figure 3.6A, B). No difference was seen in food intake between WT and KO mice (Figure 3.6C).

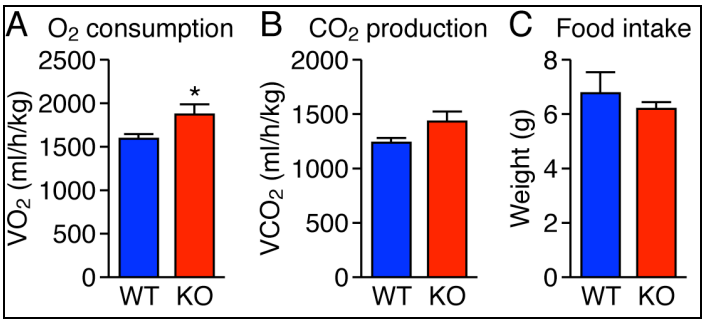


Figure 3.6. Metabolic phenotyping of miR-378/378* knockout mice. Measurement of (A) oxygen consumption, (B) CO_2 production and (C) food intake by metabolic chamber analysis in miR-378/378* knockout (KO) and wild-type mice (WT) after 5 weeks of high fat diet. *$P < 0.05$.

The importance of PGC-1β in mitochondrial biogenesis is evident based on the dramatic decrease of mitochondrial number in heart, liver and other tissues of mice with genetic deletion of PGC-1β (Lelliott et al., 2006; Sonoda et al., 2007; Vianna et al., 2006). We assessed mitochondrial content in liver, heart and skeletal muscle of miR-378/378* KO and WT mice and observed no difference in mitochondrial number, as measured by the ratio of mitochondrial and genomic DNA (Figure 3.7A-C).

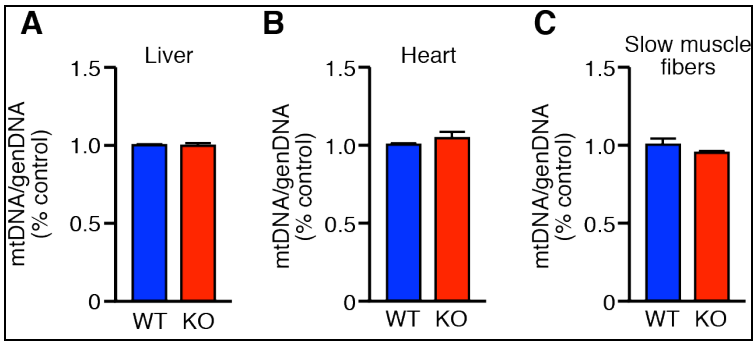


Figure 3.7. Analysis of mitochondrial content in miR-378/378* knockout mice. (A-C) Assessment of mitochondrial number, expressed as ratio of mitochondrial DNA/genomic DNA, in (A) liver, (B) heart, and (C) slow skeletal muscle isolated from miR-378/378* knockout (KO) and wild-type (WT) mice.

Following isolation of mitochondria from liver and slow muscle fibers of miR-378/378* KO and WT mice following 5 weeks on high fat diet, we observed that the retention of mitochondrial function and oxidative capacity was significantly increased in miR-378/378* mutant mice compared to WT mice (Figure 3.8D-E). Indeed, the CO_2 produced by isolated mitochondria was higher in the miR-378/378* KO mice, as well as the production of acid soluble metabolites (ASMs) (Figure 3.8D-E).

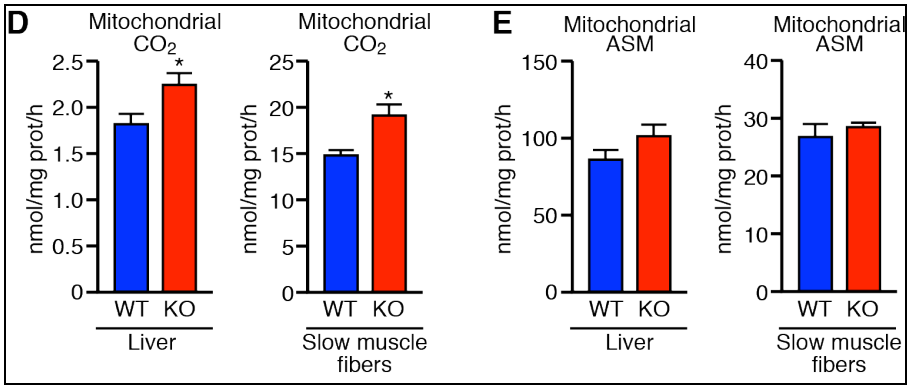


Figure 3.8. Analysis of mitochondrial function in miR-378/378* knockout mice. (D-E) Measurement of (D) CO_2 and (E) acid soluble metabolites (ASM) production in mitochondria isolated from the liver and slow skeletal muscle of miR-378/378* knockout (KO) and wild-type mice (WT) after 5 weeks of high fat diet. *$P < 0.05$.

The fact that ASMs were not increased as much as the CO_2 production in the mutant mice suggested a parallel increase in the utilization of the products of fatty acid β-oxidation through the TCA cycle in the miR-378/378* KO mice compared to WT mice (Koves et al., 2005; Vergnes et al., 2011). Thus, we examined the expression of genes of the TCA cycle in mice lacking miR-378/378* after 5 weeks of high fat diet. As expected, we observed the up-regulation of two different enzymatic components of the TCA cycle, succinate dehydrogenase complex subunit d (Sdhd) and fumarate hydratase 1 (Fh1) (Figure 3.9).

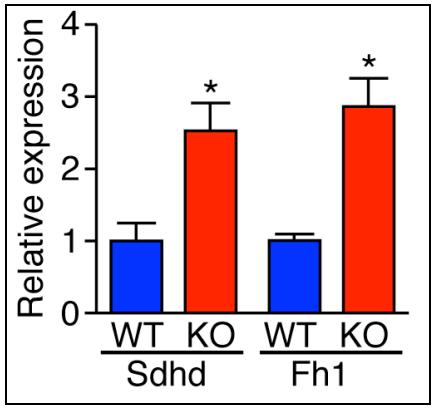


Figure 3.9. Up-regulation of genes of the TCA cycle in miR-378/378* KO mice. Quantitative real time RT-PCR shows up-regulation of Sdhd and Fh1 in miR-378/378* KO mice compared to WT mice. *P < 0.05.

Crat and Med13 are targets of miR-378/378*

To identify mRNA targets that contribute to the observed increased energy expenditure and mitochondrial oxidative capacity seen in the KO mice, we used Miranda and PicTar prediction algorithms to search for evolutionarily conserved targets for miR-378 and miR-378*. Although miR-378 and miR-378* are encoded by a common hairpin located

in the first intron of the Ppargc1b gene, they have different "seed" regions and thus target different mRNAs (Figure 3.10).

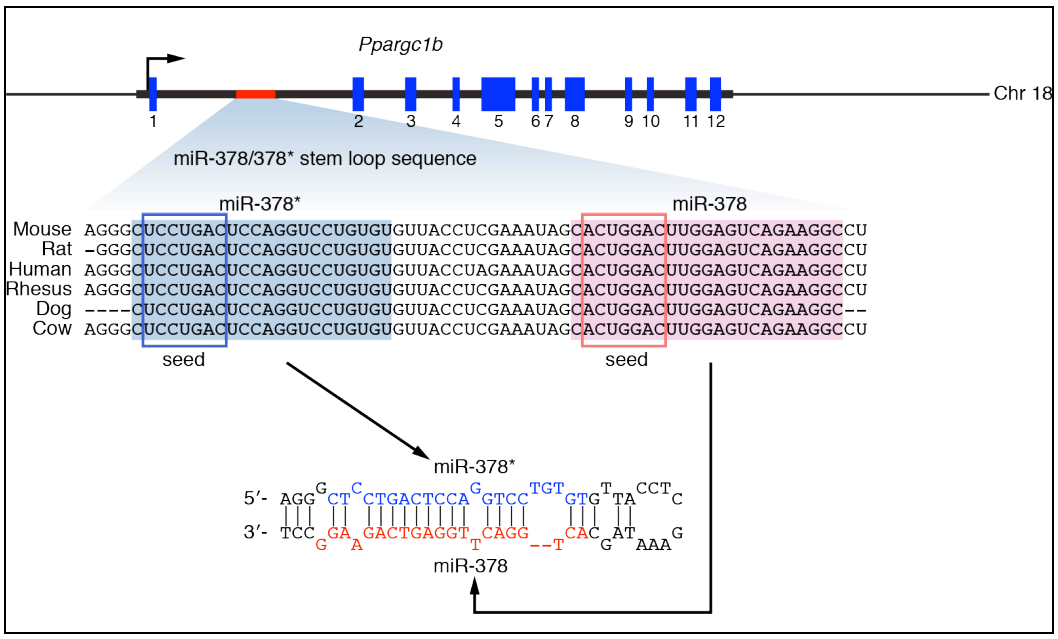


Figure 3.10. MiR-378/378* hairpin RNA structure. Schematic representation of the genomic locus encoding *Ppargc1b*. miR-378 and 378* are intronic miRNAs embedded in a conserved region of the first intron of the *Ppargc1b* gene.

Bioinformatic analysis revealed that the carnitine acetyltransferase (*Crat*) gene displays a miR-378 site in its 3' UTR (Figure 3.11A). CRAT is responsible for channeling the products of β-oxidation to the TCA cycle, away from mitochondrial efflux, thus coupling mitochondrial fatty acid uptake to oxidative metabolism (Cordente et al., 2004; Noland et al., 2009; O'Donnell et al., 2002). To validate *Crat* as a miR-378 target we placed its 3'UTR downstream of a CMV-driven luciferase reporter and performed reporter assays in COS-1 cells transfected with varying amount of expression plasmid for miR-378/378* or a control plasmid encoding an unrelated miRNA. Luciferase assays showed a dose

response repression of the Crat 3'UTR reporter by miR-378/378* (Figure 3.11B). Mutation of the seed region in the Crat 3'UTR reporter partially reversed the repression of miR-378/378* (Figure 3.11B). Consistent with these findings, quantitative real time RT-PCR analysis confirmed up-regulation of Crat in miR-378 KO mice (Figure 3.11C).

PGC-1β is an inducible co-regulator of nuclear hormone receptor signaling, which modulate metabolism via the Mediator complex (Boube et al., 2002; Finck and Kelly, 2006; Fondell et al., 1996; Ge et al., 2002; Grueter et al., 2012; Wang et al., 2002). Med13, a regulatory component of the Mediator complex, is a predicted target of miR-378*. The *Med13* gene has three conserved sites recognized by miR-378* in its 3'UTR (Figure 3.11D). Luciferase reporter assays using the *Med13* 3'UTR showed a dose-dependent repression of luciferase activity upon cotransfection of COS-1 cells with increasing amount of pCMV-miR-378/378* (Figure 3.11E). Mutation of the sequences recognized by miR-378* was sufficient to reverse the repression of luciferase activity (Figure 3.11E). Quantitative real time RT-PCR confirmed that *Med13* expression is up-regulated in the liver of mice lacking miR-378* (Figure 3.11F).

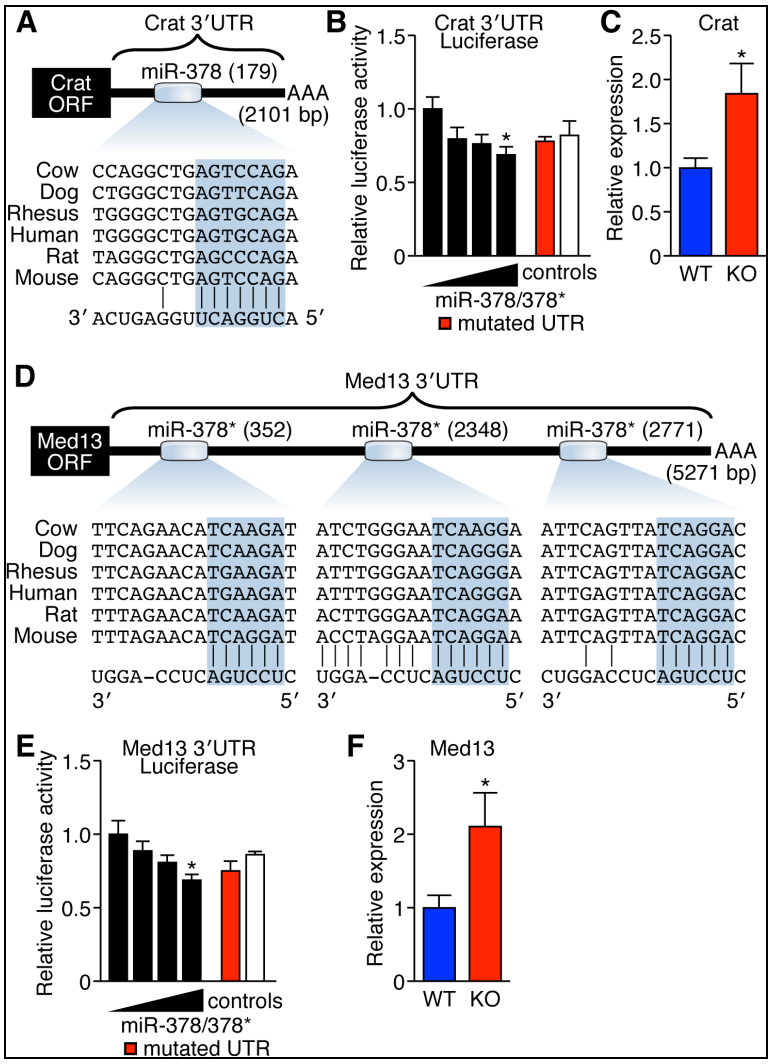


Figure 3.11. Identification and validation of miR-378/378* target genes. (A) Schematic representation of the 3'UTR of the *Crat* gene, highlighting the conserved miR-378 site. (B) Luciferase assay using the *Crat* 3'UTR and increasing amounts of pCMV-miR-378/378*. Mutation of the miR-378 site in the *Crat* 3'UTR (red) and an unrelated miRNA (white) are used as controls. (C) Quantitative real time RT-PCR of *Crat* expression in the liver of miR-378/378* knockout (KO) compared to wild-type (WT) mice after 8 weeks of high fat diet. (D) Schematic diagram of the 3'UTR of the *Med13* gene, highlighting the three conserved miR-378* sites. (E) Luciferase activity of pMIR-reporter vector containing the *Med13* 3'UTR in the presence of increasing amounts of pCMV-miR-378/378*. Mutation of the miR-378* sites in the *Med13* 3'UTR (red) and an unrelated miRNA (white) are controls. (F) Quantitative real time RT-PCR of *Med13* expression in the liver of miR-378/378* KO compared to WT mice after 8 weeks of high fat diet. *$P < 0.05$.

DISCUSSION

In this study we showed that miR-378 and miR-378* govern mitochondrial metabolism of fatty acids *in vivo*. In so doing, we revealed a novel mechanism for the regulation of the PGC-1β transcriptional circuitry, which functions in conditions of metabolic stress to control the overall oxidative capacity and energy expenditure of insulin-target tissues.

MiR-378, miR-378* and *Ppargc1b* are part of a common regulatory circuitry

MiR-378 and 378* are distinct miRNAs embedded in the *Ppargc1b* gene. We demonstrated that miR-378 and miR-378* are co-expressed and co-regulated with PGC-1β, supporting the hypothesis that intronic miRNAs act in concert with their host gene to control the same biological pathways, especially in conditions of stress. *Ppargc1b*, miR-378 and miR-378* are highly expressed in tissues rich in mitochondria, indicative of their fundamental roles in mitochondrial biogenesis and mitochondrial metabolism. We showed that miR-378/378* function in a regulatory circuitry centered on PGC-1β to control fatty acid utilization and energy expenditure in insulin-target tissues. Our data suggest that miR-378/378* acts as a brake on the PGC-1β-mediated activation of mitochondrial oxidative pathways under altered availability of energy substrates, such as HFD. Alternatively, miR-378/378* might divert excessive energy sources, such as the pyruvate derived from glucose, away from the TCA cycle into alternative pathways (i.e. glycolysis) in conditions of metabolic stress or abundance of certain energy sources (i.e. fatty acids). This would avoid overextending the mitochondrial reducing system and the accumulation of acetylated intermediates within the cell. Consistent with this hypothesis,

over-expression of miR-378* has been shown to promote tumorigenesis and the Warburg effect in cancer cells by increasing glycolytic metabolism (Eichner et al., 2010; Lee et al., 2007). The co-expression and co-regulation of *Ppargc1b*, miR-378 and miR-378* suggest that they have a synchronized role in tissues which elevated mitochondrial content. In addition, miR-378 and 378* might play an important role in white adipocytes, as we observed reduced adipocyte size in the miR-378/378* KO mice after high fat diet. Consistently, previous studies report that overexpression of miR-378 is associated with enhanced lipogenesis and fat depots size (Gerin et al., 2010a; Ortega et al., 2010).

MiR-378 and miR-378* function in conditions of metabolic stress to regulate energy expenditure

Metabolic chamber analysis revealed that oxygen consumption is elevated in miR-378/378* KO mice compared to WT littermates after high fat diet feeding, indicating an overall increase in energy expenditure in KO mice compared to WT controls. These findings imply that an increase in energy expenditure occurs in response to the loss of miR-378/378* in tissues where PGC-1β is highly expressed (such as brown adipose tissue, heart, skeletal muscle) or up-regulated (such as liver, white adipose tissue) upon high fat diet and lipid accumulation.

MiR-378 and miR-378* control mitochondrial oxidative metabolism

Free fatty acids present in the cytosol are esterified to fatty acyl-CoA in the outer mitochondrial membrane and transported into the mitochondria. Once inside the

mitochondrial matrix, fatty acids undergo β-oxidation. Pyruvate obtained from glucose can also be used to generate acetyl-CoA which enters the TCA cycle to produce reducing equivalents subsequently utilized in oxidative phosphorylation. PGC-1β is a known activator of genes of the oxidative phosphorylation chain, TCA cycle and fatty acids β-oxidation (Finck and Kelly, 2006; Handschin and Spiegelman, 2006). While mitochondrial number is not altered in KO compared to WT mice, the CO_2 produced as a by-product of the TCA cycle, as well as mitochondrial ASMs are elevated in KO mice, revealing increased oxidative capacity in the miR-378/378* mutant animals. Although detectable, the up-regulation of ASMs is not significantly higher in the KO mice, indicating the absence of incomplete fatty acid oxidation and suggesting the ability of mitochondria to clear the increased formation of products of β-oxidation via the TCA cycle. Indeed, the expression of different components of the TCA cycle is up-regulated in the mutant mice. It is likely that the altered regulation of the TCA cycle genes observed in the miR-378/378* KO mice is indirect, as such genes do not display a seed sequence recognized by miR-378 or miR-378* in their 3'UTRs. Consistent with our findings, it was previously reported that overexpression of miR-378* *in vitro* causes increased lactate production due to a shift from oxidative to glycolytic metabolism in cancer cells (Eichner et al., 2010).

MiR-378 and miR-378* target genes involved in mitochondrial metabolism

The products of β-oxidation are preferentially channeled to the TCA cycle, away from mitochondrial efflux via carnitine O-acetyltransferase (CRAT). CRAT is a matrix enzyme that catalyzes the reversible conversion of acetyl-CoA and carnitine to

acetylcarnitine and free CoA, which can enter the TCA cycle and accept more acetyl groups produced by β-oxidation, thereby allowing the oxidation to proceed. Thus, CRAT plays an essential role in the oxidation of fatty acids and in energy metabolism in eukaryotes. Carnitine insufficiency and reduced CRAT activity have been identified as reversible components of the metabolic syndrome (Noland et al., 2009). It has been previously reported in rodents that perturbation in metabolic homeostasis induced by high fat diet is associated with compromised mitochondrial fuel metabolism and incomplete fatty acid oxidation due to reduced carnitine levels (Noland et al., 2009; O'Donnell et al., 2002). Furthermore, CRAT overexpression in primary human skeletal myocytes has been shown to increase glucose uptake and attenuate lipid-induced suppression of glucose oxidation (Noland et al., 2009). Bioinformatics analysis revealed the presence of a miR-378 binding site in the 3'UTR of the *Crat* gene. Quantitative real time RT-PCR confirms increased Crat expression in the liver of miR-378/378* KO mice compared to WT littermates.

PGC-1β is a well-characterized inducible co-regulator of nuclear receptor signaling (Finck and Kelly, 2006; Handschin and Spiegelman, 2006; Knutti and Kralli, 2001; Meirhaeghe et al., 2003; Puigserver and Spiegelman, 2003). Transcriptional control of energy metabolism by nuclear hormone receptors is dependent on the Mediator, a multiprotein complex that differentially regulates the transcription of multiple target genes by recruiting RNA polymerase II (Boube et al., 2002; Fondell et al., 1996; Ge et al., 2002; Wang et al., 2002). One of the subunits of the Mediator complex, MED13, has been linked to the maintenance of global energy homeostasis in different animal models, including mice (Grueter et al., 2012; Pospisilik et al., 2010). The 3'UTR

of the gene encoding MED13 displays three conserved sites recognized by miR-378*. An additional conserved site recognized by miR-378 was identified towards the 5' end of the *Med13* 3'UTR but it was not further analyzed because of the presence of an additional nucleotide in the UTR sequence compared to the actual miR-378 seed sequence. Luciferase reporter assays confirmed that the 3'UTR region of Med13 serves as a target of miR-378* and *Med13* expression was up-regulated in the liver of miR-378/378* KO.

It appears that miR-378 and miR-378* exert their biological functions in the different insulin target tissues where they are highly expressed, in particular BAT, liver, skeletal muscle and even brain and WAT. While the actions of miR-378 and miR-378* are confined in the setting of metabolic stress, they likely have multiple targets, other than *Crat* and *Med13*, which are involved in different metabolic pathways. In conclusion, our findings indicate that miR-378 and miR-378* are integral parts of the transcriptional network formed by PGC-1β and the nuclear hormone receptors, which govern mitochondrial metabolism and energy homeostasis (Figure 3.12).

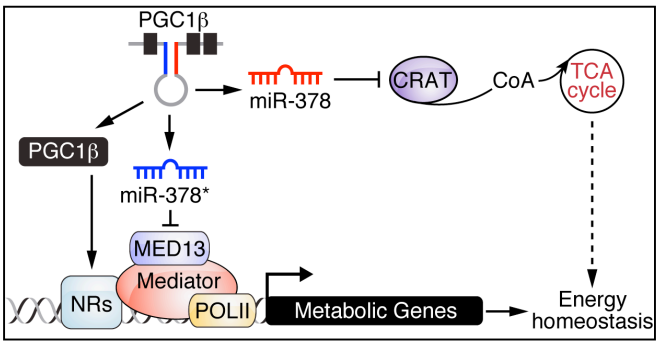


Figure 3.12. Schematic diagram of the role of miR-378 and miR-378*. The diagram shows the links that place miR-378, miR-378* and their target genes within the PGC-1β-controlled transcriptional network. During metabolic stress, miR-378/378* participate in the regulation of mitochondrial functions and global energy homeostasis via repression of their target genes. NRs, nuclear receptors

METHODS

Thoracic aortic constriction

Eight-week old male C57BL/6 mice were subjected to thoracic aortic banding to induce pressure overload in the left ventricle of the heart according to previously described procedures (Hill et al., 2000; Tatsuguchi et al., 2007). Mice were sacrificed after 3 weeks of banding, and hearts were harvested for RNA extraction.

Isolation and differentiation of primary adipocytes

Primary adipocytes from subcutaneous fat pads were prepared as previously described (Hansen et al., 1998). Cells were cultured to confluence and adipocyte differentiation was induced with 1.72 μM insulin (Sigma), 625 nM dexamethasone (Sigma), and 2 μM rosiglitazone for 8 days.

Generation of miR-378/378* knockout mice

The targeting vector used to generate the null allele of miR-378 and miR-378* was constructed using the pGKNEO-F2DTA vector, which contains a neomycin resistance gene driven by the pGK promoter, flanked by loxP sites, and a diphtheria toxin gene cassette. The miR-378/378* targeting strategy was designed to replace the pre-miR sequence with the neomycin resistance cassette flanked by loxP sites. For the targeting, the 5′ and 3′ arms of homology were generated by TAKARA Taq LA PCR amplification (TAKARA) of 129SvEv genomic DNA. The targeting vectors were electroporated into 129SvEv-derived ES cells. Five-hundred ES cell clones for each

targeting vector were isolated and analyzed for homologous recombination by Southern blotting. Three clones with a properly targeted miR-378/378* allele were injected into 3.5-d C57BL/6 blastocysts, and high-percentage chimeric male mice were crossed to C57BL/6 females to achieve germline transmission of the targeted allele. Heterozygous neo/+ mice were intercrossed with Cre-transgenic mice under the control of the CAG promoter to remove the neo cassette. All experimental procedures involving animals in this study were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center.

RNA isolation and quantitative real time RT-PCR

Total RNA was isolated from homogenized tissues with Trizol reagent (Invitrogen) according to the manufacturer's protocol. Single-strand cDNAs were synthesized by RT-PCR performed using random hexamer primers (Invitrogen). MiRNA levels were measured using TaqMan miRNA real time probes (Applied Biosystems). Quantitative real time RT-PCR was performed using TaqMan probes (ABI) or Sybr Green probes on an ABI-PE Prism 7000 sequence detection system (Applied Biosystems) according to the protocol provided by the manufacturer. The relative amounts of mRNA were measures using comparative CT method. The detected CT values were normalized against 18s for mRNA or RNU6 for miRNA.

High fat diet, glucose tolerance test, glucose and triglycerides measurements

Mice were fed with a rodent high fat diet containing 45% kcal from fat (Open Source Diets) for the indicated time. Glucose tolerance test was performed by intraperitoneal

injection of glucose (1.5 g/Kg) following an overnight fast. Blood glucose levels were measured before glucose injection and at 20, 40, 60, 80 and 120 minutes after injection. Blood glucose levels were measured using an Accu-Chek Compact Plus system. Serum triglycerides levels were measured using the Vitros 250 system. Blood was collected and serum obtained by allowing the blood to clot at room temperature for 30 minutes and then centrifuging at 4000 rpm for 10 minutes. To measure triglycerides in the liver, the tissue was frozen immediately once it was extracted from the animal, and 100-200 mg were used to extract the triglycerides. Serum and tissue triglycerides levels were measured by the Mouse Metabolic Phenotyping Core at UT Southwestern.

Histological analysis

Fat pads were harvested, fixed in 4% paraformaldehyde for 24 hours and processed for routine paraffin histology. The sections were stained with H&E using standard procedures. For neutral lipid staining, the liver was harvested and flash frozen in embedding medium containing a 3:1 mixture of Tissue Freezing Medium (Triangle Biomedical Sciences) and gum tragacanth (Sigma-Aldrich). Frozen sections were cut on a cryotome, stained with oil red O, and counterstained with hematoxylin. For quantification of white adipocytes size, pictures of the H&E sections of visceral fat pads were taken using the rhodamine channel on a Leica DMRXE fluorescence microscope.

Metabolic chambers and whole body composition analysis

Metabolic phenotyping of wild-type and miR-378/378* knockout mice was performed using TSE metabolic chamber analysis by the Mouse Metabolic Phenotyping Core at UT

Southwestern. The general experimental protocol includes an initial 5 days in acclimation chambers, followed by a 4-day experimental period with data collection. Whole body composition parameters were measured using a Bruker Minispec mq10 system.

Mitochondria isolation from liver

Mitochondria were isolated from liver of 10 weeks old wild-type and miR-378/378* knockout mice as previously described (Frezza et al., 2007), with modifications. Tissue samples were collected in buffer containing 67 mM sucrose, 50 mM Tris/HCl, 50 mM KCl, 10 mM EDTA/Tris, and 10% bovine serum albumin. Samples were minced and digested in 0.05% trypsin for 30 minutes. Samples were then homogenized, and mitochondria were isolated by differential centrifugation.

Fatty acid metabolism in isolated mitochondria

Fatty acid oxidation was assessed in isolated mitochondria by measuring and summing 14CO2 production and 14C-labeled acid-soluble metabolites from the oxidation of [1-14C]-palmitic acid as previously described (Frisard et al., 2010; Hulver et al., 2005).

Cell culture, transfection and luciferase assay

A DNA fragment containing full-length 3′ UTRs for putative miR-378 and miR-378* targets were cloned into pMIR-REPORT vector (Ambion). Mutagenesis of the miR-378 or miR-378* binding sites, cell culture and luciferase assays were performed as described previously (van Rooij et al., 2008). Briefly, COS cells were transfected using Fugene 7

reagent (Roche) according to the manufacturer's instructions. pCMV-lacZ was used as transfection control. Whole cell extracts were assayed for luciferase expression forty-eight hours after transfection using a luciferase assay kit (Promega). Relative reporter activities are expressed as luminescence units normalized for β-galactosidase expression.

Statistics

Data are presented as mean ± SEM. Differences between groups were tested for statistical significance using the unpaired 2-tailed Student's t test. P values less than 0.05 were considered significant.

Chapter IV

Redundant Control of Adipogenesis by Histone Deacetylases 1 and 2

ABSTRACT

Adipocyte differentiation is a well defined process that is under the control of transcriptional activators and repressors. We show that histone deacetylase inhibitors efficiently block adipocyte differentiation *in vitro*. This effect is specific to adipogenesis, as another mesenchymal differentiation process, osteoblastogenesis, is enhanced upon HDAC inhibition. Through the systematic genetic deletion of HDAC genes in cultured mesenchymal precursor cells, we show that deletion of HDAC1 and HDAC2 leads to reduced lipid accumulation, revealing redundant and requisite roles of these class I HDACs in adipogenesis. These findings unveil a previously unrecognized role for HDACs in the control of adipogenesis.

INTRODUCTION

In humans, unused caloric energy is converted to triglycerides and stored in fat depots. In principle, the fat mass of these depots can increase either by hypertrophy (an increase of adipocyte size) or by hyperplasia (and increase in adipocyte number). It has been recently demonstrated that fat cell number is primarily determined by early adulthood and that subsequent changes in fat mass occur mainly through increases in adipocyte volume (Spalding et al., 2008). However, 10% of fat cells are renewed annually in adults. The molecular mechanisms driving the turnover of adipocyte tissue in adults are incompletely understood, but it has been speculated that a combination of cell death and neoadipogenesis from mesenchymal precursor cells is responsible for maintaining the fat cell number pre-set in early adulthood (Spalding et al., 2008).

Adipogenesis is a tightly orchestrated process in which mesenchymal precursor cells differentiate into mature adipocytes and express batteries of genes encoding enzymes involved in lipid biosynthesis, transport, and storage. This process is under the control of a series of well characterized transcription factors, including C/EBPβ, SREBPs, and PPARγ (Lefterova et al., 2008). Studies in cultured cells have shown that these adipogenic core transcription factors interact with histone acetyltransferases, which stimulate transcription by acetylating nucleosomal histones, thereby relaxing chromatin structure (Farmer, 2006). HDACs also associate with these adipogenic transcription factors (Farmer, 2006), counteracting the functions of histone acetyltransferases. Thus, in the classic model of adipocyte differentiation, HDACs are thought to inhibit the

adipogenic program by directly repressing the transcriptional activity of pro-adipogenic transcription factors (Rosen and MacDougald, 2006).

Among the five classes of HDACs, the activity of class I HDACs can be efficiently blocked by pharmacological inhibitors (such as SAHA), and this inhibition is well tolerated in humans (Jiang et al., 2002). In this regard, HDACi prevent pathological cardiac growth and remodeling in response to numerous forms of stress (Antos et al., 2003; Kee, 2006; Kong et al., 2006). Recently, the FDA approved the HDACi SAHA for the treatment of cutaneous T-cell lymphoma (Mann, 2007). Many other clinical trials have been performed to test the efficacy of different HDACi as anticancer agents (Dokmanovic, 2007). HDACi also enhance long term memory in animal models of dementia and improve the symptoms in several models of neurodegenerative disease (Fischer et al., 2007; Hockly et al., 2003; Ryu et al., 2003; Steffan et al., 2001). Remarkably, HDAC inhibition has also been reported to enhance lifespan in lower eukaryotes (Jiang et al., 2002; Kim et al., 1999; Rogina et al., 2002). The precise mechanisms and molecular targets that mediate these actions of HDACi *in vivo* remain to be defined and represent a major issue in the field.

In the course of studying the role of different HDAC isoforms in development, multiple studies found that deletion of HDAC3 leads to a profound pro-adipogenic phenotype in liver and heart, indicative of an inhibitory role of this HDAC in adipogenesis (Knutson et al., 2008; Montgomery, 2008). This prompted us to study the role of the different HDAC isoforms in this process. We showed that pharmacological HDAC inhibition leads to a robust block of adipogenesis *in vitro*. By genetic deletion of

class I HDACs in mesenchymal precursor cells, we demonstrated that HDAC1 and HDAC2 play redundant roles as positive regulators of adipogenesis.

RESULTS

HDAC inhibitors block adipogenesis *in vitro*

Deletion of HDAC3 in the heart have previously been shown to cause the dysregulation of a gene program associated with fatty acid uptake and oxidation that leads to dramatic myocardial lipid accumulation (Montgomery, 2008). This phenotype is not seen in cardiomyocytes deficient for either HDAC1, HDAC2, or HDAC8, indicating that fatty acid and adipocyte homeostasis might be under the control of distinct HDAC isoforms (Bakker, 2003; Montgomery et al., 2007; Montgomery, 2008; Tiscornia, 2006).

As a first step toward exploring the potential role of HDAC activity in adipogenesis, we treated the 3T3-L1 pre-adipocyte cell line with the pan-HDACi TSA. Adipogenic induction of 3T3-L1 cells with isobutylmethylxanthine (IBMX), insulin, and dexamethasone results in robust adipogenesis within 8 days, which can be monitored by oil red O staining (ORO) of lipid droplet accumulation. In contrast, the treatment of 3T3-L1 cells with TSA led to a complete block of adipogenesis as measured by the lack of ORO-positive fat accumulation and the failure to up-regulate the expression of adipocyte-specific marker genes, including hormone-sensitive lipase (*Lipe*), adiponectin (*AdipoQ*), and adipocyte lipid-binding protein 2 (*aP2*) (Figure 4.1A-C). This inhibition was dose-dependent and occurred at concentrations of TSA as low as 10–50 nM (Figure 4.1D-E).

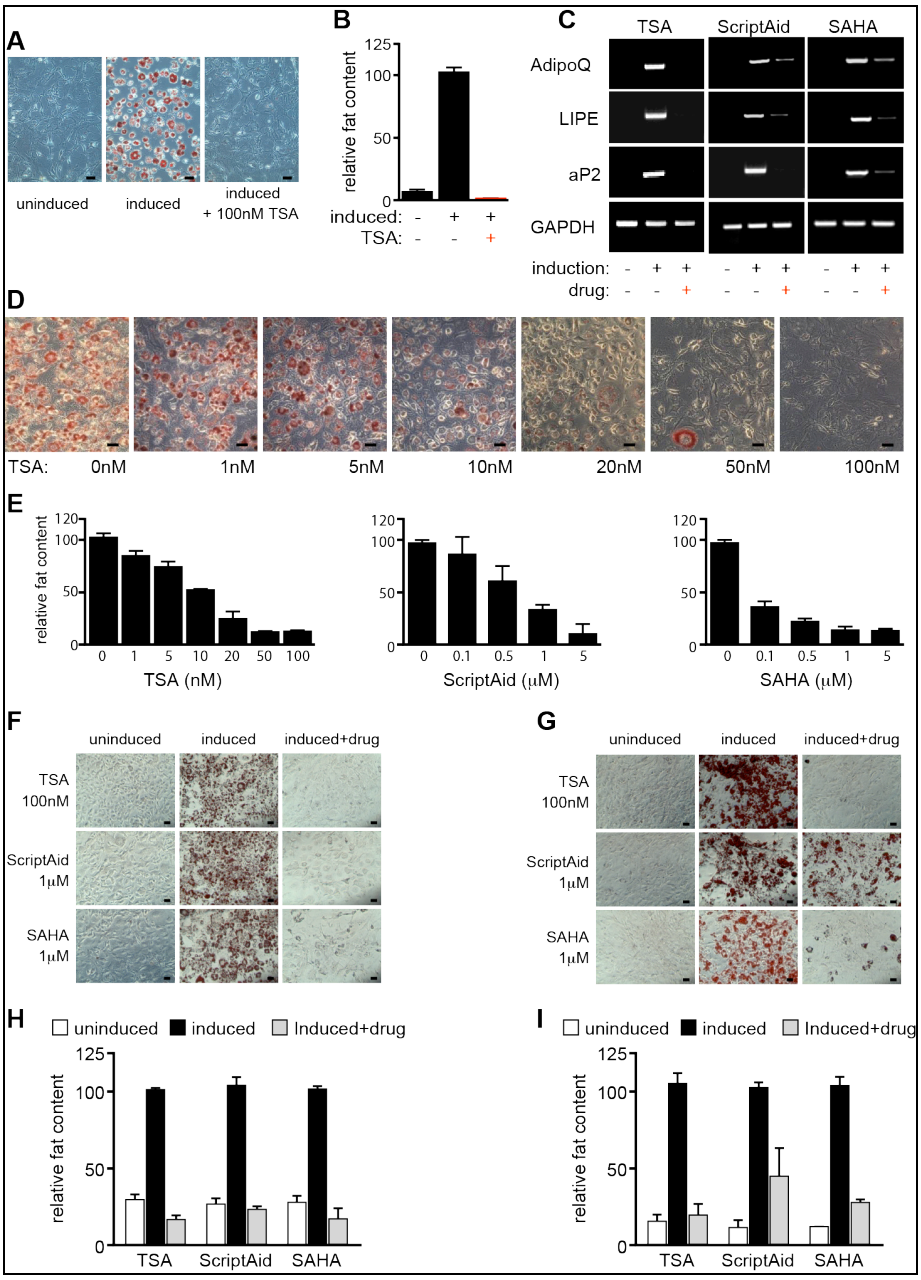


Figure 4.1. HDAC inhibitors block adipogenesis *in vitro*. (A) Staining with ORO of 3T3-L1 cells. (B) Quantification of A. (C) RT-PCR for adipogenic marker genes in 3T3-L1 cells treated as indicated. GAPDH was detected as a control. (D) Dose response of reduced ORO staining in 3T3-L1 cells treated with various amounts of TSA. (E) Quantification of ORO staining in 3T3-L1 cells treated as indicated. (F, G) Images of ORO staining in either 3T3-L1 cells (F) or MEFs (G) treated as indicated. (H, I) Quantification of ORO staining in either 3T3-L1 cells (H) or MEFs (I). Size bars: 60 μm.

We next tested whether inhibition of adipogenesis is specific to TSA and 3T3-L1 cells or if it extends to different inhibitors and different *in vitro* systems. In addition to TSA, the HDACi Scriptaid as well as the recently FDA-approved inhibitor SAHA robustly blocked adipocyte differentiation in 3T3-L1 cells (Figure 4.1E-G) as well as in mouse embryonic fibroblasts (MEFs) (Figure 4.1H, I).

Fatty acids such as sodium butyrate enhance adipogenesis

There is disagreement as to the role of HDACi in adipogenesis. Contrary to various findings, a report described an enhancement of adipogenesis upon HDAC inhibition (Yoo et al., 2006). Notably, in these report the authors use the short-chain fatty acid (SCFA) sodium butyrate as an HDACi (Wiper-Bergeron et al., 2003; Yoo et al., 2006). Because SCFAs have been described as pro-adipogenic (Hong et al., 2005; Xiong et al., 2004), we examined the effect of butyrate and other SCFAs on adipocyte differentiation (Figure 4.2A). Indeed, treatment of 3T3-L1 cells with sodium butyrate increased adipogenesis as measured by ORO quantification (Figure 4.2B-D). This effect, however, was completely blocked by co-incubation with TSA (Figure 4.2D). In addition, treatment of 3T3-L1 cells with other SCFAs such as sodium propionate and sodium acetate increased adipogenesis, and again this effect was completely abolished by TSA (Figure 4.2D).

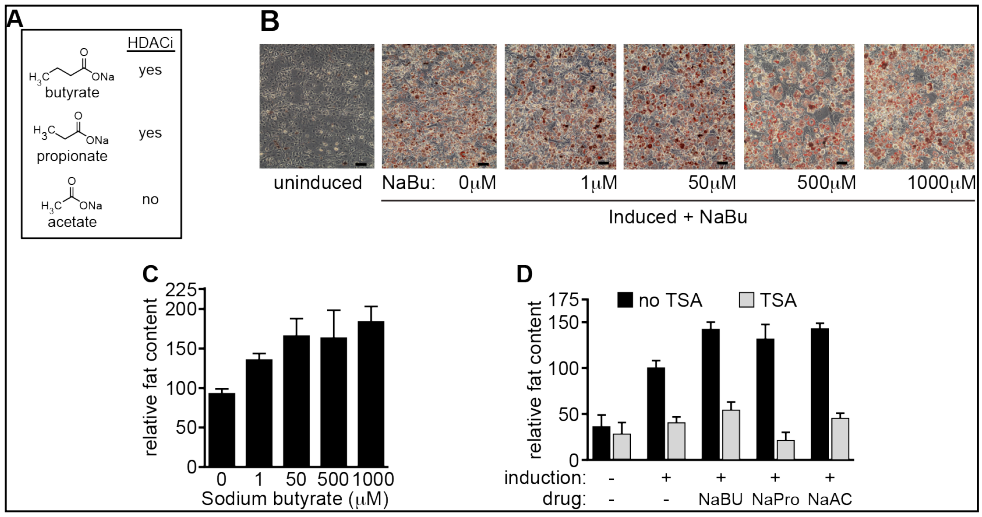


Figure 4.2. Effects of fatty acids on adipogenesis. (A) Structures of the short-chain fatty acids sodium butyrate (NaBu), sodium propionate (NaPro) and sodium acetate (NaAc). NaBu and NaPro are also HDACi. (B) NaBu enhances adipogenesis in 3T3-L1 cells as measured by ORO staining. (C) Quantification of B. (D) Treatment of 3T3-L1 cells with NaBu (1 mM), NaPro (1 mM) and NaAc (1 mM) enhances adipogenesis. This effect is completely abolished by TSA (100 nM).

Of the tested SCFAs, sodium propionate had little, and sodium acetate had a negligible effect on HDAC activity (Ohata et al., 2005; Waldecker et al., 2008), indicating that the enhancing effect of SCFAs on adipogenesis is independent of an effect on HDAC activity.

HDAC inhibition enhances osteogenesis

To test whether the inhibitory effect of HDACi was specific to adipogenesis or represented a more general block of mesenchymal cell differentiation, we examined the effect of TSA on primary pre-osteoblasts, which terminally differentiate and secrete mineralized matrix *in vitro*. Treatment of pre-osteoblasts with TSA enhanced differentiation, as measured by the production of silverstainable calcified matrix (Figure

4.3A). Additionally, expression of the osteoblast markers runt-related transcription factor 2 (*Runx2*), osterix, alkaline phosphatase, and osteocalcin was increased upon TSA treatment (Figure 4.3B).

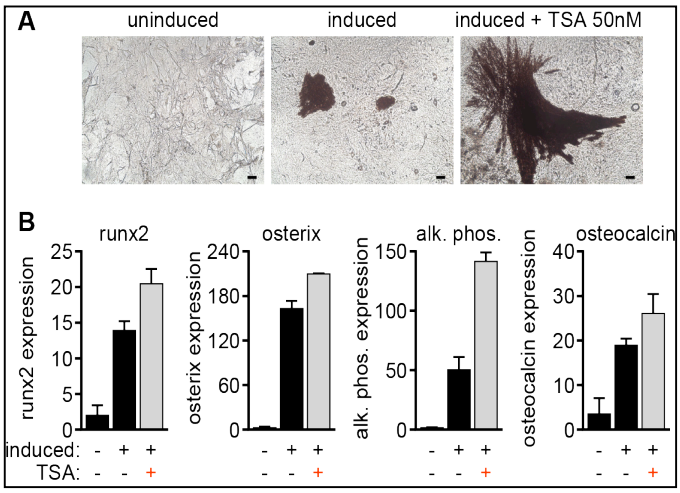


Figure 4.3. Effects of HDAC inhibition on osteoblastogenesis. (A) Primary, calvarial pre-osteoblasts were isolated and induced to differentiate. Calcified matrix deposited by mature osteoblasts was visualized using silver nitrate staining. Size bars: 60 μm. (B) Expression of osteoblast-specific markers was determined by quantitative real-time PCR.

HDAC inhibitors act upstream of *Pparγ* to block adipogenesis

To define the temporal window in the pathway of adipogenesis in which HDACi act, we performed a detailed time-course analysis of the actions of TSA on 3T3-L1 cells. Treatment for the first 48 h after induction almost completely blocked adipocyte differentiation, whereas treatment at later time points had only minor effects (Figure 4.4A, B). In support of the evidence that TSA acts early in the cascade of events that define the adipogenic program, TSA treatment of 3T3-L1 cells led to a dramatic reduction of proliferation during the clonal expansion phase of adipocyte differentiation, as measured by BrdUrd incorporation (Figure 4.4C, D).

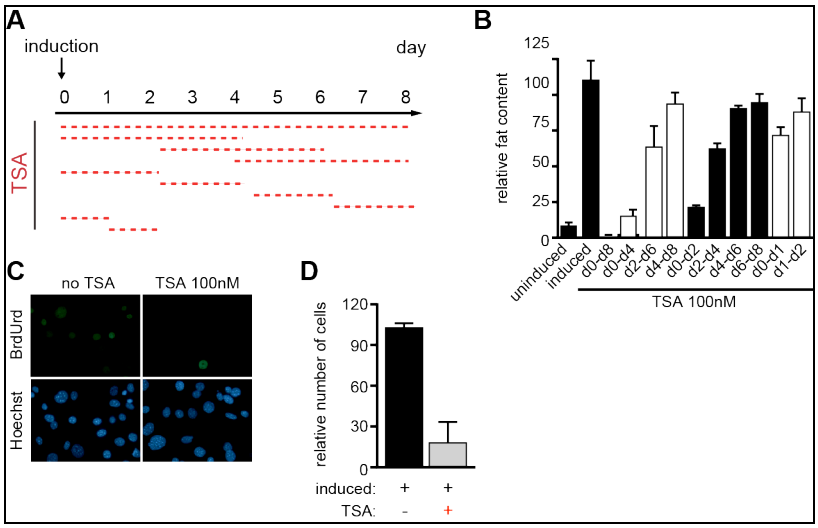


Figure 4.4. Time course analysis of adipogenic inhibition by TSA. (A) Schematic representation of the duration of TSA treatment in 3T3-L1 cells. (B) Quantification of the ORO staining in 3T3-L1 cells treated with TSA for the time indicated. (C) Immunofluorescence in 3T3-L1 cells induced to differentiate into adipocytes using anti-BrdUrd antibody after a 4-h pulse with BrdUrd. Hoechst staining was used to visualize cell nuclei. (D) Quantification of the BrdUrd positive cells in C.

We next profiled the expression of several adipocyte markers by quantitative real time RT-PCR. Treatment with TSA led to a significant down-regulation of the late adipocyte marker *aP2* (adipocyte lipid-binding protein, also known as *Fabp4*). The expression of *Pparγ*, a mid to late marker of adipogenesis, was also significantly reduced by TSA treatment. However, the expression of *Srebp-1c*, as well as the expression of *C/EBPβ*, an early marker of adipogenesis was relatively insensitive to TSA (Figure 4.5A). Similar results were obtained with the other HDACi Scriptaid and SAHA (Figure 4.5B, C). As expected, treatment of 3T3-L1 cells with the short chain fatty acid sodium butyrate did not result in the down-regulation of the adipocyte markers examined (Figure 4.5D). We conclude that HDACi act upstream of *Pparγ* but downstream of *C/EBPβ* to control adipogenesis.

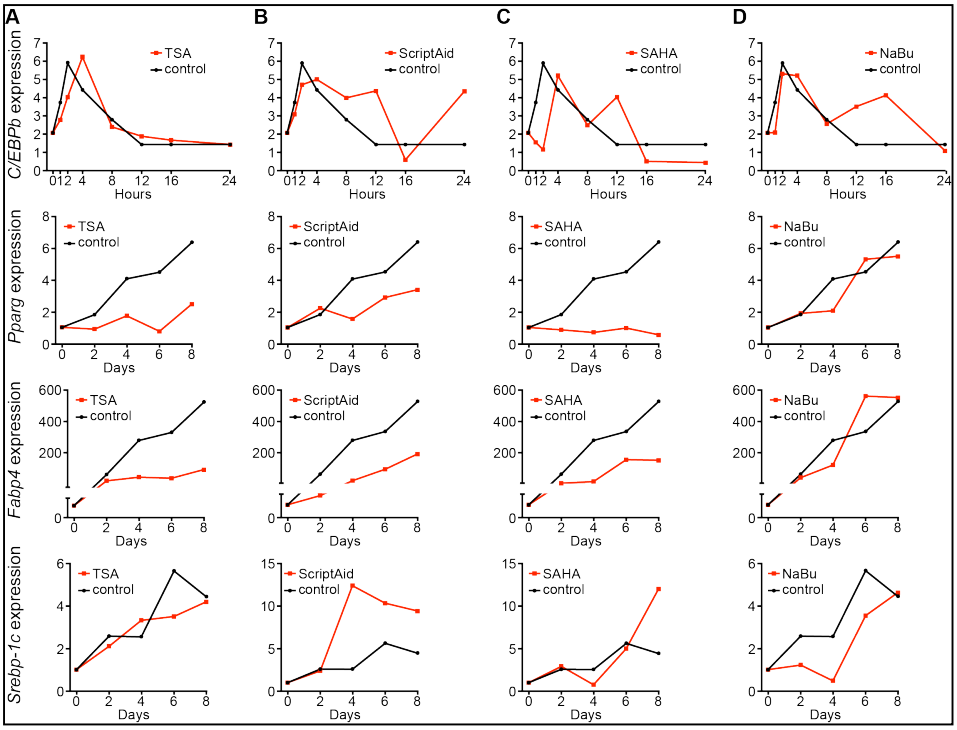


Figure 4.5. Transcriptional profiling of adipogenic marker genes in 3T3-L1 cells treated with HDACi. (A-D) Quantitative real time RT-PCR shows that treatment of 3T3-L1 cells with TSA (A), Scriptaid (B) or SAHA (C) blocks the activation of the transcription of *Pparγ* and *Fabp4* but does not reduce the extent of the upregulation of *Srebp-1c* or *C/EBPβ* transcription. Sodium butyrate does not reduce the activation of the expression of adipogenic marker genes.

Redundant control of adipogenesis by HDAC1 and HDAC2

In an effort to identify the specific HDAC isoforms responsible for the actions of HDACi on the adipogenic program, we generated embryonic fibroblasts from mice with conditional alleles for HDAC1, HDAC2, and HDAC3. After deletion using lentiviral Cre delivery, cells were differentiated using isobutylmethylxanthine, dexamethasone, insulin, and rosiglitazone. Deletion of any of these three HDACs individually had no discernable influence on adipogenesis (Figure 4.6A). Because HDAC1 and HDAC2 have been

shown to act redundantly to regulate various gene programs in other cell types, we also generated fibroblasts from mice with compound conditional alleles encoding these isoforms. Deletion of HDAC1 and HDAC2 together led to an almost complete block of the adipogenic program. In contrast, adipogenesis occurred normally when only three of the four alleles of HDAC1 and HDAC2 were deleted (Figure 4.6A-D). These results demonstrate that HDAC1 and HDAC2 redundantly control adipogenesis.

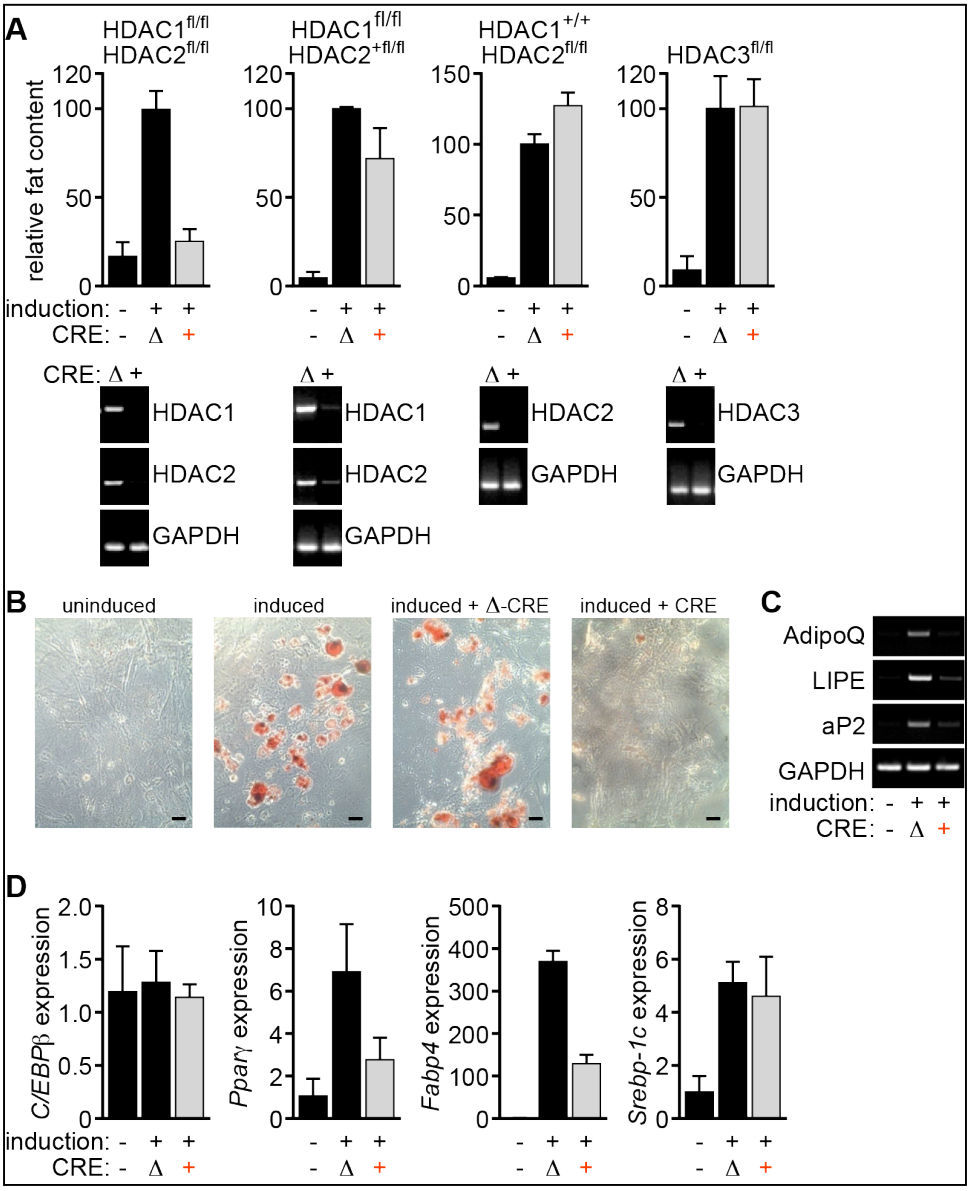


Figure 4.6. Genetic deletion of HDAC1 and HDAC2 blocks adipogenesis. (A) MEFs with the indicated genotypes were generated and genetic deletion was achieved using lentiviral Cre delivery. Deleted Cre-expressing lentiviruses were used as control. The MEFs were induced to differentiate into adipocytes and lipid accumulation was quantified by ORO staining. Efficiency of Cre-mediated take-out was tested by RT-PCR (lower panels). (B) Images of the ORO staining of MEFs treated as indicated. Size bars: 60μm. (C, D) The up-regulation of adipogenic marker genes in MEFs is blocked upon deletion of HDAC1 and HDAC2 as shown by RT-PCR (C) and quantitative real time RT-PCR (D). GAPDH was detected as a control.

DISCUSSION

The results of this study reveal a unique and unexpected role for HDACs in adipogenesis. We have shown that HDACi potently block adipocyte differentiation. Their action is mediated, at least in part, by HDAC1 and HDAC2, as deletion of these isoforms leads to a complete block of adipogenesis *in vitro*.

HDAC inhibitors specifically block the early stage of adipogenesis

We have previously observed that deletion of HDAC3 leads to a profound pro-adipogenic phenotype in cardiomyocytes (Montgomery, 2008). To further explore this phenotype, and the involvement of HDACs in lipid homeostasis, we initially tested pharmacological HDAC inhibition in an *in vitro* model of adipogenesis. Unexpectedly, we did not see an enhancement of adipogenesis but a complete block of adipocyte differentiation. This is in contrast to previously published reports of enhanced adipogenesis in pre-adipocytes treated with the HDACi sodium butyrate and valproic acid (Yoo et al., 2006). We confirmed our initial observation, namely that TSA blocks adipogenesis in the 3T3-L1 model, by using different HDACi (*i.e.* TSA, SAHA, and Scriptaid) in two different *in vitro* models (3T3-L1 cells and mouse embryonic fibroblasts).

When trying to reconcile our data with previously published results we realized that valproic acid and sodium butyrate are short chain fatty acids, a class of chemicals that has previously been demonstrated to enhance adipogenesis. We thus hypothesized that the adipogenic effect of valproic acid and butyrate is due to their SCFA nature and not to the fact that these molecules are also HDACi. Indeed, when 3T3-L1 cells were treated with valproic acid and butyrate we also observed increased adipogenesis.

However, adipogenesis was completely blocked upon co-incubation with TSA, indicating that the pro-adipogenic effect was not due to HDAC inhibition but to the SCFA nature of valproic acid and sodium butyrate.

We next tested if HDACi non-specifically block mesenchymal differentiation or if their effects are specific to adipogenesis. We used osteogenesis as an alternative mesenchymal differentiation model and treated primary osteoblasts with TSA. Strikingly, TSA treatment had no detrimental effect on osteoblastogenesis but actually increased *in vitro* osteoblast differentiation as measured by extracellular matrix calcification and the up-regulation of osteoblastic marker genes.

Based on the time course for the inhibition of adipogenesis by HDACi, which seem to exert their action within the first 48 h of induction, we conclude that this inhibition occurs downstream of C/EBPβ but upstream of PPARγ. We speculate that HDACi can block adipogenesis by affecting the acetylation state of C/EBPβ, consequently causing C/EBPβ to be sequestered in transcriptional inactive chromatin regions (Figure 4.7). Alternatively, HDACi could block adipogenesis by ultimately reducing C/EBPβ DNA-binding affinity and its transcriptional activation potential. To rule out that the observed phenotype is due to toxicity of the used drugs we used a genetic

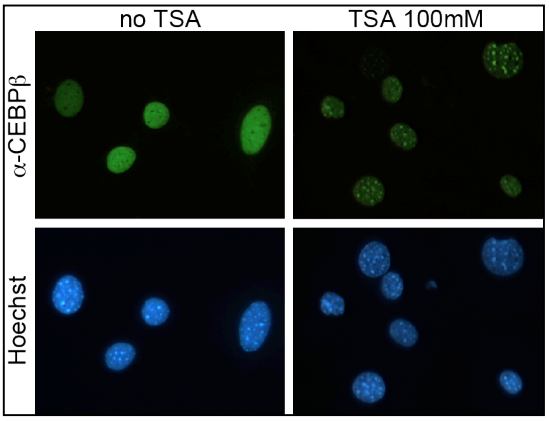


Figure 4.7. TSA induces redistribution of C/EBPβ in transcriptionally inactive chromatin regions. Immunofluorescence shows redistribution of C/EBPβ in the nucleus of TSA-treated 3T3-L1 cells after 18 h of induction of differentiation.

approach to delete HDAC isoforms in mesenchymal cells. Previous studies have shown that HDAC1 and HDAC2 often redundantly control gene programs that govern cellular differentiation (Haberland et al., 2009a; Haberland et al., 2009c; Montgomery et al., 2007; Montgomery, 2008). We thus used MEFs with conditional alleles for HDAC1 and HDAC2 to delineate the genetic requirement for HDACs in adipogenic differentiation. The fact that only the deletion of HDAC1 and HDAC2 together, but not the deletion of either HDAC1 or HDAC2 alone, causes a potent inhibition of lipid accumulation within the MEFs upon induction of adipogenesis supports the conclusion that HDAC1 and HDAC2 regulate adipocyte differentiation in a redundant fashion.

HDACi are one of the most surprising classes of drugs, as they interfere with a core transcriptional process but nevertheless display therapeutic benefit in a wide variety of clinical disease models. Additionally, they are well tolerated by humans, even in formulations that non-specifically inhibit all class I HDACs (Jiang et al., 2002). It is highly likely that the development of isoform-specific inhibitors will lead to even better therapeutic efficacy. In summary, we provide evidence that adipogenesis and adipocyte homeostasis are under the control of HDAC1 and HDAC2. It would be interesting to determine if this previously unrecognized function of HDAC1 and HDAC2 could be effectively targeted by isoform-selective HDACi within a clinical setting and whether lipid storage or metabolism are altered in human patients undergoing HDACi therapy.

METHODS

Cell culture and adipocyte differentiation

3T3-L1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics. Mouse embryonic fibroblasts were prepared from embryonic day (E) 12.5 embryos and cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics. Lentiviral infections of mouse embryonic fibroblasts were performed according to a modified version of previously described methods (Tiscornia, 2006). Briefly, 293T cells were cultured in DMEM supplemented with 10% FBS and antibiotics. The cells were then transfected with either GFP-CRE or GFP-deleted CRE fusion vectors using FuGENE (Roche Applied Science). After 12 h of transfection, the cells were incubated with fresh medium. At 36 h post-transfection, the medium containing the lentiviruses was collected, added with Polybrene (4 μg/ml), filtered, and transferred to the target cells. Primary, calvarial pre-osteoblasts were isolated as previously described (Bakker, 2003). In brief, murine preosteoblasts were isolated from 1.5-day-old pups and cultured in DMEM containing 10% FBS. After reaching confluence, the pre-osteoblasts were induced to differentiate for 14 days using 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate, and 100 nM dexamethasone. The calcified matrix deposited by the mature osteoblasts was visualized using silver nitrate staining. Briefly, cells were fixed in 10% formalin for 10 min, stained with 5% silver nitrate, and refixed in 5% sodium thiosulfate for 5 min. To induce adipogenesis and accumulation of lipid droplets in 3T3-L1 and mouse embryonic fibroblasts, cells were cultured to confluence, and after 48 h (time 0) they were incubated

with 1.72 μM insulin (Sigma), 625 nM dexamethasone (Sigma), 2 μM rosiglitazone (Cayman Chemical), and 0.5 mM isobutylmethylxanthine (Sigma) for 2 days. The cell culture medium was then supplemented with only 1.72 μM insulin, 625 nM dexamethasone, and 2 μMrosiglitazone for 6 additional days, changing the medium every 48 h. Eventually, either TSA (Sigma), Scriptaid (Calbiochem), SAHA (Calbiochem), sodium butyrate, sodium propionate, or sodium acetate (Sigma) were added to the medium as stated in the text. Lipids accumulated within the cells were visualized by oil red O staining. Briefly, cells were fixed in 10% formalin for 10 min and stained for 2 h. Quantification of the amount of fat accumulated in the cells was performed by resolubilization of the oil red O with isopropyl alcohol and spectrophotometric reading of the obtained solution at 515 nm.

Indirect immunofluorescence

3T3-L1 cells were plated onto glass coverslips and cultured until confluence. To detect C/EBPβ, post-confluent cells were induced to differentiate as described above for 18 h and eventually incubated with 100 nM TSA (Sigma). Cells were then fixed, blocked with bovine serum albumin (BSA), and incubated with anti-C/EBPβ primary antibody (mouse monoclonal, 1:200 dilution, Santa Cruz Biotechnology) for 1 h at room temperature. For BrdUrd labeling, post-confluent cells were induced to differentiate and incubated with 30 μg/ml BrdUrd for 4 h. Cells were then fixed, treated with 1.5 M HCl, permeabilized, blocked with BSA, and incubated with anti-BrdUrd primary antibody (mouse monoclonal, 1:200 dilution, Roche Applied Science) for 1 h at room temperature.

Coverslips were then washed 5 times with phosphate-buffered saline, and incubated with fluorescein isothiocyanate-conjugated secondary antibody (1:200 dilution, Vector Laboratories). After washing, coverslips were mounted on glass slides using VectaShield mounting medium with 4,6-diamidino-2-phenylindole (Vector Laboratories), and visualized with a fluorescence microscope.

Gene expression profiling and RT-PCR

Total RNA was purified from cells using Trizol reagent according to manufacturer's instructions. Quantitative real time RT-PCR was performed using TaqMan probes purchased from ABI. A probe for 18s cDNA was used to normalize the amount of starting template entering the amplification step. For RT-PCR, total RNA served as template for reverse transcription using random hexamer primers.

Chapter V

Conclusions and Future Remarks

MiR-378/378* knockout mice are resistant to obesity in a setting of metabolic stress but do not display overt phenotypes under normal laboratory conditions. MiR-378 and 378* thus represent intriguing targets for disease modulation and pharmacological intervention in the treatment of metabolic syndrome. Similarly, the dynamic nature of histone modification, as well as the relative safety of HDACi observed in multiple clinical trials, points to HDACs as attractive therapeutic targets in the prevention and treatment of diabetes and obesity.

MiR-378 and miR-378* regulate mitochondrial metabolism and energy homeostasis

In the present study, we investigated the functions of miR-378 and miR-378* in mice by deleting these miRNAs and leaving the host gene *Ppargc1b* intact. We show that mice lacking miR-378 and miR-378* are protected against diet-induced obesity *in vivo*. The phenotype we observed is likely due to the combinatorial effects that miR-378 and miR-378* have on multiple targets in the different cell types and tissues where miR-378/378* are highly expressed (BAT, heart skeletal muscle) or up-regulated in response to different stimuli (white adipocytes). Crat and Med13 represent only two of the molecular effectors of the biological functions of miR-378/378*. However, the regulation of numerous other genes is undoubtedly required to explain the global amelioration of the metabolic syndrome that we detected in the miR-378/378* mutant mice. It will be of particular interest to dissect the role of miR-378 and miR-378* in insulin-target tissues, and define the relative contribution of each microRNA in the different tissues. In the same way, miR-378 and miR-378* might have important functions in tissues that do not directly respond to insulin signaling but that still have a

relevant contribution in maintaining the overall energy homeostasis in mammals, such as the brain. Even if we did not observe differences in behavior or food consumption in the miR-378/378* KO mice we cannot exclude a role for these microRNAs in modulating different aspects of the humoral response, in particular under stress conditions, such as fasting or cold exposure. Another possible unrecognized function of miR-378 and miR-378* that requires further investigation is the dramatic reduction of adipocytes size in the mice lacking miR-378/378*. It is possible that miR-378 and miR-378* are required for efficient hypertrophy and lipid uptake in white adipocytes, making them potential targets for therapeutic intervention in the treatment of obesity.

The modulation of fatty acid metabolism is crucial not only in the context of adipose tissue and adipogenesis but also in the maintenance of cardiac function. The heart primarily utilizes fatty acids as an energy substrate. Following different types of injury or stress, such as myocardial infarction or pressure overload, cardiac metabolism switches from predominantly oxidative to glycolytic. The heart is a plastic organ and it responds to injury through a switch in energy substrate preference that limits the production of damaging intermediates, such as oxygen reactive species. However, the long term effects of a reduced mitochondrial oxidative capacity might be deleterious for cardiac tissue. We surmise that being able to modulate the ability of the heart to utilize fatty acids following injury could preserve cardiac function. Interestingly, miR-378, as well as the host gene *Ppargc1b*, is down-regulated in different murine models of heart disease (Figure 3.2) (van Rooij et al., 2008). In addition, microRNA profiling of human infarcted hearts revealed that miR-378 is consistently down-regulated during heart failure (data not shown).

In conclusion, our findings identify miR-378/378* as components of a regulatory network that control the oxidative function of insulin-target tissues under conditions of metabolic stress. MiR-378 and 378* thus serve as intriguing targets for disease modulation and pharmacological intervention in the treatment of obesity and metabolic syndrome.

Inhibition of miR-378 and miR-378* in the treatment of obesity

The findings described in this study point to miR-378 and miR-378* as potential targets for pharmacological inhibition in the treatment of obesity and related diseases. The key findings are that mice lacking miR-378 and miR-378* are resistant to high fat diet-induced obesity and display improved insulin sensitivity in the setting of metabolic stress. Design and delivery of anti-miR molecules directed against miR-378, miR-378* or both represent a valuable therapeutic approach in the treatment of obesity, potentially free of deleterious side effects. In fact, miR-378/378* knockout mice do not display overt phenotypes under normal laboratory conditions, whereas their phenotype becomes apparent under conditions of metabolic stress.

The use of anti-miR molecules also offers the possibility to distinguish the relative biologic contributions of single microRNAs. While genetic deletion of either miR-378 or miR-378* alone is not possible, the implementation of a pharmacological approach to suppress the expression of miR-378, 378* (or both) offers numerous advantages in terms of temporal and spatial inhibition of a specific microRNA. In addition, given the potentially important role that miR-378 and 378* play in the liver, a pharmacological approach would likely be efficacious, as delivery of anti-miR to the

liver is highly efficient. In this regard, different experimental paradigms can be designed in mice in the setting of high fat diet. For example, anti-miRs against miR-378, miR-378* or both can be administered after 12 weeks of high fat diet, to determine if inhibition of different combinations of miR-378 and miR-378* is sufficient to revert the obese phenotype. The reduced adipocyte hypertrophy observed in the miR-378/378* KO mice is only one of the reasons why targeting of miR-378 and miR-378* could be beneficial in the treatment of obesity. MiR-378 and miR-378* could be considered either markers or determinants of lipid content in the different tissues, such as liver, heart, skeletal muscle, and WAT. In this regard, it has been previously described that overexpression of miR-378/378* in ST2 cells is sufficient to increase lipid uptake. Conversely, knock-down of miR-378 or miR-378* reduces fat accumulation and enhances the transcriptional activity of C/EBPα and C/EBPβ (Gerin et al., 2010a). Further defining the role of miR-378/378* in adipogenesis will be valuable to better design new therapeutic approaches for treatment of obesity.

HDAC1 and HDAC2 redundantly control adipogenesis

The results of this study reveal an unrecognized role for HDAC1 and HDAC2 in adipogenesis. We demonstrated that inactivation of both HDAC1 and HDAC2 through either pharmacological inhibition or genetic deletion are required for differentiation of mesenchymal precursors into mature adipocytes. Further studies were conducted *in vivo* to determine if white fat tissue-specific knockout of HDAC1 and HDAC2 is sufficient to confer resistance to high fat diet-induced obesity. To this end, we crossed mice carrying

floxed alleles for HDAC1 and HDAC2 with mice expressing Cre-recombinase under the control of the *aP2* promoter. The results of the study, even if promising, did not permit us to make definitive conclusion about the efficacy of HDAC1 and HDAC2 genetic deletion for treatment of obesity *in vivo* in a high fat diet setting. Lack of a clear reduction in body weight and white fat content in HDAC1 and HDAC2 knockout mice can be ascribed to multiple reasons. In addition to mouse strain variability, it is commonly accepted that only about 10% of the entire adipocyte population in mammals renews every year (Rigamonti et al., 2011; Spalding et al., 2008), implying that a block in early differentiation of progenitor fat cells into mature adipocytes can affect only 10% of the entire white fat tissue mass *in vivo*. This complicates the interpretation of our results with the HDAC1 and HDAC2 knockout mice. Furthermore, deletion of HDAC1 and HDAC2 with the *aP2*-driven Cre was achieved late in the cascade of molecular events that characterize adipocyte differentiation, although we showed that HDACi blocks the early stages of adipogenesis, upstream of *Pparγ*. Further studies where deletion of HDAC1 and HDAC2 is mediated by different Cre systems in syngenic mouse strains will provide additional *in vivo* evidence for the role of HDACs in the control of adipogenesis.

HDACi block adipogenesis: possible therapeutic implications

Our findings open a new chapter in the potential clinical uses of HDACi, making them intriguing candidates for pharmacological intervention in the treatment of obesity. In particular, we recapitulated the block of adipogenesis operated by broad-spectrum HDACi *in vitro* with the genetic deletion of HDAC1 and HDAC2, strongly suggesting that the inhibition of these two class I HDAC isoforms is sufficient to suppress adipocyte

differentiation. In addition, we showed that short chain fatty acids, which have HDAC inhibitory activity, behave differently from other HDACi, as they enhance adipogenesis. We sought to determine if administration of HDACi in mice is beneficial in the setting of high fat diet feeding. However, the poor solubility, stability and delivery of commercially available HDACi made our experimental study problematic. The only HDACi soluble in aqueous solution is valproic acid, a short chain fatty acid. Valproic acid is not efficacious in blocking diet-induced obesity (data not shown and Verrotti et al., 2011). In addition, TSA is not stable at room temperature and is poorly soluble in aqueous solution. Both these characteristics made the delivery of TSA in mice utilizing osmotic pumps not effective in reducing body weight following high fat diet.

Design of novel class I-specific HDACi seems to be required in order to achieve an effective block of adipogenesis *in vivo*. The hydroxamate-based inhibitor Vorinostat (Zolinza) was the first HDACi to be approved by the Food and Drug Administration in October 2006 for the treatment of refractory cutaneous T-cell lymphoma (Wagner et al., 2010). Many more HDACi have been tested in clinical trials designed to test the safety and efficacy of the compounds in patients with different types of tumors (Wagner et al., 2010). Given our findings, it will be important to determine the possible effects of the HDACi treatment on body composition during future clinical trials. Furthermore, in a clinical setting, it would be extremely informative to determine if inhibition of HDAC1 and HDAC2 is sufficient to revert the obese phenotype induced by high fat diet. Furthermore, treatment of differentiated 3T3-L1 cells with TSA increases lipolysis, as measured by the detection of free fatty acids released in the culture media (data not shown). In conclusion, given the loss-of-function phenotype associated with HDAC1

and HDAC2, the design of class I-specific HDACi may be beneficial for treatment of obesity.

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