

Dissertation

PhD – Thesis

Role of Lipolysis in Cancer Associated Cachexia and Regulation of Lipolysis by microRNA 124a



submitted by

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Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of “Good Scientific Practice”.

A handwritten signature in blue ink, followed by the date 01/08/2011.

Graz, [date & signature]

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Zusammenfassung

Die Lipolyse von Triglyceriden und die Fettsäureoxidation (beta-Oxidation) sind zentrale Stoffwechselvorgänge, die die Lipid-Homöostase regulieren. Eine ausgewogene Balance ist daher von großer Bedeutung um negative Effekte wie Lipotoxizität, Diabetes und andere metabolische Erkrankungen zu verhindern.

Unter Kachexie versteht man multifaktoriell bedingtes Geschehen, das am häufigsten bei Patienten mit malignen Erkrankungen auftritt und durch einen unkontrollierbaren Verlust von Fettgewebe und Skelettmuskulatur gekennzeichnet ist. Bei der Krebs-assoziierten Kachexie (CAC) wurde die Lipolyse als ein wichtiger pathophysiologischer Prozess identifiziert, der durch erhöhte Werte freier Fettsäuren und von Triglyceriden charakterisiert ist. In Adipozyten von Patienten mit CAC ist die Aktivität der hormonsensitiven Lipase (HSL) und der „adipose triglyceride lipase (ATGL)“ erhöht. Wir untersuchten daher, ob der Verlust einer Lipase den Beginn oder das Fortschreiten der CAC verhindern kann. Es zeigte sich, dass die Inhibierung der Lipolyse durch Verlust der ATGL oder HSL die Hauptsymptome der CAC verhindern kann. In wild-Typ C57BL/6 Mäusen führt die Injektion von Lewis lung carcinoma oder B16 melanoma Zellen zu Tumorwachstum und völligem Verlust des weißen Fettgewebes sowie zu einer starken Reduktion der Skelettmuskulatur. Im Gegensatz dazu sind ATGL defiziente Mäuse vollständig vor gesteigerter Lipolyse im Fettgewebe, Apoptose in Skelettmuskelzellen und Verlust der Skelettmuskulatur durch Aktivierung der Proteasomen geschützt. Das Fettgewebe und die Skelettmuskulatur bleiben vollständig erhalten. HSL defiziente Mäuse waren hingegen nur partiell geschützt. Die Lipolyse ist daher ein essentieller Bestandteil der Pathogenese der CAC. Eine pharmakologische Inhibierung dieser Lipasen könnte dazu beitragen, die Entstehung der CAC zu verhindern.

Um die Regulation des Lipidstoffwechsels durch miRNAs zu untersuchen, untersuchten wir die Sanger Datenbank um miRNAs zu identifizieren, die als derartige Regulatoren fungieren könnten. Hierbei identifizierten wir MiRNA-124a, die den ersten und geschwindigkeitsbestimmenden Schritt des TG Abbaus beeinflussen kann indem sie ATGL und „comparative gene identification 58 (CGI-58/ABHD5)“ auf Protein- und mRNA-Ebene negativ reguliert. miR-124a reguliert weiters die wichtigen Komponenten der Regulation der beta-Oxidation PPAR α auf Protein- und mRNA-Ebene und beeinträchtigt die Translation von RXR α mRNA. Daher reguliert miRNA 124a nicht nur den Abbau von TG zu Fettsäuren sondern auch deren Abbau durch beta-Oxidation.

Summary

Triglycerol hydrolysis (lipolysis) and fatty acid oxidation (β -oxidation) are central metabolic processes regulating lipid homeostasis. Thus, a precise balance between the two processes is imperative to prevent adverse affects such as lipotoxicity, obesity, diabetes and other metabolic disorders.

Cachexia is a multifactorial wasting syndrome most common in patients with cancer that is characterized by the uncontrolled loss of adipose and muscle mass. Increased lipolysis in cancer-associated cachexia (CAC) has been implicated as a pathophysiological process and is inferred by enhanced circulating fatty acids and glycerol concentrations. In adipocytes of patients suffering from tumor-associated cachexia, expressions of hormone-sensitive lipase (HSL) and to some extent adipose triglyceride lipase (ATGL) are enhanced. Therefore, we investigated if ablation of lipases can prevent the initiation or progression of CAC. We found that the inhibition of lipolysis through genetic ablation of *Atgl* or *Hsl* ameliorates key features of CAC. In wild-type C57BL/6 mice, injection of Lewis lung carcinoma or B16 melanoma cells causes tumor growth, complete loss of white adipose tissue (WAT), and a marked reduction of skeletal muscle. In contrast, *Atgl*-deficient mice with tumors were protected from increased WAT lipolysis, myocyte apoptosis, and proteasomal muscle degradation and maintained normal adipose and gastrocnemius muscle mass. *Hsl*-deficient mice with tumors were also protected, although to a lesser degree. Thus, lipolysis is essential in the pathogenesis of CAC. Pharmacological inhibition of metabolic lipases may help prevent cachexia.

To investigate the regulation of lipolysis by micro RNAs (miRNAs), we screened the Sanger miRNA database for suitable miRNAs and identified miR-124a as a potential key regulator of lipid metabolism. miR-124a influences the first and rate limiting step of TG catabolism by negatively regulating ATGL and comparative gene identification 58 (CGI-58/ABHD5) at mRNA as well as protein level. miR-124a also downregulates Peroxisome proliferator-activator receptor alpha (PPAR α) at mRNA and protein level as well as impairs the translation of Retinoid X receptor alpha (RXR α) mRNA, key components regulating β -oxidation. Thus, miR-124a not only governs the catabolism of TG and generation of fatty acids but also regulates fatty acid oxidation.

Abbreviations

ABCA1	ATP-binding cassette, sub-family A member 1
ADH8	Alcohol dehydrogenase, iron containing, 1 (ADHFE1)
AGO	Argonaute
AIDS	Acquired immune deficiency syndrome
α -MSH	Proopiomelanocortin (POMC)
APP	Acute phase protein
APR	Acute phase response
ARF1	ADP-ribosylation factor 1
ATGL	Adipose triglyceride lipase
BAT	Brown adipose tissue
BKCD	Branched chain α -keto acid dehydrogenase
BMI	Body-Mass Index
CAC	Cancer associated cachexia
cAMP	Cyclic adenosine monophosphate
CE	Cholesteryl ester
CFP-CGI-MIR	Plasmid carrying a Cyan fluorescent Protein (CFP) gene with miR-124a binding site, (from at 3' UTR of CGI-58) cloned at the 3' UTR.
CGI-58	Comparative gene identification 58; Abhydrolase domain containing 5 (CGI-58/ABHD5)
CIDEA	Cell death-inducing DFFA-like effector α
CO-IP	Co-Immunoprecipitation
COPD	Chronic obstructive pulmonary disease
CPT	Carnitine palmitoyltransferase
CRP	C-reactive protein
DG	Diglyceride
DGCR8	DiGeorge syndrome critical region gene 8
DMEM	Dulbecco's Modified Eagle Medium
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FA	Fatty acids/Free fatty acids
FACS	Fluorescence-activated cell sorting

FAD	Flavin adenine dinucleotide
FATP 1	Solute carrier family 27 (Slc27a1)
FBS	Fetal bovine serum
G0S2	G0/G1switch 2
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSL	Hormone sensitive lipase
IFNg	interferon gamma
IL	Interleukin
KO	Knockout
LLC	Lewis lung carcinoma
LMF	Lipid mobilizing factor
LPAAT	Lysophosphatidic acid acyltransferase
LPL	Lipoprotein lipase
LUC-MIR-ATGL	Luciferase plasmid with miR-124a binding site, (from at 3'UTR of ATGL) cloned at the 3'UTR
LUC-MIR-CGI	Luciferase plasmid with miR-124a binding site, (from at 3'UTR of CGI) cloned at the 3'UTR
LUC-SCR-ATGL	Luciferase plasmid with scrambled control cloned at the 3'UTR
LUC-SCR-CGI	Luciferase plasmid with scrambled control cloned at the 3'UTR
Mc3r	Melanocortin 3 receptor
Mc4r	Melanocortin 4 receptor
MCH	Melanin-concentrating hormone
MEM α	Minimum Essential Medium alpha modification
MG	Mono glyceride
MGL	Mono glyceride lipase
MIC1	chromosome 18 open reading frame 8 (C18orf8)
miRNA	micro RNA
mRNA	messenger RNA
NMR	Nuclear magnetic resonance
NPY	Neuropeptide Y
O/N	Overnight
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction

PGC 1 α	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PPARGC1A)
PIF	Proteolysis inducing factor/ dermcidin (DCD)
PKA	Protein kinase A
PKC	Protein kinase C
PKR	Eukaryotic translation initiation factor 2-alpha kinase 2 (EIF2AK2)
POMC	Proopiomelanocortin
PPAR	Peroxisome proliferator-activated receptor
PPRE	Peroxisome proliferator-activated receptor response element
pre-miRNA	pre micro RNA
pri-miRNA	primary micro RNA
qPCR	Quantitative polymerase chain reaction
RE	Retinoic acid
RISC	RNA-Induced Silencing Complex
RXR	Retinoid X receptor
TG	Triglyceride
TGF	Transforming growth factor
TNF α	Tumor necrosis factor alpha
UCP	Uncoupling protein
UTR	Untranslated region
WAT	White adipose tissue
Wt	Wild type
XPO5	Exportin 5
YFP-ATGL-MIR	Plasmid carrying a Yellow fluorescence protein (YFP) gene with miR-124a binding site, (from at 3'UTR of ATGL) cloned at the 3'UTR
ZAG	alpha-2-glycoprotein 1, zinc-binding (ZAG)

Introduction...

Fatty acid metabolism

Triglyceride (TG) hydrolysis and fatty acid (FA) oxidation are key metabolic processes for energy supply and homeostasis. Fatty acid metabolism becomes even more important during fasting and stress responses. Moreover, TG hydrolysis provides important signaling molecules for several other processes. This involves multiple factors and regulators which act in a synchronized fashion. The intricate regulation of various components involved in a central pathway is certainly essential in various biological and pathological situations (Tomlins et al., 2006). Overproduction of non-esterified fatty acids and chronic exposure of non-adipose cells and tissues to elevated levels of free fatty acids triggers adverse effects subsumed in the concept of “lipotoxicity” (Duncan, 2008; Lelliott *et al.*, 2004; Prieur *et al.*; Shimabukuro, 2006; Vidal-Puig *et al.*; Weinberg, 2006). Therefore, knowledge of the various regulatory components governing lipolysis and the co-ordination between different pathways of energy metabolism is imperative to understand the physiological balance between different pathways and to conceptualize possible intervention against the metabolic disorders.

Lipolysis

FA levels are mainly controlled by two processes: lipolysis and FA esterification. Hydrolysis of TG to FA and glycerol is termed as lipolysis and is controlled by a cascade of various enzymes and co-factors (Holm, 2003; Lass et al., 2006). Adipose triglyceride lipase (ATGL) catalyses the first step of lipolysis by degrading TG into one FA and diacylglycerol (DG) (Zimmermann et al., 2004). Hormone sensitive lipase (HSL) can hydrolyze TG, DG as well as monoacylglycerol (MG). However, it is mainly responsible for the hydrolysis of DG and in the process generates one FA and monoglyceride (MG) (Haemmerle et al., 2002; Osuga et al., 2000). Monoglyceride lipase (MGL) catalyses the last step of lipolysis by hydrolyzing MG to one FA and glycerol (Karlsson et al., 1997). The FA molecules generated are used as energy substrates for β -oxidation and also orchestrate various signaling events in the cell.

Adipose triglyceride lipase (ATGL, PNPLA2)

ATGL, a patatin-like phospholipase, is transcribed from its gene present in chromosome 11 of human (Zimmermann et al., 2004). Various orthologues of ATGL have been reported in mouse, rat, chicken, pig, yeast and plants (Athenstaedt *et al.*, 2005; Chen *et al.*, 2006; Eastmond, 2006; Gronke *et al.*, 2005; Kurat *et al.*, 2006; Saarela *et al.*, 2008). The transcriptional regulation of ATGL is still elusive; however PPAR γ has been reported to modulate its expression via the PPAR response element (PPRE) present in the promoter of the murine *Atgl* gene by directly binding to it (Kim et al., 2006). ATGL is mainly expressed in white adipose tissue (WAT) and brown adipose tissue (BAT), however detectable level of *Atgl* could be found in all tissues (Kershaw et al., 2006; Lake et al., 2005; Villena et al., 2004; Zimmermann et al., 2004). ATGL selectively hydrolyzes TG to form DG and FA as the first step of lipolysis (Zimmermann et al., 2004). The substrate preferences of ATGL and the specific mode of action are still to be discovered.

Activators and inhibitors of the enzyme have recently been reported. ATGL activity is significantly induced by its co-activator, comparative gene identification-58 (CGI-58) (Granneman et al., 2009; Gruber et al., 2010; Lass et al., 2006; Schweiger et al., 2008). The gene encoding CGI-58 is located on chromosome 3. CGI-58 is a member of the α/β hydrolase-fold containing subfamily, which lacks intrinsic hydrolase activity because of the absence of nucleophilic serine residue (Lass et al., 2006). CGI-58 was shown to interact with the patatin domain of ATGL and enhances its hydrolase activity several fold (Lass et al., 2006). However, the mode of activation by CGI-58 is still unclear. Interactions of CGI-58 with ATGL as well as with the lipid droplets are essential for activation of the enzyme as CGI-58 variants lacking either of the two interactions failed to activate lipolysis (Granneman et al., 2007; Gruber et al., 2010; Yang et al., 2010a). In addition to its role in activating ATGL, CGI-58 also possesses acyl-CoA dependent lysophosphatidic acid acyltransferase activity (LPAAT) *in vitro* (Ghosh et al., 2008; Montero-Moran et al., 2010). The N-terminus of the patatin domain of ATGL is responsible for the activation of the

enzyme by CGI-58, whereas the C-terminal region has an inhibitory effect as revealed by mutation studies (Schweiger et al., 2008).

Recently, Yang et. al. showed that G0/G1 switch protein 2 (G0S2) selectively inactivates ATGL activity by direct interaction with the N-terminal region of the patatin domain. However the interaction does not compete with CGI-58 (Yang et al., 2010b). G0S2 is ubiquitously expressed with high expression in adipose tissue, liver, muscle, ovary and kidney (Yang et al., 2010b; Zandbergen et al., 2005). Expression of G0S2 is positively regulated by PPAR γ and insulin, whereas negatively regulated by PPAR α , TNF α and isoproterenol (Yang et al., 2010b; Zandbergen et al., 2005).

Regulation of lipolysis by ATGL also depends on the interaction of the enzyme with lipid droplets. ARF1, SAR1, guanine nucleotide exchange factor, COP I and II are responsible for the transport of ATGL from the endoplasmic reticulum membrane to lipid droplets (Soni et al., 2009). Recently, Ahmadian et. al., showed that murine ATGL is activated by AMPK *via* phosphorylation at serine 406 (Ahmadian et al., 2011). ATGL is also upregulated at mRNA level by glucocorticoids, PPAR γ agonists as well as fasting (Festuccia et al., 2006; Kershaw et al., 2007; Kim et al., 2006; Liu et al., 2009; Villena et al., 2004), whereas TNF α , insulin, mTOR complex 1 and feeding downregulates ATGL expression (Chakrabarti et al., 2010; Jocken et al., 2007; Kershaw et al., 2006; Kim et al., 2006; Kralisch et al., 2005).

Hormone sensitive lipase (HSL)

HSL was discovered in WAT and had been regarded as the main enzyme orchestrating the lipolytic pathway for decades (Bjorntorp *et al.*, 1962; Hollenberg *et al.*, 1961; Rizack, 1964; Vaughan *et al.*, 1964). Unlike ATGL, HSL can hydrolyze TG, DG, MG, cholesterol ester (CE) and retinol ester (RE), however, with varying rates of hydrolysis (1:10:1:4:2, TG:DG:MG:CE:RE) (Lass et al., 2011). Human HSL mRNA is transcribed from the gene located on chromosome 19 and shows a similar expression pattern as ATGL with highest expression in

adipose tissue. An alternative transcription start site leads to various isoforms of HSL in both human and mouse resulting in tissue specific expression (Langin *et al.*, 1993; Laurin *et al.*, 2000). The precise physiological role of HSL isoforms is yet to be determined.

HSL is known to be induced by fasting and catabolic hormonal stimulation (Bjorntorp *et al.*, 1962; Hollenberg *et al.*, 1961; Rizack, 1964; Vaughan *et al.*, 1964). In response to β -adrenergic stimulation, protein kinases including cAMP-dependent protein kinase A (PKA), glycogen synthase kinase-4 and Ca^{2+} /calmodulin-dependent kinase II phosphorylate serine residues present in HSL, leading to enhanced enzyme activity (Donsmark *et al.*, 2004; Garton *et al.*, 1989; Kraemer *et al.*, 2002; Krintel *et al.*, 2008; Langfort *et al.*, 2003; Olsson *et al.*, 1986; Watt *et al.*, 2004). Phosphorylated HSL translocates to the lipid droplet and interacts with lipid droplet bound perilipin-1.

Monoglyceride lipase (MGL)

Human MGL gene is present on chromosome 3. MGL was discovered in adipose tissue of rat but is ubiquitously expressed in most human and murine tissues (Tornqvist *et al.*, 1976). MGL was shown to hydrolyze MG but not TG or DG (Tornqvist *et al.*, 1976). MGL catabolizes the final step of the lipolytic pathway by converting MG to FA and glycerol, thus completing the cascade. Recently, Dinh *et al.* showed that MGL is also responsible for the degradation of 2-arachidonoyl glycerol (2-AG), a bioactive MG, thus establishing a critical role of MGL in the endocannabinoid signaling pathway by altering the ligands of cannabinoid receptor(s) (Dinh *et al.*, 2002; Taschler *et al.*, 2011).

The physiological function and relevance of MGL in adipose tissue still remains unclear because in adipose tissue, HSL also participates in the catabolism of MG. Since MGL expression is constitutively high in all tissues, the regulation of MGL at expression or enzyme level remains unclear (Karlsson *et al.*, 1997).

Beta oxidation

FAs are metabolized mainly *via* mitochondrial β -oxidation. The fatty acids in the cytoplasm are converted into long-chain acyl-CoA esters by fatty acyl-CoA synthetase. The long chain fatty acyl-CoA is subsequently converted to long chain acylcarnitine by carnitine palmitoyltransferase 1 (CPT1). Carnitine:acylcarnitine translocase (CT) exchanges acylcarnitine of long chain acylcarnitine with carnitine and translocates it across the inner mitochondrial membrane. Resultant free carnitine is then provided to CPT 1 for subsequent reactions. CPT2 present in the matrix side of the inner mitochondrial membrane converts acylcarnitine back to long-chain acyl-CoA, which finally enters the β -oxidation pathway.

β -oxidation is a process regulated by acyl-CoA dehydrogenase, enoyl-CoA hydratase, L-3-hydroxyacyl CoA dehydrogenase and 3-ketoacyl CoA thiolase enzymes in a sequential fashion producing acetyl CoA, flavin adenine dinucleotide (FADH₂) and nicotinamide adenine dinucleotide (NADH) at every step thereby shortening the fatty acyl moiety by two carbons.

Transport of FA into the mitochondria and subsequent β -oxidation is under control of PPARs (α , β and γ) and PGC 1 α , which regulates the transcription of many members of the pathway (Desvergne *et al.*, 2006; Finck *et al.*, 2007; Huss *et al.*, 2004; Madrazo *et al.*, 2008; Yang *et al.*, 2007). However, loss or gain of function of different PPARs displayed differential phenotypes and thus the targets are not equally distributed between the regulators (Burkart *et al.*, 2007; Cheng *et al.*, 2004; Finck *et al.*, 2002; Son *et al.*, 2007; Watanabe *et al.*, 2000). In addition, the regulation is highly dependent on the tissue and organism.

Chapter 1

Introduction...

Definition and History

Cachexia (Gr. kakos, 'bad'; hexis, 'habit')... It is chiefly used in regard to chronic diseases, where the general nutrition is defective, and the blood in an unhealthy state. Thus cancerous cachexia indicates the peculiar impoverished state of the blood and general debility which are associated with the deposits of cancer in various parts of the body...

– Chambers Encyclopaedia, 1901

Cachexia is a multifactorial syndrome often associated with chronic infection such as tuberculosis or the acquired immune deficiency syndrome (AIDS), other diseases including chronic obstructive pulmonary disease (COPD), chronic kidney disease, or chronic heart failure. However, it is most prevalent in cancer patients since a high proportion of cancer patients suffer from cancer associated cachexia (CAC). The highest frequency of CAC occurs in pancreatic and gastric cancer (Dewys et al., 1980; Morley et al., 2006; Tisdale, 2002). CAC is an important adverse prognostic factor and the immediate cause of death in an estimated 15% of all cancer patients (Deans et al., 2005; Dewys et al., 1980; Fearon et al., 2002).

Cachexia has been reported throughout history in different forms and definitions. However, due to lack of knowledge regarding the cause and consequences, the consensus definition and symptoms for cachexia still remains elusive. Hippocrates (460-377 BC) described about a particular illness whose symptoms can be matched with cachexia as we know today.

The flesh is consumed and becomes water... the abdomen fills with water, the feet and legs swell, the shoulders, clavicles, chest and thighs melt away... This illness is fatal. (Doehner et al., 2002)

The famous Greek physician Aretaeus of Cappadocia mentioned Hippocrates's excerpt to describe the symptoms of a patient with pulmonary tuberculosis (vaghan et. al. 1922). Cachexia was described and classified as a third group disease by William Cullen's in 1769 as published in *Synopsis nosologiae methodicae* (Sebastian A., 1999). Since then cachexia has been associated with various conditions and has been defined separately as cachexia virginum for chlorosis, cachexia strumpriva for myxoedema or pituitary cachexia for Simmond's disease (Sebastian A., 1999; Magalini and Magalini, 1997).

King David (more than 3500 years ago) who possibly was suffering from cancer, described his conditions as *"I forgot to eat my bread... My strength failed... My bones wasted away... My bones cleave to my skin... I am feeble and depressed"* (Ben-Noun, 2004). He linked the syndrome to the inability of work, depression, isolation and resulting psychological ill-being.

Reports suggest that cachexia was first studied in its own form and was given its name to separate it from other conditions on the island of Kos in Greece. It was derived from two Greek words, kakós (bad) and hexis (habit) (Ben-Noun, 2004). The first ever definition of cachexia could be found in the Chambers Encyclopedia which also mentioned cancer cachexia but in very broad terms. William Osler described the symptoms associated with cachexia in "The principles and practice of medicine" published in 1901 (Osler W., 1901).

Progressive emaciation is one of the most constant features... Loss in strength is usually proportionate to the loss in weight... Anemia is present in a large proportion... and with emaciation gives the picture of cachexia... Anorexia, loss of desire for food, is a frequent and valuable symptom, more constant perhaps than any other.

Dr. G.R. Butler in 1906 described cachexia in general and cachexia associated with cancer in “The diagnostics of Internal Medicine. 2nd ed.” as follows:

“A yellow, waxy face, associated with anaemia, general debility, and more or less emaciation, are the characteristic signs of a condition which is spoken of as “cachectic” or a “cachexia”... it expresses very graphically a state which would otherwise require many words of description, and the recognition of which is often of diagnostic value... cancer cachexiae: debility, emaciation, anaemia, and a dirty yellowish-brown or brown-green complexion.” (Butler GR., 1906)

In 1913, Prof. Rudolf Schmidt from Vienna defined cachexia associated with cancer as:

“There is a combination of general symptoms, namely, the trio: emaciation, adynamia and an unhealthy colour of the face... Occasionally those surrounding the patient have noticed the falling of the cheeks, prominence of the zygomatic arches, and the ‘pointed’ appearance... emaciation, this is, the loss of flesh and, later on, of muscle tissue... this symptom (adynamia) will appear earlier and more pronouncedly in asthenic constitutions and vice versa manifest itself late in stronger constitutions... Adynamia shows itself... not only in general fatigue but also in a lowering of circulatory force.” Schmidt R., 1913)

Hubert French tried to define cachexia in 1912 in “An index of differential diagnosis of main symptoms” as:

"[Cachexia is] an ill-defined term used to include almost any depraved condition of the body in which nutrition everywhere is defective... It is generally applied to patients who exhibit at the same time progressive loss of weight, and change of complexion in the direction of sallowness or actual anaemia. The word is generally prefixed by a qualifying adjective, such as cancerous cachexia..."

– Herbert French, *An index of differential diagnosis of main symptoms*, 1912

The first ever detailed description of cachexia associated with cancer are found in a book chapter written by the founder of the American Society for the control of Cancer (known today as American Cancer Society), Howard Canning Taylor, in 1915. He described cachexia as "late constitutional manifestation of the malignant process". He also described the occurrence of cachexia as progressive along with the tumor progression and associated the syndrome with some particular types of cancer such as stomach or liver (Bennani-Baiti *et al.*, 2009). Moreover he described the cause and symptoms of cachexia:

"The cause of the cachexia cannot be assigned to a single influence. By the ancient writers the cachexia of a malignant growth was looked upon as a separate disease and not as a result of the cancer... With our present beliefs, parts of which are based on theoretical knowledge and part on clinical experience, the causes of the cachexia can practically be arranged under two headings: (1) Absorption of decomposition products, toxins, etc., from primary tumor and its metastases, and (2) interference with the functions of the various organs... The rapidity with which they develop is subject to wide variations... The change in color is due mostly to the diminution of the hemoglobin in the red blood

cells. The emaciation is a late symptom, but is usually present before the termination of the disease... The emaciation is due to disturbances of the gastro-intestinal tract, with the resulting loss of appetite, nausea, vomiting, etc.”(Taylor, 1915)

A study conducted in 1980 with more than 3000 cancer patients, found the descriptions given by Taylor were true and could define cancer cachexia to amazing detail (Dewys et al., 1980).

In 1970 the American Cancer Society held a conference to discuss the symptoms and definitions in detail and concluded that cancer cachexia results in the unknown futile cycles which make the food intake insufficient for the energy requirements of the tumor bearing host. Also they enlisted various metabolic abnormalities associated with cancer associated cachexia such as systemic inflammation, insulin resistance, increased protein and lipid degradation (De Wys, 1970).

Roubenoff and colleagues in 1977 suggested that wasting is different from cachexia (Roubenoff et al., 1997). They defined cachexia as “... *involuntary loss of body cell mass or fat-free mass when this compartment is reduced with little or no weight loss*”. They also said that “...*everyone with wasting has cachexia but the opposite is not true*” (Roubenoff et al., 1997). However, the definition faced a strong disagreement as cachectic patients show a rapid and involuntary weight loss (Bennani-Baiti et al., 2009).

First effort to enlist the mechanism of cancer cachexia was attempted by MacDonald et. al. in 2003.

“a wasting syndrome involving loss of muscle and fat directly caused by tumor factors, or indirectly caused by an aberrant host response to tumor presence”(MacDonald et al., 2003)

However, the authors did not attempt to address the complexity of cachexia and the numerous tumor factors or the precise tumor-host responses. T. Ohnuma in 2003 (Ohnuma T., 2003) and Evans et. al. in 2008 (Evans et al., 2008) distinguished cachexia from starvation and regarded cachexia as multiorgan syndrome which cannot be reversed by re-feeding. Ohnuma described cachexia as *“anorexia, early satiety, wasting of muscle and adipose tissues, weight loss, fatigue, anemia, hyperlipidemia, systemic inflammatory response, and other metabolic abnormalities”* (Ohnuma T., 2003). However, Evans et. al. reported that cachexia

can be characterized by loss of muscle mass with or without loss of fat mass (Figure 1) (Evans et al., 2008).

After several years of research by prominent research groups, the clear and concise definition of cachexia which can define all aspects of the complex syndrome is still not available (Bennani-Baiti et al., 2009).

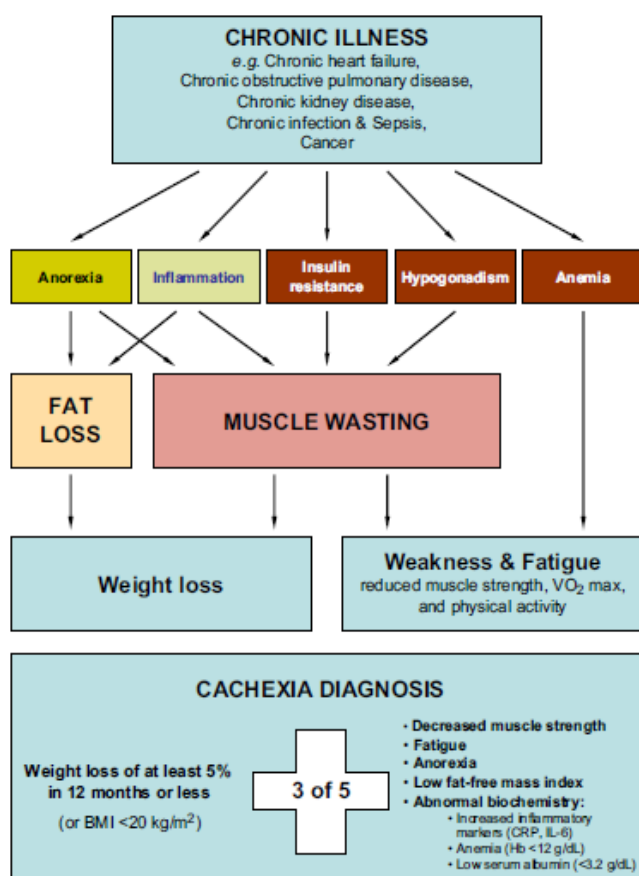


Figure 1. Symptoms and diagnostic criteria for cachexia (Evans et al., 2008). With permission for reuse from Clinical Nutrition.

It is therefore of utmost importance to find a clear definition of cachexia as the diagnosis and the treatment relies on the definition and clear distinction of the symptoms from other disorders.

Cancer associated cachexia

Occurrence and symptoms

The majority of cancer patients lose weight depending on the tumor type and its progression as well as intervention. More than 15% of cancer patients lose greater than 10% of the initial body weight during the progression of cancer (Dewys, 1986). Patients with gastro-intestinal track tumors especially pancreatic or gastric cancer counter highest frequency of weight loss (Dewys, 1986). Whereas patients suffering from sarcomas, non-Hodgkin's lymphoma, acute nonlymphocytic leukemia and breast carcinoma show reduced occurrence of body mass decrease (Dewys, 1986). Occurrence and severity of cachexia varies based on the tumor phenotype (Monitto et al., 2001) and/or host genotype. Weight loss is mostly progressive with the progression of cancer leading up to 24.5% median of weight loss in pancreatic cancer patients (Wigmore et al., 1997). Unexplained weight loss has been reported in 84% of disease-free breast cancer patients, who developed recurrence (Tisdale, 2009).

One of the most prominent prognostic factors of cachexia is weight loss, which results from depletion of skeletal muscle and adipose tissue mass (Fearon, 1992). However in cancer cachexia, the non-muscle protein is less affected and this distinguishes cachexia from simple starvation (Tisdale, 2009). Therefore, feeding nutritional supplements to patients suffering from cancer cachexia proved to be futile (Lainscak et al., 2008). Patients suffering from cachexia lose on average 32% of their weight, 75% of skeletal muscle as well as 85% of total body fat (Figure 2) (Fearon, 1992). The loss of skeletal muscle and adipose tissue mass along with loss of mineral from bones leads to immobility in patients and associated deterioration of quality of life (Windsor *et al.*, 1988). Cachexia is reported to be directly responsible for the death in 10% of cancer patients.

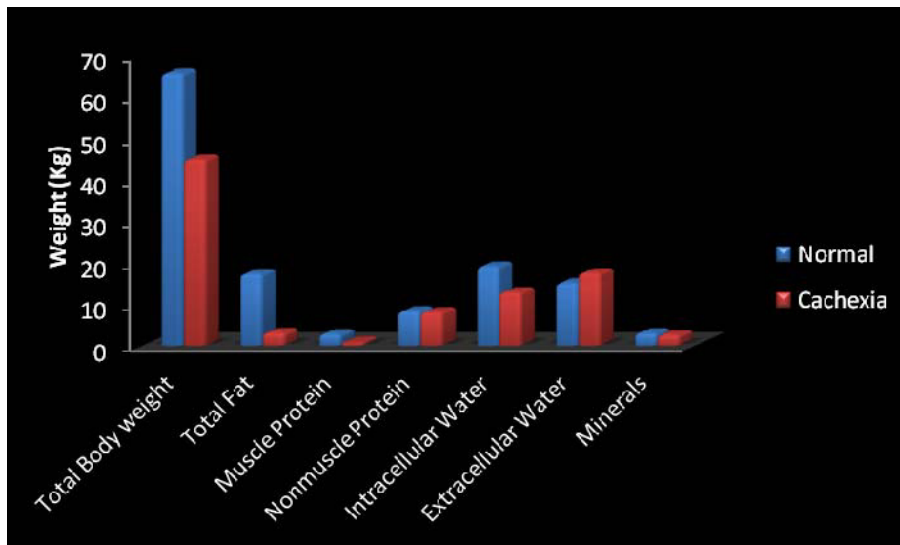


Figure 2. Comparison of body mass between normal and cachexia patients (Fearon, 1992). With permission for reuse from Proceedings of the Nutrition Society (Figure displayed in modified form)

Adipose tissue

Adipose tissue acts as a repository of TG and is the main energy reservoir. Lipolysis is mediated by a cascade of enzymes namely ATGL, HSL and MGL. ATGL catalyzes the first step of lipolysis by converting TG to DG. DG is subsequently degraded by HSL.

Adipose tissue is wasted in patients with the progression of tumor in cachexia patients (Fouladiun et al., 2005). The loss of adipose tissue is more rapid than the skeletal muscle wasting (Fouladiun et al., 2005). Adipose tissue is actually not degraded in cancer cachexia but increased lipolysis reduces the TG reserves in the fat cells (Ryden et al., 2008). Increased fibrosis and deformation of adipocytes was observed in cachectic mice (Bing et al., 2006). Cancer patients with rapid weight loss show increased glycerol and FA concentrations compared to weight stable controls (Shaw *et al.*, 1987). Increased FA concentrations and FA oxidation is reported in cancer cachexia patients (Legaspi et al., 1987). Increased sensitivity towards lipolytic stimuli was observed in cachexia patients (Agustsson et al., 2007). However no increase in basal lipolytic rates could be observed (Agustsson et al., 2007). Expression of HSL is increased significantly

in cancer cachexia patients corresponding to the elevated serum FA and TG levels. Expression of ATGL and lipoprotein lipase (LPL) is not altered in cancer cachexia (Agustsson et al., 2007; Unmehopa et al., 2005).

Along with increased de-lipidation through lipolysis, adipogenesis is also hindered during cachexia. Expression of PPAR γ , SREBP-1c, CEBP α and β are markedly decreased in cachexia thus obstructing adipocyte differentiation (Bing et al., 2006).

Skeletal muscle

Increase of protein degradation and decrease in protein synthesis were both shown to be responsible for the outcome of cachexia in cancer patients. Initially it was suggested that impaired protein synthesis is mainly responsible for the skeletal muscle atrophy (Eley et al., 2007b; Lundholm et al., 1982; Smith et al., 2004b). Both in murine cachexia models and cancer patients, elevated levels of phospho-PKR and phospho-eIF2a were detected (Eley et al., 2008; Eley et al., 2007b). However, exceptionally high protein degradation in skeletal muscle of hepatocellular cancer patients was regarded to be the cause of skeletal muscle loss (O'Keefe et al., 1990). Fast twitch muscles (type I) are more affected in CAC compared to slow twitch muscles (type II) (Yu et al., 2008).

The ubiquitin-proteasome system was reported to be activated in CAC and can play a pivotal role in muscle atrophy (Khal et al., 2005a). MAC16 tumor-bearing mice show increased proteasome proteolytic activities when the mice lost weight up to 16% of their body weight (Chand et al., 2005). Expression of both α and β -proteasome subunits was increased in MAC16 tumor-bearing mice (Khal et al., 2005b).

Along with increased proteosomal activities, caspase-1, 3, 6, and 9 activities were shown to be increased in gastrocnemius muscle of tumor-bearing mice (Belizario et al., 2001). Higher rate of apoptosis was also observed in skeletal muscle of Lewis lung carcinoma and Yoshida AH-130 ascites hepatoma-bearing

mice (van Royen et al., 2000). Muscle biopsies of weight losing gastrointestinal cancer patients showed increased DNA fragmentations confirming the role of apoptosis in muscle atrophy leading to CAC (Busquets et al., 2007a).

Molecular mechanism(s)

Cachexia is a net phenotype originating from tumor-host interactions. An understanding of the causative factors and the role of various tumor-host interactions is indispensable in order to decipher the underlying mechanisms leading to cachexia. During the past decade, many research groups suggested various key factors and processes which might play critical roles during the development of cachexia.

Neuropeptide Y:

Patients suffering from cancer cachexia often report loss of taste and smell which leads to anorexia (DeWys *et al.*, 1975). Anorexia can be a result of imbalance in neuropeptides governing orexigenic signals (neuropeptide Y [NPY]) and anorexigenic signals (proopiomelanocortin [POMC]) (Davis et al., 2004). NPY release is reduced in tumor-bearing rats compared to controls (Chance et al., 1994) and hence intrahypothalamic injection of NPY was less potent in stimulating the rats for feeding in rats with methylcholanthrene-induced sarcoma (Chance et al., 1996). Thus the factors originating from tumors or cytokines might interfere with the NPY release or downstream targets (Bing et al., 2006).

Leptin

Serum leptin concentration decreases drastically in patients suffering from cachexia (Aleman et al., 2002). Leptin maintains a balance with the adipose tissue mass and a decrease in total body fat might be instrumental in decreasing the leptin levels in patients.

Melanocortin

An increase of CNS melanocortin (α -MSH) levels has been reported in most of the cancer patients suffering from anorexia (Wisse et al., 2001). Therefore administering antagonists against melanocortin receptors (Mc3r and Mc4r) in rats with prostate cancer, increased food intake and resulted in significant weight gain (Wisse et al., 2001). However, the increase or restoration of the adipose and skeletal muscle mass has not been investigated.

Macrophage inhibitory cytokine-1 (MIC-1)

MIC-1 is a member of the transforming growth factor (TGF)- β superfamily. Serum MIC-1 levels were found to be significantly correlated with weight loss in advanced prostatic cancer patients (Johnen et al., 2007).

Melanin concentrating hormone (MCH)

MCH is a leptin-sensitive orexigenic neuropeptide which stimulates food intake (Ludwig et al., 2001). The receptor of MCH, MCH1R, was reported to be significantly upregulated in the infundibular nucleus of cachexia patients (Unmehopa et al., 2005).

Acute phase proteins (APP)

Acute phase proteins include CRP, fibrinogen, serum amyloid A, 2-macroglobulin and α -1 antitrypsin. APPs are generated during the acute phase response (APR) from liver in response to infection, inflammation or tissue injury (Tisdale, 2009). APP levels were found to be high in patients with lung, gastrointestinal, non-small-cell lung, as well as pancreatic cancer (Falconer et al., 1995; McKeown et al., 2004; McMillan et al., 1998). APPs were found to correlate with loss of body weight and progression of cachexia (McMillan et al., 1999).

Uncoupling Proteins (UCP)

Uncoupling proteins, UCP1, 2 and 3 mediate the thermogenic effects in BAT and skeletal muscle and thus regulate the total and resting energy expenditure (Qualliotine-Mann et al., 1993). UCP1 mRNA expression in BAT was increased in mice and rat bearing cancer which induced cachexia (Bing et al., 2000; Bing et al., 2006). UCP2 and UCP3 mRNA expression did not show any change in BAT but UCP3 mRNA expression in skeletal muscle was increased in tumor-bearing mice (Bing et al., 2000; Bing et al., 2006). Also in cancer patients, UCP3 mRNA levels were found to be significantly elevated in rectus abdominis muscle (Collins et al., 2002).

Lipid mobilizing factor (LMF)/Zinc α 2-glycoprotein (ZAG)

Lipid mobilizing factor (LMF)/Zinc α 2-glycoprotein (ZAG) was purified from the urine of cancer patients as well as from MAC16 tumor bearing mice (Todorov et al., 1998). Sequence and immunoreactivity of LMF was been shown to be identical to a protein encoded by the AZGP1 gene which has a high degree of similarity to the class I major histocompatibility complex gene(s) (Todorov et al., 1998). Both ZAG/LMF induces lipolysis and glycerol release from fat pads, most probably by cAMP mediated mechanisms (Hirai et al., 1998). ZAG also increases UCP1, 2 and 3 expression most probably through the β 3-adrenoreceptor and thus is blocked by using a receptor antagonist (Sanders et al., 2004a; Sanders et al., 2004b; Yoshitomi et al., 1998). ZAG expression is significantly induced in both mice and human with cancer cachexia (Bing et al., 2004; Dahlman et al., 2005).

Tumor necrosis factor alpha (TNF α)

TNF α has long been known as cachectin or the principal causative agent for CAC. TNF α levels were found to be increased in cancer patients suffering from cachexia (Karayiannakis et al., 2001). However, this finding is still controversial as other studies did not find a clear correlation between circulating TNF α levels

and weight loss (Mader et al., 1995). $\text{TNF}\alpha$ induces lipolysis through the activation of MAPK and JNK (Ryden et al., 2004; Zhang et al., 2002). It also reduces the mRNA and enzyme activity of lipoprotein lipase (LPL); however the LPL activity remains intact in the adipose tissue of cancer patients (Berg et al., 1994; Price et al., 1986; Thompson et al., 1993). Mechanisms mediating increase of lipolysis by $\text{TNF}\alpha$ still remain unclear but $\text{TNF}\alpha$ decreases the expression of perilipin 1 (PLIN) and might increase the access of lipolytic enzymes including HSL and ATGL to lipid droplets (Laurencikienė et al., 2007; Ryden et al., 2004; Zhang et al., 2002). However, $\text{TNF}\alpha$ also reduces the expression of HSL in human adipocytes (Laurencikienė et al., 2007). $\text{TNF}\alpha$ levels in cancer patients are dependant on diurnal fluctuations, sensitivity of technique used for measurement as well as the type and grade of tumor. Therefore it is hard to find clear evidence for the role of $\text{TNF}\alpha$ in the development of CAC.

Interleukins

$\text{TNF}\alpha$ induces pro-inflammatory cytokines like IL-6 and 1. IL-6 induces the liver acute phase response (APR), but it does not cause anorexia, whereas $\text{TNF}\alpha$ induces decrease in food and water intake in mice (Moldawer et al., 1988). IL-6 levels were correlated to the development of cachexia in mouse bearing C-26 colon carcinoma (Strassmann et al., 1992). Neutralizing antibody against IL6 could suppress the development of cachexia, however anti- $\text{TNF}\alpha$ or $\text{IFN}\gamma$ antibody could not protect the mice from wasting syndrome (Strassmann et al., 1992) and clones of C-26 tumors which did not induce cachexia also showed similar serum IL-6 levels (Soda et al., 1995). The role of IL-6 as a causative agent for the development of cachexia remains controversial as its levels do not correlate with the weight loss in advanced or terminal cancer patients (Maltoni et al., 1997).

Proteolysis inducing factor (PIF)

PIF is a glycoprotein family member, originally isolated from MAC16 tumor (Todorov et al., 1996). PIF was detected in urine of cancer patients suffering from cachexia (Cariuk et al., 1997; Wang et al., 2003; Williams et al., 2004). PIF isolated from urine of cachectic patients and from MAC16 tumor injected in mice potentiated rapid weight loss (Cariuk et al., 1997; Todorov et al., 1996). The weight loss was not induced by decrease in food or water intake. The depletion of lean body mass was a result of both decreases in protein synthesis as well as increase in protein degradation (Cariuk et al., 1997). PIF depletes specifically the myosin and not actin filaments in mice (Wyke *et al.*, 2005). PIF also increases the cytokine production and hence APR in the liver (Watchorn et al., 2002).

Ghrelin

Ghrelin is released from the stomach and stimulates food intake. Plasma ghrelin levels showed a significant increase in cancer patients suffering from cachexia (Wolf et al., 2006). Ghrelin signaling and function is impaired in cachectic patients and hence its infusion increased food intake in anorexic patients (Neary et al., 2004; Wolf et al., 2006).

Treatment options of cancer cachexia

Megestrol acetate (megace)

Megace is a synthetic progestin which acts in the ventromedial hypothalamus and stimulates appetite via NPY as well as decreases pro-inflammatory cytokines (Mantovani et al., 1997; Mantovani et al., 2001). However the drug failed to increase or restore the lean mass (Loprinzi et al., 1993). Similar results were observed with other nutritional supplements and insulin (Evans et al., 1985; Lundholm et al., 2007; Simons et al., 1998).

RC-1291

RC-1291 is a ghrelin mimetic which when given to cancer patients increased the hand grip strength and also lean body mass. It failed however to increase body weight of cachexia patients (Tisdale, 2009). In tumor bearing mice, it increased the food intake and fat mass (Wang et al., 2006).

EPA

EPA, a polyunsaturated FA, when administered to cancer patients, stabilized body weight. However it failed to reverse the decrease in protein synthesis in the lean mass (Whitehouse et al., 2001). EPA decreases the expression of ZAG as well as the expression and thus the activity of ubiquitin-proteasome (Russell et al., 2005; Whitehouse et al., 2001). EPA stabilizes the I κ B/NF κ B complex and prevents the activation of NF κ B by PIF (Whitehouse et al., 2003). Further clinical trials are needed to prove the efficacy of the treatment in patients suffering from CAC.

β -hydroxy- β -methylbutyrate (HMB)

HMB downregulates the ubiquitin-proteasome pathway and hence decrease the protein degradation (Smith et al., 2004a). HMB inhibits PKC activation and thus decreases also NF κ B activation (Smith et al., 2004a). Both protein degradation and the inhibition of protein synthesis are rescued by HMB in cachectic mice (Eley et al., 2007a). Cancer patients showed improved body weight and lean mass without any significant effect on fat mass when administered with L-glutamine and L-arginine along with HMB (May et al., 2002).

Infliximab

Infliximab is an inhibitor of TNF α . Since TNF α has been implicated to be one of the major mediators of protein degradation and loss of lean mass in cancer cachexia patients, TNF α inhibitors were a promising drug option to treat cancer

cachexia. However, cancer patients lacked any significant increase in lean mass when administered with Infliximab (Wiedenmann et al., 2008).

Thalidomide

Thalidomide also reduces $\text{TNF}\alpha$ by degrading $\text{TNF}\alpha$ mRNA (Keifer et al., 2001). It also blocks I κ B kinase (IKK) activity and thus decreases the expression of NF κ B regulated genes (Keifer et al., 2001). When administered to cancer patients, thalidomide increased body weight and also restored the lean mass compared to the placebo group (Gordon et al., 2005; Khan et al., 2003).

Anti-inflammatory agents

Ibuprofen inhibits the activation of NF κ B and IKK and thus might inhibit proteasome activity in cancer cachexia patients (Palayoor et al., 1999). It also reduces the APR and serum CRP levels (Wigmore et al., 1995). When administered in patients along with megestrol acetate, ibuprofen increased total body weight and also improved the quality of life (McMillan et al., 1999). However, the body mass analysis has not been performed so far after treatment with Ibuprofen.

AIM...

The pathogenesis of CAC is multifactorial (Tisdale, 2009). Central mechanisms regulate appetite, food intake, and energy consumption. Contributing peripheral mechanisms control lipid and carbohydrate metabolism in various tissues. Severe lipid loss in CAC is driven by changes in lipid catabolism (Agustsson *et al.*, 2007; Hyltander *et al.*, 2000; Klein *et al.*, 1990; Legaspi *et al.*, 1987; Ryden *et al.*, 2008; Shaw *et al.*, 1987) and, possibly, lipogenesis (Jeevanandam *et al.*, 1986). The concept that increased TG degradation may contribute decisively to CAC is strongly underscored by increased plasma levels of FA and glycerol, increased lipolytic rates upon epinephrine stimulation, and increased expression of lipid mobilizing factors, such as ZAG, TNF- α , and IL-1 and -6 (Argiles *et al.*, 2009; Bing *et al.*, 2004). The breakdown of fat requires lipolysis of TG stored in cellular lipid droplets and is mediated by ATGL and HSL (Zechner *et al.*, 2009). This led to our hypothesis that disruption of fat catabolism may prevent the initiation and/or progression of CAC. In vertebrates, lipolysis is most active in adipose tissue with ATGL predominantly being responsible for the initial step of TG hydrolysis (formation of DG) and HSL for the hydrolysis of DG. In the current study we investigated whether one or both of these lipases are essential for CAC.

Materials & Methods...

Animal care

Female C57BL/6 mice were maintained on a regular light-dark cycle (12 hours (h) light, 12 h dark) and kept on a standard laboratory chow diet (4.5 % w/w fat). *Hsl^{-/-}* and *Atgl^{-/-}* mice were generated by targeted homologous recombination (Haemmerle et al., 2006; Haemmerle et al., 2002). Mice used for injection of tumor cells as well as control litter mates were 8-9 weeks of age. During experiments, food intake as well as body weight was monitored. All studies were performed with littermates of the same genetic background as controls. All animal studies were performed in accordance with the guidelines and provisions of the Commission for Animals Experiments of the Austrian Ministry of Science and Research.

Tumor implantation and tissue harvesting

Mice were anesthetized using isoflurane and blood samples were collected by retro-orbital puncture. Animals were randomly divided into groups. 4×10^6 LLC cells or 2×10^6 B16 melanoma cells, obtained from an exponentially proliferating cell culture, maintained in conventional Dulbecco's Modified Eagle Medium (DMEM) high glucose cell culture medium containing 10% fetal bovine serum (FBS) [v/v] (PAA, Pasching, Austria), or saline solution as negative control were injected subcutaneously at *dorsum* just below the neck. After 14 or 21 d (for LLC) and 16 d (for B16 melanoma cells), overnight (o/n)-fasted or non-fasted mice were anaesthetized using isoflurane and blood samples were taken as described above. Mice were kept at an unconscious stage using 1% isoflurane. *M. gastrocnemius* and *M. soleus* were excised rapidly, weighed and either frozen in liquid nitrogen or stored in 4% neutral buffered formalin for subsequent preparation of paraffin blocks. Thereafter, mice were sacrificed by cervical dislocation and tissues were rapidly excised, weighed, and either frozen in liquid nitrogen or stored in formalin. Tissue weights were normalized to *tibia* length.

***In vivo* nuclear magnetic resonance measurement of fat content**

Whole body fat mass was determined using a Minispec mq NMR analyzer (Brucker Optics, Woodlands, TX). Whole body fat mass was obtained as percentage of body weight and presented as gain or loss of fat mass compared to initial values.

Magnetic Resonance Imaging (MRI) **(performed with Mag. Clemens Diwoky)**

MR images were acquired at a 3T human MRI system (Siemens Tim-Trio, Erlangen, Germany) with an 8-channel multipurpose coil (Noras MRI products, Hoechenberg, Germany) to maximize signal-to-noise ratio. For fat-measurements a T1 weighted 2D Turbo Spin Echo (TSE) in coronal plane with the following parameters was used (TR, 743 ms; TE, 39 ms; TF, 5). A field of view 90x45 mm with a 384x192 matrix was used to provide an in-plane resolution of 230x230 μ m with a slice thickness of 0.9 mm. 24 excitations were averaged. For the final images a maximum intensity projection (MIP) was performed over all coronal slices with a self-developed software written in IDL (ITT Visual Information Solutions, Boulder, CO).

Lipase activity assay (triacylglycerol hydrolase assay)

Gonadal WAT was removed surgically as described above, rinsed in phosphate-buffered saline (PBS) containing 1 mM ethylene diamine tetraacetic acid (EDTA) and homogenized in lysis buffer (0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol (DTT), 20 μ g/ml leupeptin, 2 μ g/ml antipain, 1 μ g/ml pepstatin, pH 7.0) using a Magna Lyser (Roche Diagnostics GmbH, Mannheim, Germany). The WAT lysate was centrifuged at 100,000 g for 1 h at 4 °C. The lipid-free infranatant (cytosolic fraction) was collected and used for TG hydrolase assays. The substrate for determining lipase activity contained non-labeled triolein and [9,10-³H(N)-triolein] (NEN Life Science Products, Boston, MA) as radioactive tracer that was emulsified with phosphatidylcholine/phosphatidylinositol using a conventional ultrasound sonicator. The cytosolic fractions with or without the specific HSL inhibitor 76-0079 (NNC 0076-0000-0079, Novo Nordisk, Bagsværd,

Denmark) were incubated at 37 °C for 60 min under constant shaking. The reaction was terminated by the addition of methanol/chloroform/heptane (10:9:7), 0.1 M potassium carbonate and 0.1 M boric acid (pH 10.5). After centrifugation at 800 g for 20 min the radioactivity in 1 ml of the upper phase was determined by liquid scintillation counting in a LS 6500 Multi-Purpose Scintillation Counter from Beckman Coulter (Fullerton, CA).

Ex vivo lipolysis of isolated WAT

Gonadal fat pads were dissected and washed with PBS and cut into small pieces (~20 mg). The small WAT pieces were incubated in DMEM (PAA) containing 2 % FA-free bovine serum albumin (Sigma-Aldrich, St. Louis, MO) at 37°C. Aliquots of the medium were analyzed for FA and glycerol content (release) by commercial kits (Wako Chemicals, Neuss, Germany and Sigma-Aldrich respectively).

Proteasome assay

M. gastrocnemius was homogenized in lysis buffer (20 mM Tris-HCl [pH 7.2], 0.1 mM EDTA, 1 mM 2-mercaptoethanol, 1 mM DTT, 5 mM ATP, 20 % glycerol, 0.04 % (v/v) Triton X-100) using a magna lyser (Roche Diagnostics GmbH) at 4°C. The lysate was centrifuged at 13,000 g for 15 min at 4°C. The supernatant was collected and protein concentration was determined using the RC DC Protein assay kit (BioRad, Hercules, CA). The chymotrypsin like proteasome activity was determined by incubating 40 µg protein with 0.167 µg/µl N-succinyl-Leu-Leu-Val-Try-7-amido-4-methylcoumarin (N-Suc LLVY-AMC, Sigma-Aldrich) in incubation buffer (100 mM Tris-HCl [pH 7.4], 50 mM HEPES [pH 8.0] and 5 mmol/l Ethyleneglycoltetraacetic-acid (EGTA)) for 60 min at 37°C. Fluorescence was determined using a conventional spectrofluorometer at 380 nm excitation and 460 nm emission.

Caspase activity assay

15 mg to 25 mg tissue from *M. gastrocnemius* was homogenized in 300 µl homogenization buffer (25 mM HEPES pH 7.5, 5 mM MgCl₂, 1 mM EGTA, 1 mM Pefabloc SC, 1 % protease-inhibitor cocktail [v/v] P8340 from Sigma-Aldrich) at 4°C. The lysate was centrifuged at 13,000 g for 20 min at 4°C. The supernatant was collected and the protein content was measured using the DC Protein Assay (BioRad). Caspase activity was determined by incubating 50 µg protein in 100 µl with 100 µl of Caspase-Glo 3/7 Reagent (Promega, Madison, WI, USA) for 1.5 h at 20°C and luminescence was determined using a luminometer (LUMIstar Optima BMG Labtec, Offenburg, Germany).

Blood and plasma parameters

Plasma glucose was measured using Accu-Check glucometer (Roche Diagnostics). Serum TG and FFA levels were determined using commercial kits from Thermo Electron, Thebarton, Australia and Wako Chemicals respectively.

Determination of TG content of cardiac muscle and *M. gastrocnemius*

Lipids were extracted from respective tissues by Folch extraction. The chloroform phase was evaporated using N₂ gas and the neutral lipids were solubilized in 2% Triton X-100 by sonication. TG was determined using Triglyceride FS kit (Diasys, Holzheim, Germany).

Determination of muscle and cardiac muscle protein content

M. gastrocnemius and heart samples were homogenized in RIPA lysis buffer (Fisher Scientific, Waltham, MA, USA) using a magna lyser (Roche Diagnostics GmbH) at 4°C. The lysate was centrifuged at 13,000 g for 15 min at 4°C. The supernatant was collected and protein concentration was determined using the DC Protein assay kit (BioRad).

IL-6 and TNF- α ELISA

Serum levels of TNF- α and IL-6 were measured using mouse TNF- α ELISA ReadySETGo and mouse IL-6 ELISA ReadySETGo (eBiosciences, San Diego, CA, USA). The plate was read at 450 nm using a conventional photometer.

Western blot analysis

Serum levels of zinc- α B₂-glycoprotein (ZAG) were determined by semi quantitative Western blot using ZAG (E-20) antibody (sc-11243, Santa Cruz, CA, USA) and consecutively the bands were analyzed densitometrically.

Determination of mRNA expression of fatty acid transporter and fatty acid metabolizing genes

Total RNA from frozen sections of *M. gastrocnemius* was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. cDNA was prepared from 1 μ g of total RNA using high capacity reverse transcription kit (Applied Biosystems, Carlsbad, CA) and q-PCRs were performed using primers for *CD36*, *Fatp-1*, *Cpt-1 β* and *Pgc-1 α* using Taqman Universal PCR master mix (Applied Biosystems) in triplicates. The relative expressions were calculated by the ddCt method using *TF-2B* as the housekeeping gene.

Human adipose tissue samples

Criteria for inclusion were malignant conditions. Nutritional status was assessed by body mass index (BMI). Patients were considered cachectic according to the definition of Evans et al. when BMI was < 20 and patients records indicated 3 or more of the additional criteria [decreased muscle strength, fatigue, anorexia, low fat-free mass index or abnormal biochemistry such as increased inflammatory markers (CRP, IL-6), anemia (Hb <12 g/dL) or low serum albumin (<3.2 g/dL)] (Evans et al., 2008). Non-tumor patients were used as control. Autopsies were

performed within one day after demise. Samples of adipose tissue were collected from visceral adipose tissue and immediately snap frozen. The study had been approved by the ethics committee of the Medical University of Graz (20-096 ex 08/09).

Statistical Methods

Results are presented as mean $\pm$ standard deviation (S.D.). Comparisons were performed using unpaired Students t-test and are presented as *** for $p \leq 0.001$, ** for $p \leq 0.01$, * for $p \leq 0.05$.

For patient samples, non parametric Mann Whitney U test was performed and Spearman's rho was calculated using graphpad.

Results & Discussion...

To investigate the role of lipases in CAC, we utilized two different cachexia models in mice lacking either *Atgl* or *Hsl*. Cachexia was induced in wild-type C57BL/6 (*Wt*) mice, *Atgl*-deficient (*Atgl*^{-/-}) mice (Haemmerle et al., 2006), and *Hsl*-deficient (*Hsl*^{-/-}) mice (Haemmerle et al., 2002) by subcutaneous injection of Lewis lung carcinoma (LLC) cells (van Royen et al., 2000) or B16 melanoma cells (Kawamura et al., 1999). Tumor growth was observed in 100% of treated animals. Tumor weights tended to be lower in non-fasted (Figure 3 A, B, C) and overnight (o/n)-fasted (Figure 4 A, B) *Atgl*^{-/-} and *Hsl*^{-/-} mice than in *Wt* mice. However, none of the differences reached statistical significance.

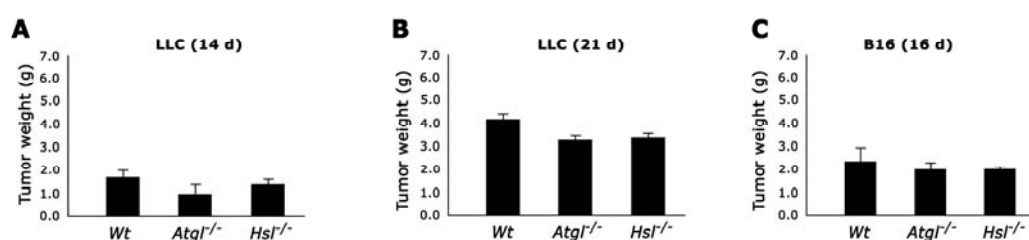


Figure 3

Tumor weights 14 days (d) and 21 d after injecting LLC and 16 d after injecting B16 melanoma (B16) cells were slightly lower in lipase deficient mice compared to *Wt*. *n* = 5 to 7 except for (A) and (B) *Hsl*^{-/-}, *n* = 3

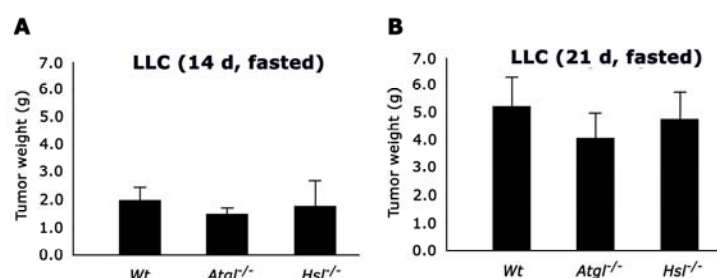


Figure 4

Tumor weights 14 d (A) and 21 d (B) post-injection of LLC in 12 h fasted lipase deficient mice were slightly lower than in *Wt* mice. *n* = 7

Next we analyzed body weight, plasma metabolite concentrations, and fat mass in mice of all genotypes with or without tumors. Since all of these parameters strongly depend on the feeding status of the mice and to account for any

nutritional bias, they were determined in non-fasted and o/n-fasted animals. Total body weight (after subtraction of tumor weight) differed drastically in lipase-deficient mouse models compared to *Wt* mice in response to LLC and B16 (Figure 5 A, B, C).

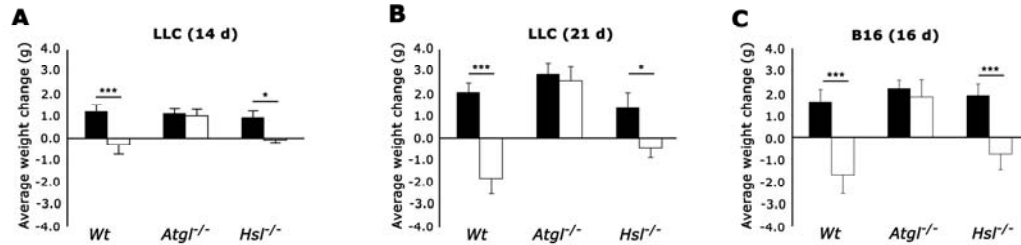


Figure 5

Wt mice significantly lost weight with tumor progression after injection of LLC and B16 tumor cells while *Atgl*^{-/-} animals did not develop cachexia. *Hsl*^{-/-} mice also lost weight but less pronounced than *Wt* mice. Black bars indicate control (saline injected) animals, white bars tumor-bearing animals. To allow direct comparison, values were determined after preparation and removal of the respective tumor. *** $p < 0.001$, * $p < 0.05$, $n = 5$ to 7 except for (A) *Hsl*^{-/-} control, $n = 2$, tumor, $n = 3$ and (B) *Hsl*^{-/-}, $n = 3$.

Whereas non-tumor bearing *Wt* mice on normal chow diet gained weight over a period of three weeks, *Wt* mice with LLC started to lose weight 14 days (d) after tumor injection resulting in an average weight loss of 1.8 g after 21 d. In contrast, body weight in *Atgl*^{-/-} mice with LLC was identical to *Atgl*^{-/-} mice without tumors at all times. *Hsl*^{-/-} mice exhibited an intermediate phenotype. Compared to non-tumor bearing *Hsl*^{-/-} mice, body weight was reduced. However, the loss was less extreme than in *Wt* mice carrying the tumor. Similar results were obtained in B16 treated mice (Figure 5 C). Compared to *Wt* mice without tumor, B16 treated mice weighed 3.3 g less 16 d after tumor injection. In contrast, B16- treated *Atgl*^{-/-} mice maintained similar weights as untreated *Atgl*^{-/-} mice. *Hsl*^{-/-} animals were less protected than *Atgl*^{-/-} mice and lost on average 2.7 g of body weight.

The differences were even more pronounced in o/n-fasted mice, a condition when lipolysis is physiologically induced (Figure 6). Whereas *Wt* mice with LLC weighed 2.1 g (after 14 d) and 5.5 g (after 21 days) less than *Wt* mice without

tumor, *Atgl*^{-/-} mice were totally resistant to weight loss and *Hsl*^{-/-} animals reached intermediate values.

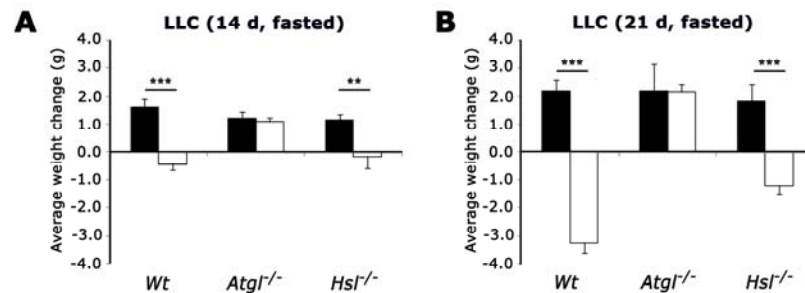


Figure 6

Ablation of *Atgl* protects mice from cancer associated weight loss

Tumor development instigated significant weight loss in o/n fasted *Wt* and *Hsl*^{-/-} mice 14 d (A) and 21 d (B) after injection of LLC tumor cells while *Atgl*^{-/-} mice retained weight despite tumor development. Black bars indicate control (saline injected) animals, white bars tumor-bearing animals. To allow direct comparison, values were determined after preparation and removal of the respective tumor. *** p<0.001, ** p<0.01, n = 7

Differences in weight loss in response to the tumors were not explained by variable food intake (Figure 7 A, B), as it was similar in all animals during the initial phase of the experiment and decreased uniformly in all tumor-carrying mice during the final 2-4 d. Thus, in the mouse, protection from CAC can be entirely conferred by the lack of ATGL and partially by the absence of HSL.

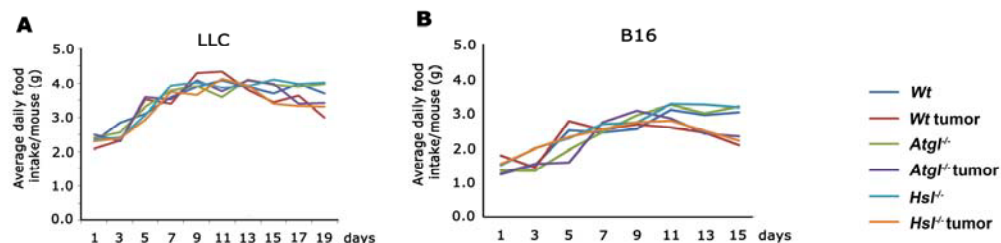


Figure 7

Changes in body weight are not attributable to dietary intake.

Food intake remained similar in LLC (A) and B16 melanoma (B) bearing mice compared to control mice of all genotypes. However, it tended to be reducing in the last 2-4 days of the experiment in all tumor bearing animals irrespective of genotype. n = 7

Consistent with previous findings (Haemmerle et al., 2006; Haemmerle et al., 2002), plasma glucose, FA, and TG levels were reduced in o/n-fasted *Atgl*^{-/-} vs. *Wt* mice (Figure 8-10). In o/n-fasted *Hsl*^{-/-} mice, FA and TG levels were also reduced (although less than in *Atgl*^{-/-} mice), whereas plasma glucose concentrations were unchanged compared to *Wt* mice. The presence of LLC for 14 d did not affect plasma glucose levels in o/n-fasted mice of any genotype (Figure 8 A). After 21 d plasma glucose levels were decreased in tumor-bearing, o/n-fasted *Wt* (-43.2 %) and *Hsl*^{-/-} mice (-27.3 %) but remained unchanged in *Atgl*^{-/-} mice when compared to mice of the same genotype without tumors (Figure 8 B). These differences in glucose levels in LLC-treated *Wt* and *Hsl*^{-/-} mice were not observed in non-fasted animals (Figure 8 C). Similarly, plasma glucose and FA levels of non-fasted B16-treated mice matched those in LLC bearing animals (Figure 8 D; 7D).

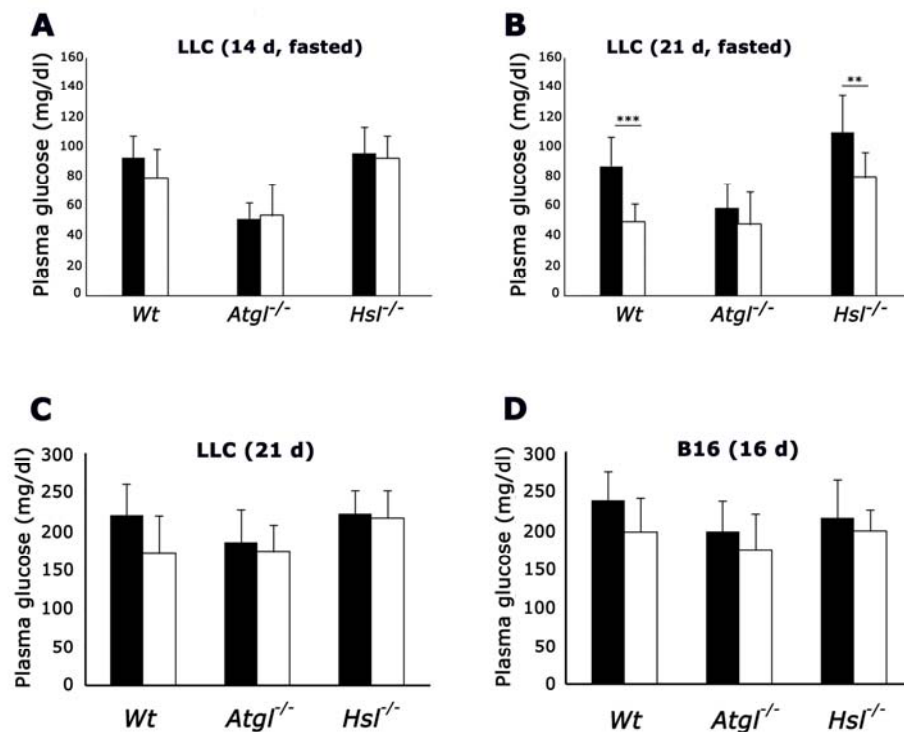


Figure 8

Plasma glucose levels in tumor bearing and control *Wt*, *Atgl*^{-/-} and *Hsl*^{-/-} mice.

(A, B) fasted glucose levels were reduced in tumor-bearing *Wt* and *Hsl*^{-/-} mice compared to non-tumor-bearing littermates 21 d post tumor cell injection. This effect was not detected after 14 d. In contrast, plasma glucose levels remained unchanged in *Atgl*^{-/-} mice. (C, D) plasma glucose levels were not significantly changed in non fasted LLC or

B16 tumor-bearing mice compared to non-tumor-bearing littermates of any genotypes. Black bars indicate control (saline injected) animals, white bars tumor-bearing animals. *** $p < 0.001$, ** $p < 0.01$, $n = 7$, except for (C), $n = 3-5$.

Presence of LLC caused an increase in FA levels in *Wt* (fasted: +24.0% at 14 d and +9.9% at 21 d; non-fasted: +54.5% at 21 d) and *Hsl*^{-/-} mice (fasted: +23.4% at 14 d and +25.0% at 21 d) (Figure 9). In contrast, FA levels in tumor-bearing *Atgl*^{-/-} mice remained unchanged compared to *Atgl*^{-/-} mice without tumor independent of the feeding status. Increased FA levels were also observed in non-fasted B16-tumor bearing *Wt* mice (+27%) (Figure 9 D). Serum TG levels were not significantly different in o/n fasted animals with or without tumors (Figure 10).

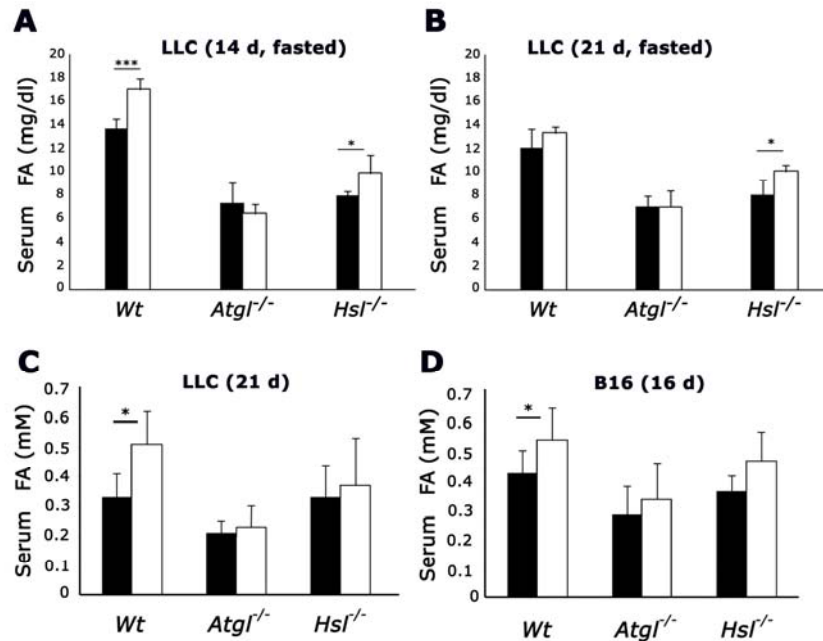


Figure 9

Serum free fatty acid levels in tumor bearing and control *Wt*, *Atgl*^{-/-} and *Hsl*^{-/-} mice.

(A, B) fasted FA levels were increased in tumor-bearing *Wt* mice after 14 d and in tumor-bearing *Hsl*^{-/-} mice after 21 d. No changes were detected in *Atgl*^{-/-} mice. (C, D) Serum FA levels of non-fasted LLC and B16 tumor bearing *Wt* mice increased after 21 d and 16 d respectively. *Atgl*^{-/-} and *Hsl*^{-/-} mice did not show significant differences in serum FA levels between tumor and non tumor-bearing mice. Black bars indicate control (saline injected) animals, white bars tumor-bearing animals. *** $p < 0.001$, * $p < 0.05$, $n = 7$ except for (C), $n = 3-5$.

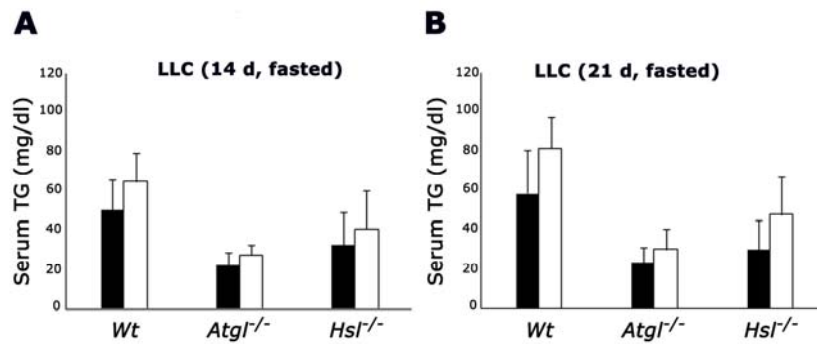


Figure 10

Serum TG levels in fasted tumor bearing and control *Wt*, *Atgl*^{-/-} and *Hsl*^{-/-} mice.

(A, B) fasted TG levels were reduced in *Atgl*^{-/-} and *Hsl*^{-/-} mice compared to *Wt* but were not significantly affected by tumor growth. Black bars indicate control (saline injected) animals, white bars tumor-bearing animals. n = 7

To assess the contribution of adipose tissue loss to the tumor-induced weight loss, WAT mass was determined by visual inspection, weighing of surgically removed adipose depots (gonadal and retroperitoneal adipose tissue), and *in vivo* NMR WAT quantitation. In non-fasted LLC-bearing *Wt* mice, WAT weight decreased by 55% after 21 d of tumor growth (Figure 11 A), which corresponded to a loss of 1.7 g of WAT in NMR analysis (Figure 12 A). Non-fasted B16-bearing *Wt* mice lost 85% of WAT weight after 16 d of tumor growth (Figure 11 B) (-2.0 g of adipose tissue mass in NMR analysis, Figure 12 B). In contrast, none of the tumors affected WAT mass of *Atgl*^{-/-} mice. In fact, weight of body fat depots and total body fat was increased in *Atgl*^{-/-} mice independent of the presence or absence of the tumor when compared to non-tumor bearing *Wt* mice (Figure 11 A, B). This confirms our earlier observation that *Atgl* deficiency causes obesity in mice kept on a normal chow diet (Haemmerle et al., 2006) and shows that WAT mass is independent of the presence of the tumor. In non-fasted *Hsl*^{-/-} mice, LLC did not affect the weight of WAT depots. However, NMR analyses revealed a total WAT reduction by 0.7 g (Figure 11 A, 12 A). B16 in *Hsl*^{-/-} mice caused a 32% reduction of adipose tissue weight and a 0.9 g WAT loss in NMR analysis (Figure 11 B, 12 B). In o/n-fasted animals the differences were even more striking (Figure 11 C, D, 12 C). After 21 d, LLC tumors caused the loss of more than 95% of WAT weight (2 g in NMR analysis) in *Wt* mice whereas adipose

mass was again completely retained in LLC-treated *Atgl*^{-/-} mice. *Hsl*^{-/-} mice exhibited an intermediate loss of 37% of WAT weight.

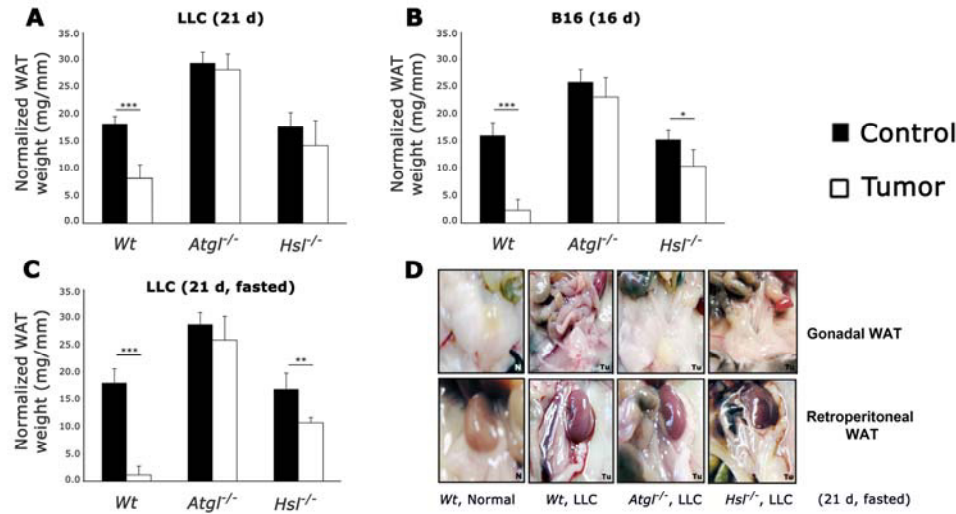


Figure 11

Ablation of *Atgl* protects mice from cancer associated loss of adipose tissue

(A) Normalized gonadal and retroperitoneal WAT was reduced approximately by 55% in non-fasted tumor-bearing *Wt* mice 21 d after LLC tumor implantation, whereas lipase deficient mice were protected from WAT loss. (B, C, D) No or minimal gonadal and retroperitoneal WAT was detected upon dissection of non-fasted *Wt* B16 tumor-bearing mice after 16 d and fasted *Wt* LLC tumor-bearing mice after 21 d compared to saline injected control mice. *Atgl*^{-/-} tumor-bearing mice retained gonadal and retroperitoneal WAT, whereas partial loss of gonadal and retroperitoneal WAT was observed in *Hsl*^{-/-} tumor-bearing mice. WAT was dissected and its mass normalized to *tibia* length as described in the methods section. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. $n = 5$ to 7 except for (A) *Hsl*^{-/-}, $n = 3$.

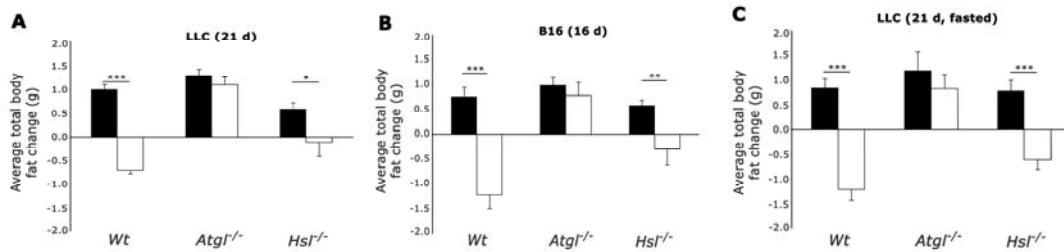


Figure 12

Ablation of *Atgl* protects mice from cancer associated loss of adipose tissue.

(A, B, C) Total body fat content was significantly reduced in LLC and B16 tumor-bearing *Wt* and *Hsl*^{-/-} mice as determined by NMR. Total body fat content of tumor bearing *Atgl*^{-/-} mice, however, remained similar to non-tumor bearing *Atgl*^{-/-} mice. Black bars indicate control (saline injected) animals, white bars tumor-bearing animals. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, $n = 7$, except (A) $n = 3-5$

Results for LLC-bearing animals were substantiated by MRI analysis (Figure 13). Taken together, these results show that independently of feeding status and tumor type, ATGL deficiency completely and HSL deficiency partially protects mice from the loss of WAT.

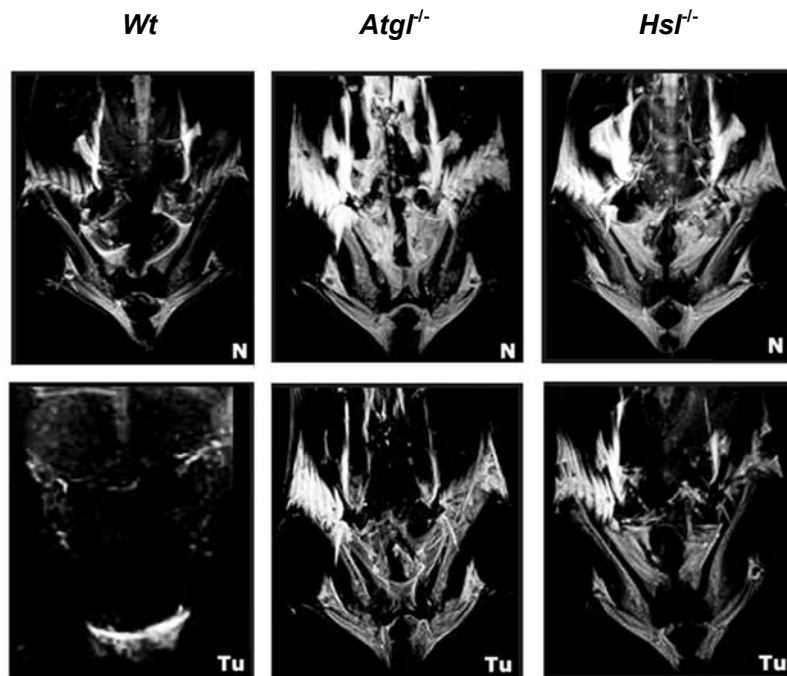


Figure 13

Lipase deficiency preserves WAT in tumor bearing mice.

MR images confirm that tumor bearing *Atgl*^{-/-} and *Hsl*^{-/-} mice retains adipose tissue while *Wt* mice lose most of the adipose tissue in response to the LLC. MR imaging was performed by Clemens Diwoky.

Tumor-associated loss of adipose tissue in animal models has been mostly attributed to an increase in WAT lipolysis (Agustsson *et al.*, 2007; Hylander *et al.*, 2000; Jeevanandam *et al.*, 1986; Klein *et al.*, 1990; Legaspi *et al.*, 1987; Ryden *et al.*, 2008; Shaw *et al.*, 1987). Consistent with these reports, the release of FA and glycerol from WAT explants was increased in *Wt* mice with LLC (38% and 31%, respectively) and B16 (39% and 21%, respectively) compared to mice without tumor (Figure 14 A, B, C, D). This increase in lipolysis did not occur in LLC or B16-tumor bearing *Atgl*^{-/-} mice. WAT lipolysis was also attenuated in tumor-bearing *Hsl*^{-/-} mice. Whereas FA and glycerol release from WAT was similar in *Hsl*^{-/-} mice with or without LLC, B16 melanoma formation caused a 28% and 19% increase, respectively.

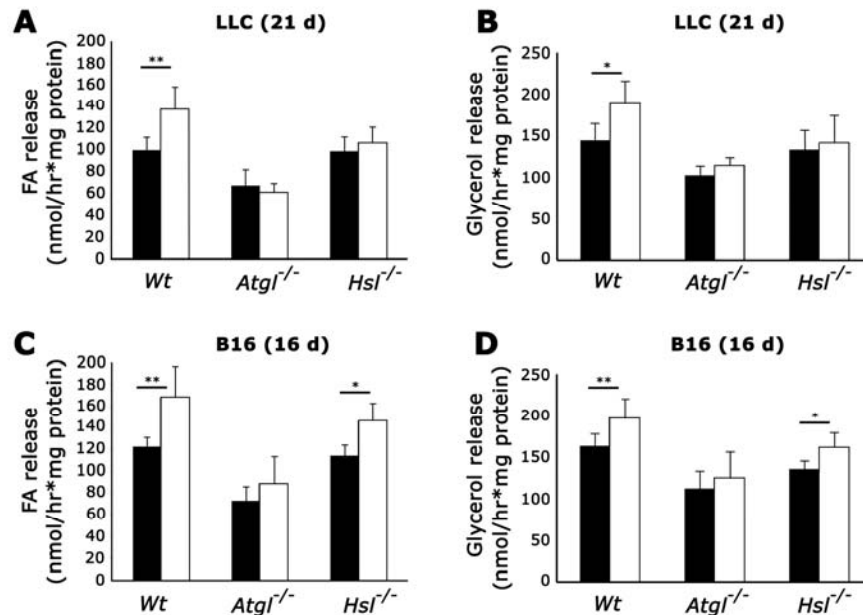


Figure 14

Loss of WAT in tumor-bearing animals is mainly attributable to lipolysis.

Both LLC and B16 tumors significantly increased FA (A, C) and glycerol (B, D) release from WAT explants in non-fasted tumor-bearing *Wt* mice. LLC-bearing lipase deficient mice did not exhibit increased FA or glycerol release from WAT explants whereas increased FA and glycerol release was observed from WAT explants of B16 tumor

bearing *Hsl*^{-/-} mice. WAT explants from B16 tumor bearing *Atgl*^{-/-} mice did not show increased FA or glycerol release. Black bars indicate control (saline injected) animals, white bars tumor-bearing animals. ** p<0.01, * p<0.05. n = 7 except for (A) and (B), n = 3 to 5.

To investigate whether changes in TG hydrolase activities underlie the observed differences in lipolytic rates, we measured ATGL and HSL enzyme activities in WAT in response to tumor growth (Figure 15). LLC in o/n-fasted *Wt* mice caused a 2- fold increase in total lipase activity due to increased ATGL and HSL activity. Notably, no tumor-induced increase in WAT lipase activity was observed in *Atgl*^{-/-} mice whereas in *Hsl*^{-/-} mice total TG lipase activity increased by 2.1-fold due to increased ATGL activity. Thus, LLC and B16 cause an induction of WAT TG hydrolase activity leading to an increased release of FA and glycerol from WAT. This induction of lipolysis is not observed in the absence of ATGL and significantly reduced in the absence of HSL. Thus, lipase deficiency blocks tumor induced WAT loss.

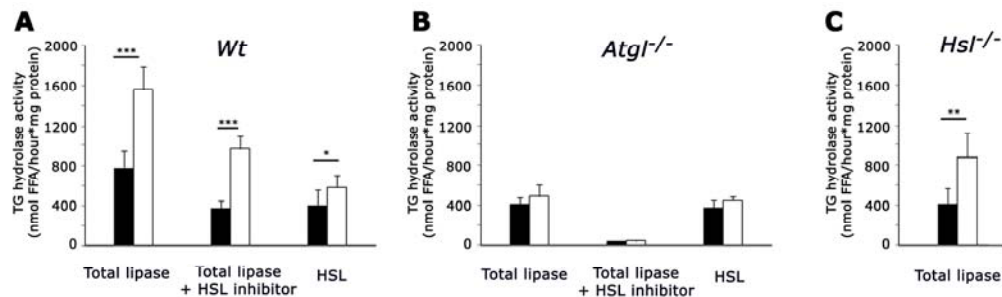


Figure 15

TG hydrolase activity is increased in WAT of LLC bearing *Wt* and *Hsl*^{-/-} mice but not *Atgl*^{-/-} mice.

(A) Total lipase activity, specifically inhibited (HSL, 76-0079) lipase activity (mainly ATGL activity), and HSL activity (determined by subtraction of inhibited lipase activity from total lipase activity) in *Wt* mice. Total lipase activity is increased mainly due to increased ATGL activity in tumor-bearing mice. (B) no significant change in lipase activity in *Atgl*^{-/-} mice. (C) total (mainly ATGL) lipase activity is increased in *Hsl*^{-/-} tumor-bearing mice. Black bars indicate control (saline injected) animals, white bars tumor-bearing animals. *** p<0.001, ** p<0.01, * p<0.05, n=7

Several reports have shown that the induction of lipolysis during CAC is mediated by inflammatory cytokines (Argiles et al., 2009), ZAG (also designated lipid mobilizing factor, LMF) (Bing et al., 2004), or cytotoxin induced death executor proteins (CIDE), such as CIDE-A (Laurencikiene et al., 2008). To investigate whether these lipolytic agonists are increased in cachexia models lacking specific lipases, we assessed their plasma levels. In response to LLC, TNF- α and IL-6 levels were increased (between 2 to 3.2-fold) in all genotypes (Figure 16 A, B). Similarly, quantitative Western blot analysis revealed that plasma ZAG levels were higher in LLC-bearing animals of all genotypes (Figure 14 C). In response to B16, the induction of cytokine release was even more pronounced (Figure 16 D, E). Plasma levels of TNF- α and IL-6 increased approximately 5 to 9-fold and 18 to 21-fold, respectively. Plasma ZAG levels were also consistently higher in B16-treated mice of all genotypes compared to untreated mice (Figure 16 F). This suggests that in *Wt* mice the increased concentration of inflammatory and lipolytic agonists induce lipolysis via ATGL and HSL leading to the uncontrolled loss of WAT and cachexia. In the absence of lipases, particularly in the absence of ATGL, this process is disrupted and WAT is retained.

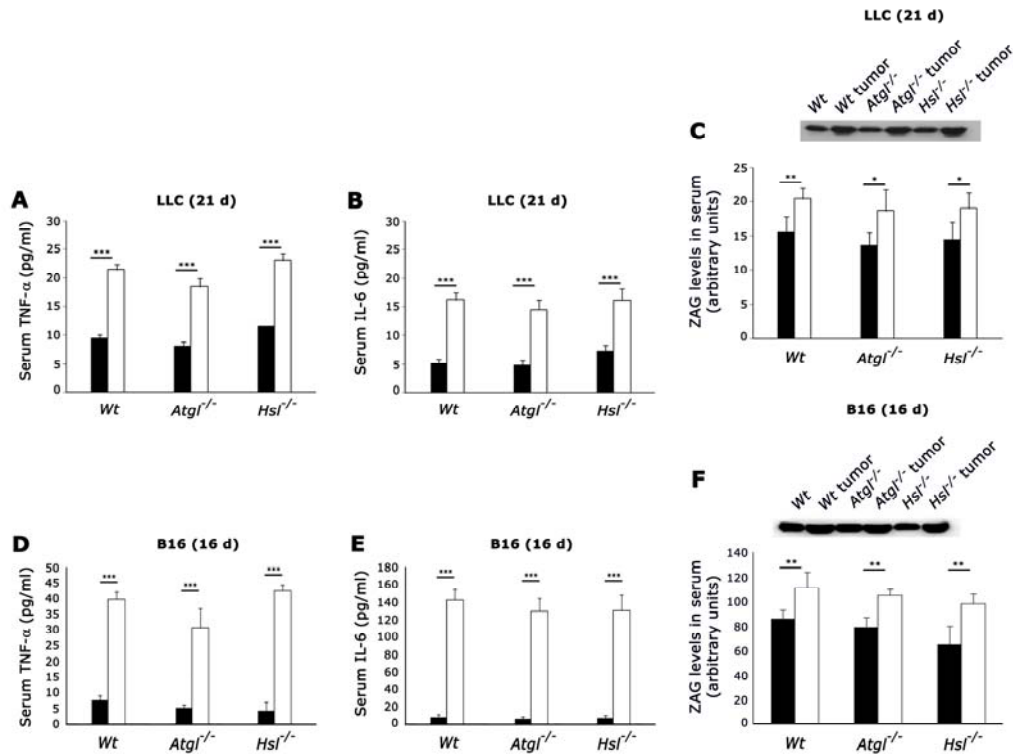


Figure 16

Lipolytic agonists (TNF- α , IL-6 and ZAG) were increased in both LLC and B16 tumor-bearing mice from all genotypes. Black bars indicate control (saline injected) animals, white bars tumor-bearing animals. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. $n = 7$.

In cancer patients, CAC not only emaciates adipose tissue but also consumes skeletal muscle and cardiac muscle (Busquets et al., 2007b; Tisdale, 2009; van Royen et al., 2000; Zhou et al., 2010). Similarly, we observed that LLC and B16 melanoma in mice lowers skeletal muscle mass and heart weight. Surgically removed *M. gastrocnemius* of *Wt* mice injected with LLC (21 d) and B-16 (16 d) weighed 36% and 25% less than of control *Wt* mice, respectively (Figure 17 A, D, E). Remarkably, *Atgl*^{-/-} mice suffered no significant loss of *M. gastrocnemius* weight in response to the tumors (Figure 17 B, D, E). Similarly as in *Wt* mice, LLC and B16 in *Hsl*^{-/-} mice diminished *M. gastrocnemius* weight, albeit to a lesser degree (-27% and -18%, respectively).

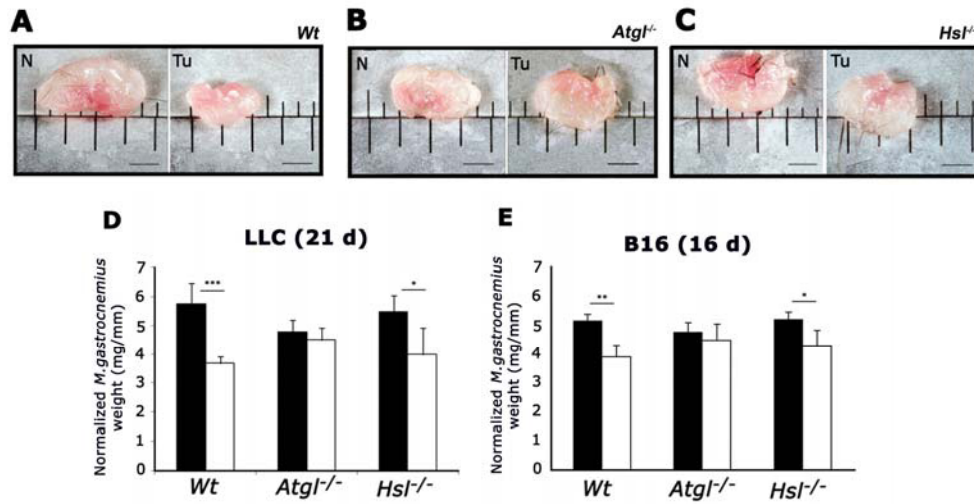


Figure 17

Macroscopic views of *M. gastrocnemius* cross sections (A, B and C) and weight change (normalized to *tibia* length) (D and E) of surgically prepared *M. gastrocnemius* illustrate skeletal muscle loss in tumor-bearing *Wt* and *Hsl*^{-/-} mice but not in *Atgl*^{-/-} mice. Black bars indicate control (saline injected) animals, white bars tumor-bearing animals. *** p<0.001, ** p<0.01, * p<0.05. n = 7.

Wasting of *M. gastrocnemius* was also reflected by a reduction of total muscle protein in *Wt* (-36%) and *Hsl*^{-/-} mice (-22%) in response to B16, whereas *Atgl*^{-/-} mice with B16 melanoma were resistant to the loss of muscle protein (Figure 18).

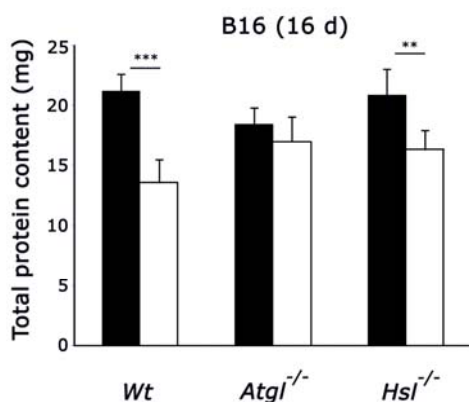


Figure 18

Total protein content of *M. gastrocnemius* is significantly reduced in B16 bearing *Wt* and *Hsl*^{-/-} mice. No significant differences could be observed in *Atgl*^{-/-} mice. Black bars indicate control (saline injected) animals, white bars tumor-bearing animals. *** p<0.001, ** p<0.01, n = 7.

The weight of *M. soleus* also decreased in LLC injected *Wt* mice after 21 d (33%, not statistically significant) and B16-injected *Wt* mice (27%) (Figure 19).

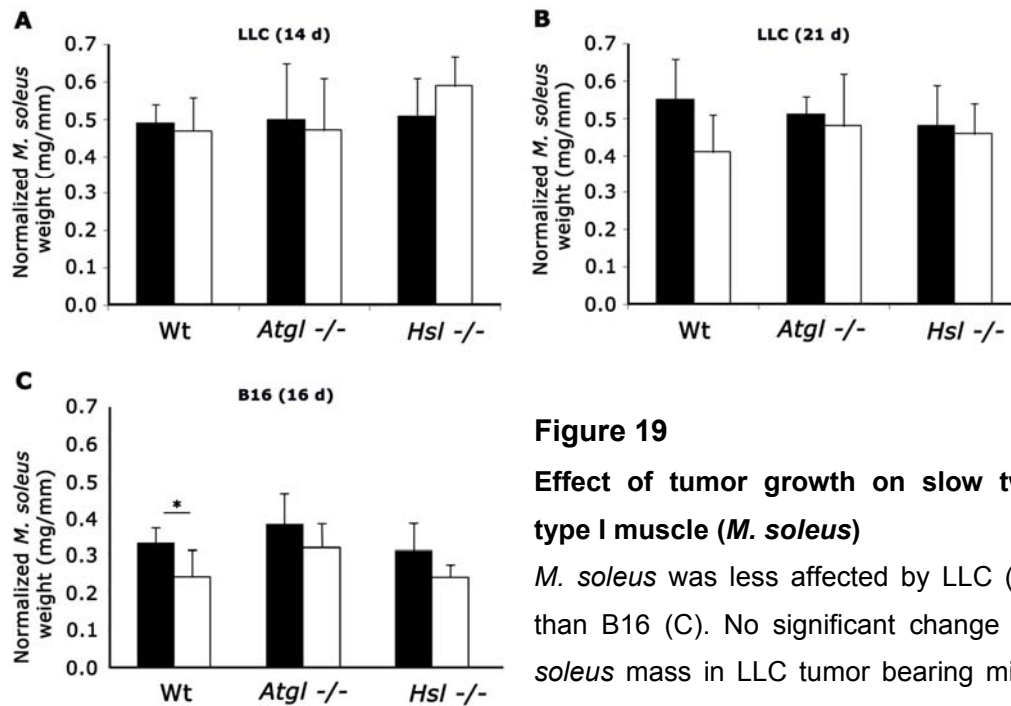


Figure 19

Effect of tumor growth on slow twitch type I muscle (*M. soleus*)

M. soleus was less affected by LLC (A, B) than B16 (C). No significant change in *M. soleus* mass in LLC tumor bearing mice of any genotypes was detected compared to

saline injected mice after 14 d and 21 d. In contrast, *M. soleus* was significantly reduced in *Wt* B16 tumor bearing mice. However, lipase deficient animals were protected from B16 tumor induced *M. soleus* loss. Muscle was dissected and its mass normalized to *tibia* length as described in the methods section. Black bars indicate control (saline injected) animals, white bars tumor-bearing animals. * $p < 0.05$, $n = 7$

Both LLC and B16 caused a moderate decrease in heart weight (-7.4% and - 9.9%, respectively) and total cardiac protein weight (-20.1% and - 24.9%, respectively) in *Wt* mice but not in *Atgl*^{-/-} or *Hsl*^{-/-} mice (Figure 20).

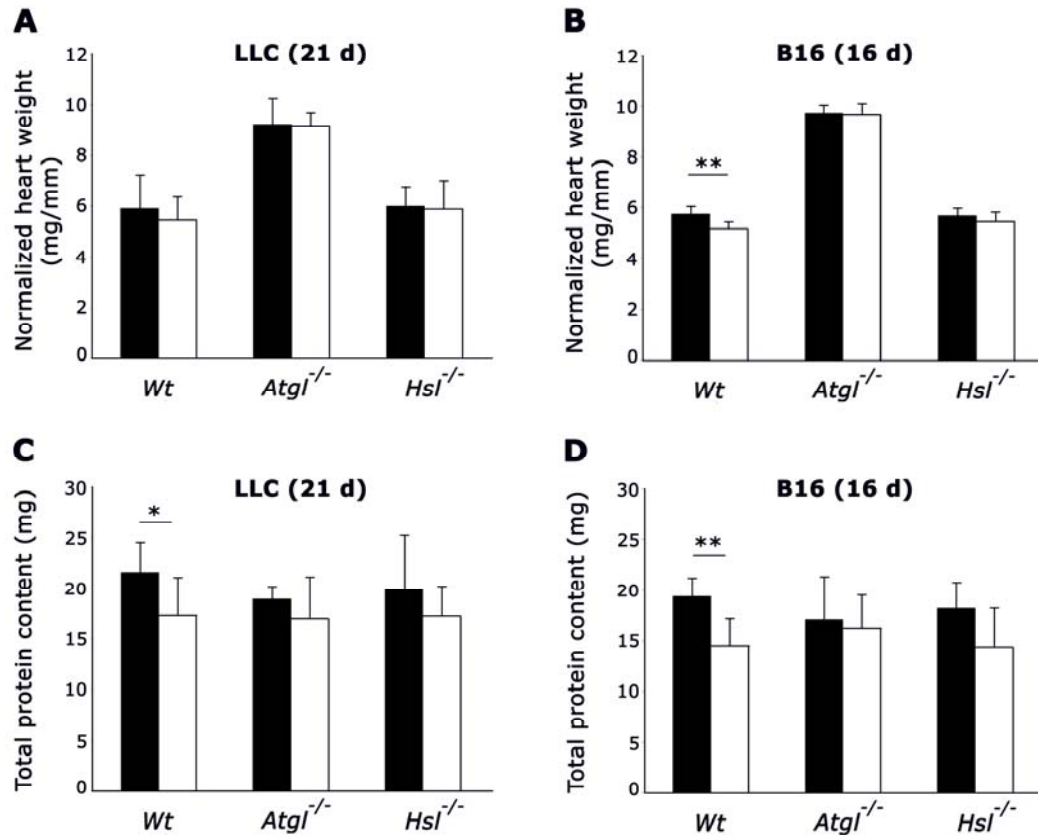


Figure 20

Lipase deficient mice were protected from reduction of cardiac mass and cardiac protein.

Heart weight tended to decrease in LLC (A) and B16 (B) bearing *Wt* mice compared to saline injected animals whereas *Atgl*^{-/-} or *Hsl*^{-/-} mice did not show significant differences. (C, D) Total protein content was reduced in LLC and B16 melanoma bearing *Wt* mice. Lipase deficient mice did not show any significant difference regardless of tumor development. Black bars indicate control (saline injected) animals, white bars tumor-bearing animals. ** p<0.01, * p<0.05, n = 7

Consistent with our previous observations (Haemmerle et al., 2006), *Atgl*^{-/-} mice exhibited a 2-fold increased TG content in *M.gastrocnemius* and a 12-15-fold increased TG content in cardiac muscle (Figure 21) compared to *Wt* animals. Muscle TG content tended to increase with response to tumor in *M.gastrocnemius* and cardiac tissue (Figure 21).

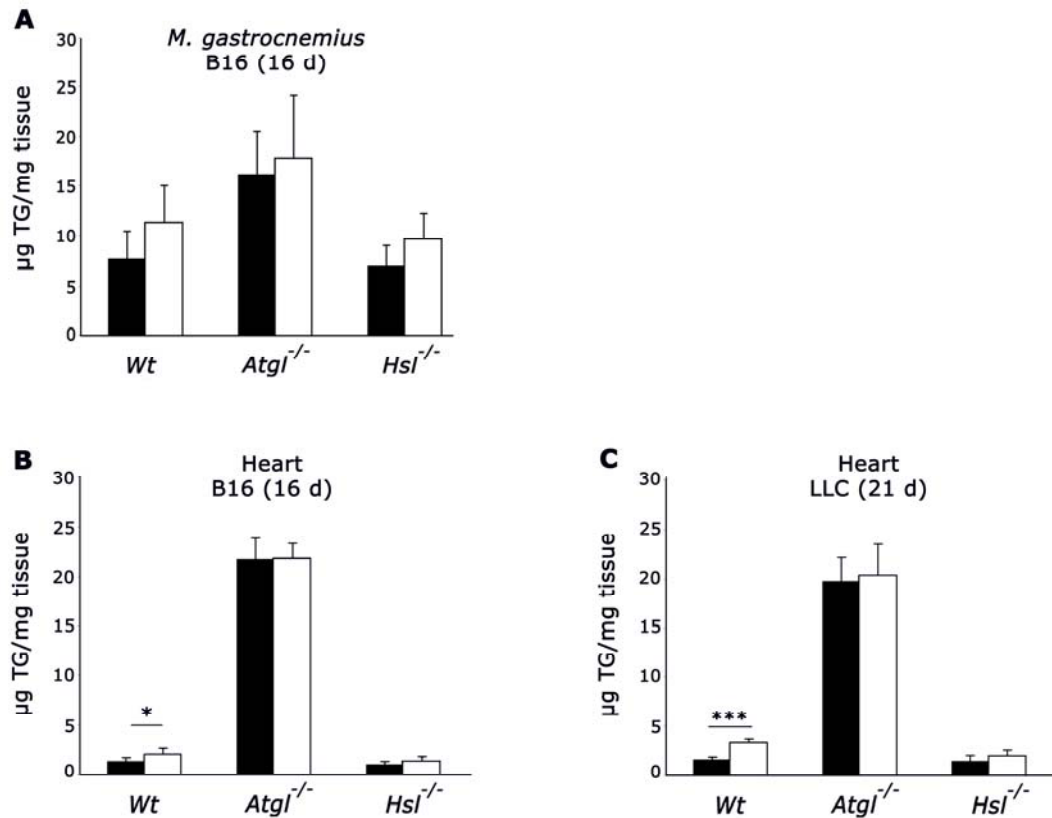


Figure 21

Increase of TG levels in *M.gastrocnemius* and cardiac tissue of tumor bearing mice.

(A) B16 melanoma bearing mice tended to have increased TG levels in *M. gastrocnemius*, the differences was, however, not significant. In *Wt* mice B16 (B) and LLC (C) tumors induced a significant TG increase in the heart whereas heart TG levels remained unaffected in lipase deficient mice. Black bars indicate control (saline injected) animals, white bars tumor-bearing animals. *** $p < 0.001$, * $p < 0.05$, $n = 7$

Muscle atrophy may originate from a decrease in protein synthesis (Rennie et al., 1983) or an increase in protein degradation (Tisdale, 2009; van Royen et al., 2000). Studies in a number of experimental models of cachexia suggest that both processes occur simultaneously (Tisdale, 2009). Animal models of cancer cachexia as well as studies in cancer patients provided evidence that the ubiquitin-proteasome pathway mainly degrades myofibrillar proteins, particularly at later stages of cachexia when patients have lost more than 10 % of their body weight (Khal et al., 2005a). Additionally, apoptotic cell death characterized by

increased activity of caspases contributes to the loss of *M. gastrocnemius* of mice bearing the cachexia-inducing MAC16 tumor (Belizario et al., 2001). Consistent with these observations, we detected a marked increase in proteasome activity (Figure 22 A, C) and an increase in caspase 3/7 activity (Figure 22 B, D) in *M. gastrocnemius* of *Wt* mice and *Hsl*^{-/-} mice 21 d after LLC injection and 16 d after B16 injection. In contrast, no significant change in proteasome or caspase 3/7 activity was observed in *M. gastrocnemius* of *Atgl*^{-/-} mice in response to both tumors (Figure 22 A, B, C, D).

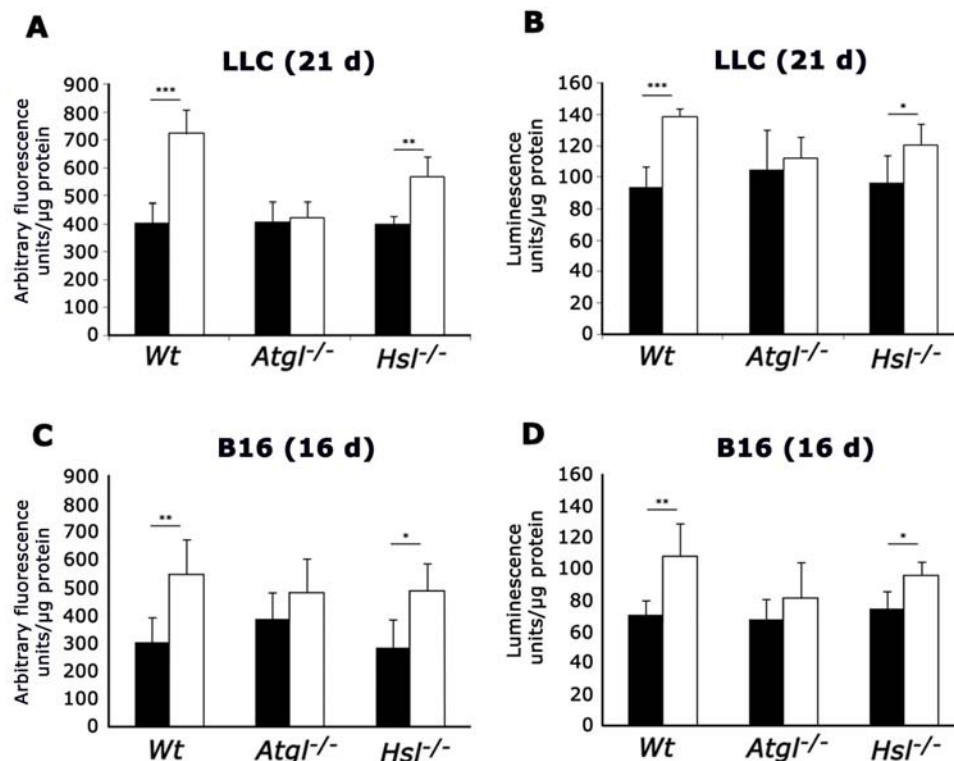


Figure 22

Increased proteasomal and caspase activity observed in tumor bearing *Wt* and *Hsl*^{-/-} mice.

(A, C) Proteasome activity in *M. gastrocnemius* homogenates is significantly increased in tumor-bearing *Wt* and *Hsl*^{-/-} mice but not in *Atgl*^{-/-} mice. (B, D) Increased caspase 3/7 activity in *M. gastrocnemius* homogenates demonstrates activation of apoptosis in tumor bearing *Wt* and *Hsl*^{-/-} mice. Caspase 3/7 activity in *M. gastrocnemius* of *Atgl*^{-/-} mice was not affected by tumor growth. Black bars indicate control (saline injected) animals, white bars tumor-bearing animals. *** p<0.001, ** p<0.01, * p<0.05, n = 7

Changes in the weight of *M. gastrocnemius*, caspase 3/7 activity, and proteasome activity were not observed in *Wt* and *Hsl*^{-/-} mice, two weeks after LLC injection (Figure 23), suggesting that loss of WAT precedes the loss of muscle mass, which is consistent with earlier observations (Agustsson et al., 2007).

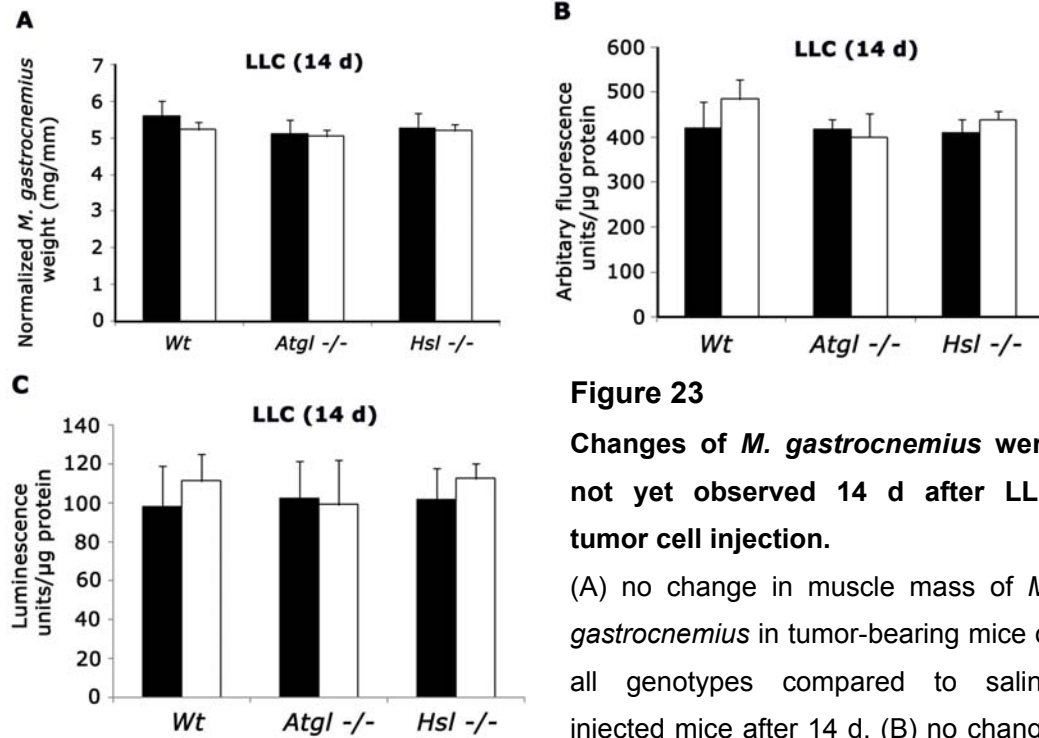


Figure 23

Changes of *M. gastrocnemius* were not yet observed 14 d after LLC tumor cell injection.

(A) no change in muscle mass of *M. gastrocnemius* in tumor-bearing mice of all genotypes compared to saline injected mice after 14 d. (B) no change

in proteasome and (C) caspase activity in homogenates of *M. gastrocnemius* in tumor-bearing mice of all genotypes. Black bars indicate control (saline injected) animals, white bars tumor-bearing animals. *n* = 7

Previous work showed that cachexia is associated with increased FA oxidation in *M. gastrocnemius* (Tisdale, 2009). Our data confirm these findings showing 1.8 to 2.5-fold increased mRNA expression levels of genes involved in the regulation of cellular FA uptake (*Cd36* and fatty acid transport protein 1, *Fatp-1*), FA transport into mitochondria (carnitin palmitoyltransferase-1 β , *Cpt-1 β*), and mitochondrial function (peroxisome proliferator-activated receptor- γ coactivator-1 α , *Pgc-1 α*) in *M. gastrocnemius* samples of *Wt* mice with LLC (Figure 24). mRNA levels in muscle samples of *Atgl*^{-/-} mice were not affected in response to LLC. In *Hsl*^{-/-} mice mRNA levels increased in the presence of the tumor although to a lesser extent than in *Wt* mice. This suggests that the catabolic state in CAC

mobilizes FA from adipose tissue leading to an energy substrate switch from glucose to FA utilization in skeletal muscle.

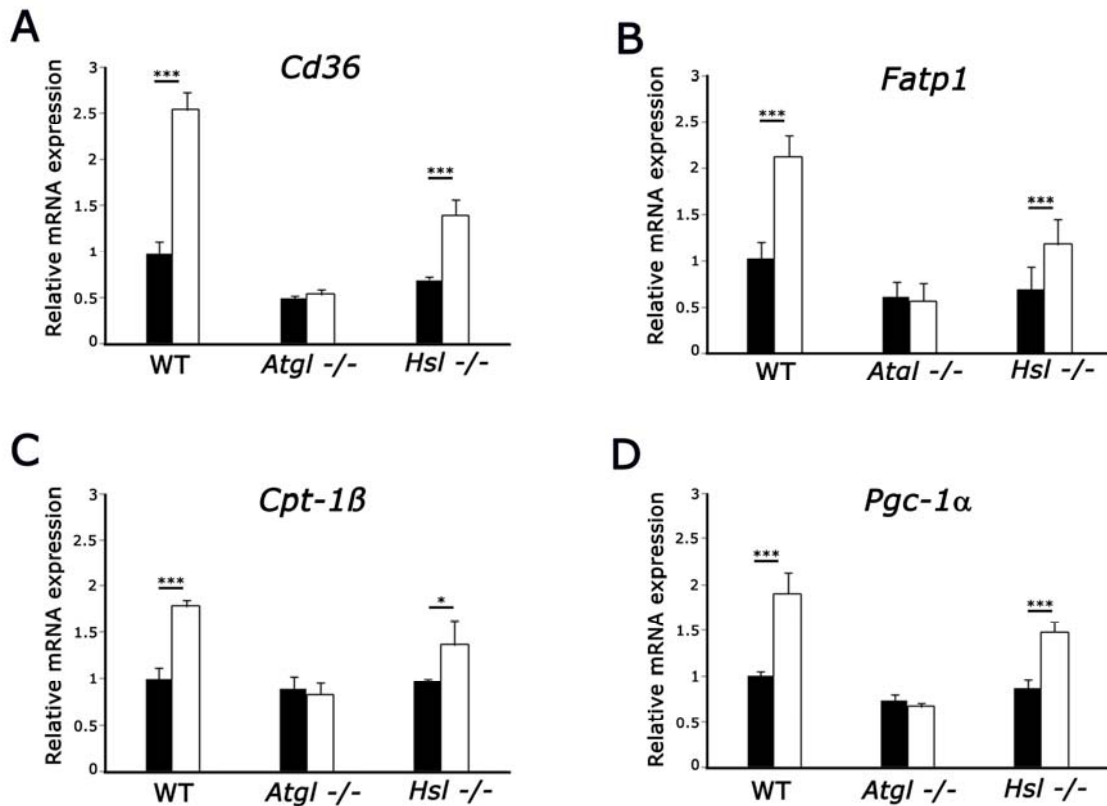


Figure 24

mRNA expression levels of FA metabolic genes were increased in *M. gastrocnemius* of tumor-bearing *Wt* and *Hsl*^{-/-} mice.

mRNA expression of (A) *Cd36*, (B) *Fatp1*, (C) *Cpt-1β* and (D) *Pgc-1α* determined by qRT-PCR was increased in tumor-bearing *Wt* and *Hsl*^{-/-} mice compared to control (saline injected) animals. No change in the expression was observed in the muscle of tumor-bearing *Atgl*^{-/-} mice. Black bars indicate control (saline injected) animals and white bars tumor-bearing animals. *** p < 0.001, * p < 0.05, n = 7

To test whether the activity of metabolic lipases in WAT also associates with CAC in humans, we determined ATGL and HSL-mediated TG hydrolase activities of visceral WAT from autopsy samples of 27 patients. 12 of these individuals had been diagnosed with various forms of malignancies (2 adenocarcinomas of the lung, 2 adenocarcinomas of the colon, 2 ductal adenocarcinomas of the breast, 2 adenocarcinomas of the prostate, 1

hepatocellular carcinoma, 1 clear cell carcinoma of the kidney, 1 squamous cell carcinoma of the esophagus and 1 malignant germ cell tumor). 6 out of the 12 patients were designated as cachectic according to the definition of Evans et al. (Evans et al., 2008). Total lipase, ATGL, and HSL activities were significantly higher in visceral WAT of cancer patients compared to individuals without cancer and significantly higher in cancer patients with cachexia compared to cancer patients without cachexia (Figure 25 A, B, C). Lipase activities in cancer patients without cachexia were similar to those of non-tumor patients. Additionally, a significant inverse correlation was found between total lipase, ATGL and HSL activities in WAT of cancer patients and their BMI (Figure 25 D, E, F).

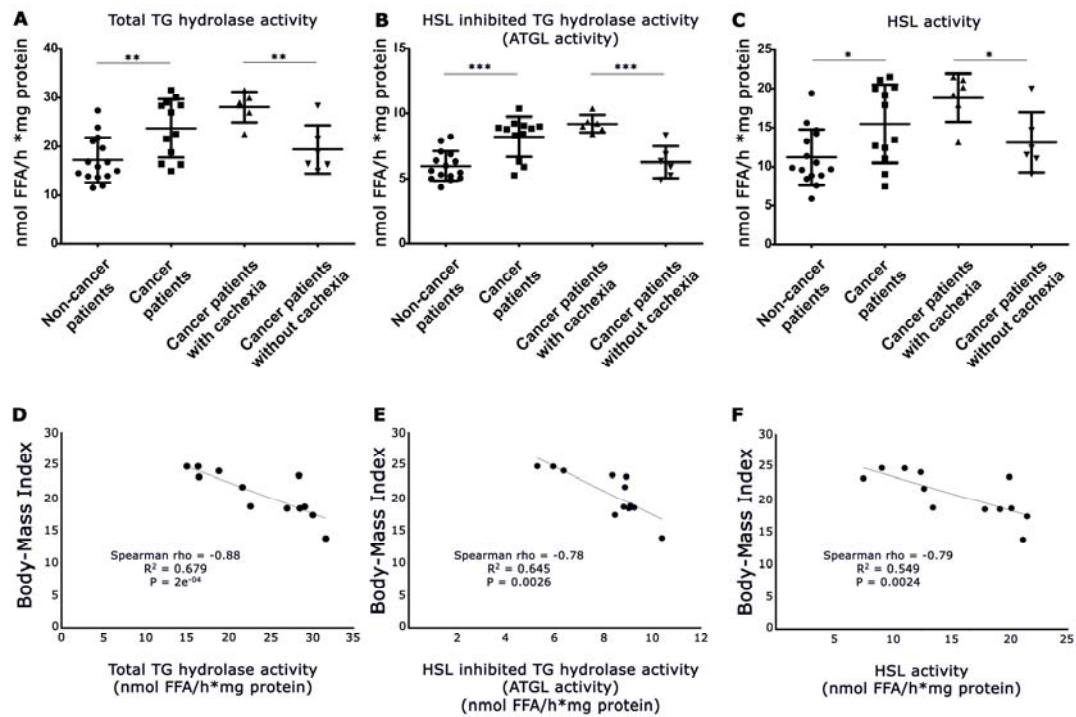


Figure 25

CAC patients show increased TG hydrolase activity compared to non-cachectic patients.

Total lipase activity (A), specifically inhibited (HSL, 76-0079) lipase activity (mainly ATGL activity) (B), and HSL activity (determined by subtraction of HSL inhibited lipase activity from total lipase activity) (C) in visceral WAT of tumor bearing patients compared to non-tumor bearing patients. (A, B, C) Lipase activities in WAT of tumor bearing cachexia patients are significantly higher than in tumor bearing non-cachexia patients.

(D, E, F) Total, HSL inhibited and HSL lipase activities show negative correlation with BMI of tumor patients.

In contrast, lipolytic activities in WAT of non-tumor patients showed no correlation with their BMI (Figure 26). Thus, our study provides compelling evidence that the previously observed increase in FA and glycerol release from WAT of patients with CAC (Agustsson et al., 2007; Tisdale, 2009) is due to upregulation of ATGL and HSL activities and that increased lipase activities strongly correlate with cachexia.

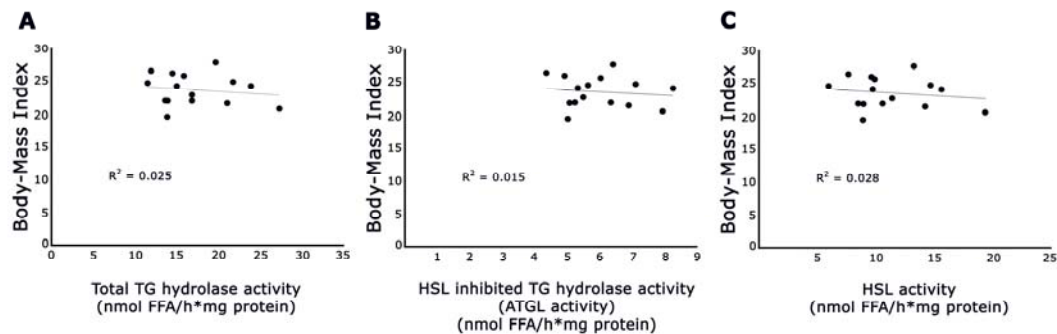


Figure 26

No significant correlation could be found between either

(A) total lipase activity, (B) specifically inhibited (HSL, 76-0079) lipase activity (mainly ATGL activity), or (C) HSL activity (determined by subtraction of inhibited lipase activity from total lipase activity) in WAT and BMI of non-tumor patients.

In summary, our data are consistent with the view that lipolysis plays an instrumental role in the pathogenesis of CAC. The increased catabolism of adipose lipid stores leads to the complete loss of WAT followed by a reduction in muscle mass. The absence of ATGL and, to a lesser degree, HSL reduces FA mobilization, retains WAT and muscle mass, and prevents CAC. Whether the protection of adipose and muscle loss in lipase-deficient mice is a consequence of defective tissue autonomous lipolysis or due to endocrine signaling from the tumor or WAT remains to be elucidated. Importantly, however, pharmacological inhibition of lipases may represent a powerful strategy to avoid the devastating condition of cachexia in response to cancer or other chronic diseases.

Chapter 2

Introduction...

Micro RNA (miRNA)

Short 22-nt long RNA species with the power of regulating the expression of thousands of genes were named as miRNA in 2001 (Lagos-Quintana et al., 2001; Lau et al., 2001). The discovery of miRNAs by Ambros and colleagues in 1993 added a great significance to the non protein coding regions of the genome. It was shown that an 21 nt RNA encoded by lin-4 gene could bind and regulate the expression of lin-14 in *C.elegans* (Lee et al., 1993). Baulcombe and colleagues showed the importance of miRNAs in plants (Jones et al., 1999). Since then more than 10000 miRNAs have been identified in various organisms and the regulation of physiological processes by these miRNAs has been a focus of research over the past decade.

Biogenesis of miRNAs

miRNA is transcribed as pri-miRNA which is a 60-90 nt RNA having a characteristic hairpin stem-loop structure. The hairpin structure is cleaved by a RNase III enzyme, Drosha and its binding partner, DGCR8 (Pasha), to form pre-miRNA having 22 bp stem, a loop and an overhang region (Denli et al., 2004; Gregory et al., 2004; Lee et al., 2003). Exportin-5 (XPO5) subsequently transports the pre-miRNA in the cytoplasm, where it is further cleaved by the next RNase III enzyme, Dicer, thus forming a ~22-nt miRNA duplex (Grishok et al., 2001; Hutvagner et al., 2001; Yi et al., 2003). The duplex is acted upon by an unknown helicase to yield a ~22-nt mature miRNA (guide strand) and a complementary miRNA* (passenger strand) (Sun et al., 2010). miRNA* is degraded rapidly by RNases (Khvorova et al., 2003; Schwarz et al., 2003). Mature miRNA is then packed into a ribonucleoprotein miRNA-induced silencing complex (miRISC) and this complex is responsible for regulation of the target genes. miR-29b, however, is synthesized in the mature form in the nucleus (Hwang et al., 2007). Argonaute (Ago) proteins present in the ribonucleoprotein complex act as endonuclease which cleaves the miRNA-mRNA duplexes but are incapable of cleaving the single stranded RNA. miRNA are also known to repress the translation of the target mRNA, however precise mechanisms governing the repression are still unclear (Figure 27).

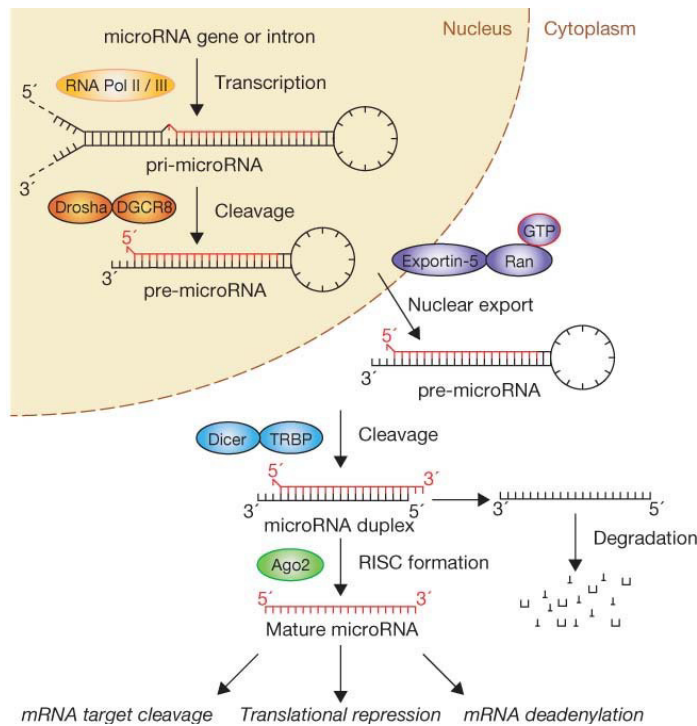


Figure 27.

Biogenesis of miRNA and regulation of target expression by miRNAs (Winter *et al.*, 2009). With permission for reuse from Nature Cell Biology.

miRNAs bind to the target mRNAs along with the ribonucleoprotein complex based on the sequence complementarity. miRNAs regulate the expression of target sequences by either mRNA cleavage or translation repression. The governing process and the factors involved in the decision of the regulation are mainly unknown. However, in plants, most of the miRNAs degrade the target mRNA, whereas in animal cells miRNAs mainly obstruct the translation process (Jones-Rhoades *et al.*, 2006; Nilsen, 2007). Also in plants, miRNAs share a high degree of sequence complementarity to the target sequence but in human cells, this high complementarity is usually absent (Jones-Rhoades *et al.*, 2006).

miRNAs mostly recognize the target sequences based on 100% complementarity in the seed region comprising 2-8 nt within the 3'UTR of the target mRNA. For stabilization, 3' miRNAs also contains a high complementarity with the target mRNA (Brennecke *et al.*, 2005; Grimson *et al.*, 2007). Other regions of the miRNA-mRNA complex might contain mismatches and bulges; however the Ago interaction site should not have any mismatches. The target mRNA might contain more than one interaction site of the same miRNA, which might act independent of each other and recruit the miRNAs associated with

ribonucleoprotein complex independently or even act in unison and influence the function of each other (Brennecke et al., 2005; Grimson et al., 2007; Lewis et al., 2005; Nielsen et al., 2007).

miRNAs can interfere with the protein translation of the target mRNA by direct or indirect mechanisms. miRNA can directly obstruct the translation of target mRNA by inhibiting the initiation, interfere with the ribosome assembly, repression of elongation, or termination (Chekulaeva *et al.*, 2006; Chendrimada *et al.*, 2007; Cho *et al.*, 2005; Kiriakidou *et al.*, 2007; Mootz *et al.*, 2004; Olsen *et al.*, 1999; Petersen *et al.*, 2006; Richter *et al.*, 2005) .

miRNAs can cause destabilization and subsequent degradation of the target mRNAs usually by deadenylation (Bagga et al., 2005; Giraldez et al., 2006; Wu et al., 2006) as a form of indirect translation repression mechanism. Some miRNA-mRNA interactions also results in the sequestration of the complex in processing bodies (P bodies), which are enriched in translation repressors (Eulalio et al., 2007; Valencia-Sanchez et al., 2006).

Regulation of miRNA expression

Most miRNAs are transcribed from their own genes and the transcription process is mediated via transcription factors including NFkB, c-myc, p53, HIF etc (Dews et al., 2006; Kluiver et al., 2007; Kulshreshtha et al., 2007; O'Donnell et al., 2005). However, very few specific transcription binding sites have been recognized experimentally so far. Expression of miRNAs is also shown to be influenced by methylation (Sun et al., 2010).

Regulation of lipid metabolism by miRNAs

Recent findings demonstrated that miRNAs are capable of regulating key members involved in various metabolic pathways including amino acid, glucose, and lipid metabolism. miR-375 negatively regulates insulin release and hence insulin secretion by pancreatic β cells is increased by inhibition of miR-375 (Poy

et al., 2004). Branched-chain α -ketoacid dehydrogenase (BCKD) involved in the branched chain amino acid catabolism is shown to be translationally repressed by miR-29b (Mersey et al., 2005). miR-122 regulates HMG-CoA reductase and hence controls plasma cholesterol levels, hepatic β -oxidation and also regulates FA and cholesterol synthesis (Krutzfeldt et al., 2005). Inhibition of miR-122 resulted in decrease of plasma cholesterol level and improvement of steatosis (Krutzfeldt et al., 2005). miR-27, 143, 203, let-7 were shown to regulate the adipogenesis of fat cell by modulating the expression of many key factors (Cawthorn *et al.*, 2008; Kobayashi *et al.*, 2008; Pittenger *et al.*, 1999; Tschop *et al.*, 2001). miR-33 regulates the expression of CPT-1a, CROT1, ADH8 as well as ABCA1 thus controls β -oxidation and cholesterol export (Gerin *et al.*, 2010b; Rayner *et al.*, 2010). Also miR-14, 143, 122 have been shown to play instrumental roles in lipid metabolism (Krutzfeldt *et al.*, 2006). miR-378 and miR-27b plays a pivotal role in regulating lipogenesis (Gerin et al., 2010a; Karbiener et al., 2009). Chen et. al. showed that miR-29a regulates LPL expression and thus controls cytokine secretion and scavenger receptor expression in dendritic cells (Chen et al., 2011).

AIM...

TG hydrolysis (lipolysis) and FA β -oxidation are central metabolic processes. The intricate regulation of the components and steps of lipid metabolism is essential in biological and pathological situations. Any deregulation of the factors in these processes might result in severe lipotoxic effects leading to tissue damage and/or organ failure. As we could identify lipolysis (especially ATGL) as a key regulator of CAC, we wanted to understand the regulation of ATGL and lipolysis. Single miRNAs are known to regulate and co-ordinate the expression of genes involved in multiple pathways. Therefore, we aimed to identify any possible regulation of key components of lipid metabolism by miRNA(s).

Materials & Methods...

Animal care and tissue harvesting:

C57BL/6 mice were kept on a regular light-dark cycle (12 hours (h) light, 12 h dark) and fed a standard laboratory chow diet. All animal studies were performed in accordance with the guidelines and provisions of the Commission for Animals Experiments of the Austrian Ministry of Science and Research recommendations.

8-10 week old mice were anesthetized using isoflurane and subsequently sacrificed by cervical dislocation. The tissues were rapidly excised and frozen in liquid nitrogen.

Cell lines and constructs used

OP9 preadipocytes were cultured in MEM- α (PAA, Pasching, Austria) supplemented with 20% FBS and differentiated in adipocytes by insulin-oleate treatment as described earlier (Wolins et al., 2006). C2C12 myoblasts were cultured in DMEM (high glucose) media (PAA) supplemented with 10% FBS and differentiated into myotubes as described earlier (Airey et al., 1991). HEPG2 cells were maintained in culture with DMEM (high glucose) supplemented with 10%FBS.

Putative miR-124a target sequence from the 3'UTR of ATGL (LUC-MIR-ATGL) or a scrambled control (LUC-SCR-ATGL) was cloned into the 3'UTR of a luciferase gene that is under the control of a CMV promoter. A CMV promoter controlled ATGL-YFP was constructed which includes the putative miR-124a interaction site in its 3'UTR (YFP-ATGL-miR). miR-124a target sequence from CGI-58's 3'UTR (LUC-MIR-CGI) or scrambled sequence (LUC-SCR-CGI) was cloned into the 3'UTR of the luciferase gene. The putative target site of miR-124a was cloned into the 3'UTR of CGI-58-CFP (CFP-CGI-miR). ATGL-YFP and CGI-58-CFP were kindly provided by Dr. Martina Schweiger. The construct with PPRE cloned in the promoter region of luciferase gene was kindly provided by Dr. Thierry Claudel.

pre-miR-124 (Ambion, Austin, TX, USA), miR-124a inhibitor (Ambion) were used to modulate the expression and function of miR-124a in the cells.

Primary adipocytes were isolated from gonadal white adipose tissue of C57BL/6 mice as described earlier (Fernyhough et al., 2004). In brief, adipose tissue slices were cut into ~ 1 mm² pieces in HBSS and the adipocytes were isolated using Collagenase type I (Invitrogen, Carlsbad, CA, USA). The resultant mixture was filtered through a sterile 300 µm mesh and centrifuged at 200 g. The fat layer was resuspended in HBSS wash buffer. After washing off collagenase, the resultant fat layer was resuspended in DMEM/F12 + 10 % FBS + 1% P/S and plated in cell culture flasks.

Transfection and Lentiviral transduction

Transient transfections were performed with the respective plasmids, pre-miRNAs and inhibitors or controls using Lipofectamine 2000 (Invitrogen) according to company's protocol.

Short hairpin clones with pre-miR-124a (GeneCopia, Rockville, MD, USA) were used to produce lentiviral particles in Hek-293T cells. The lentiviral particles were then used to transduce the cells in culture.

Triacylglycerol hydrolase assay

TG hydrolase assay was performed as described earlier (Lass et al., 2006). In short, OP9 differentiated adipocytes were homogenized in lysis buffer (0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol, 20 µg/ml leupeptin, 2 µg/ml antipain, 1 µg/ml pepstatin, pH 7.0) and centrifuged at 20,000 g for 1 h at 4 °C. The lipid-free cytosolic fraction was collected and used for TG hydrolase assays. Non-labeled triolein (Sigma aldrich, St. Louis, MO, USA) and [9,10-³H(N)- triolein] (American Radiolabeled Chemicals (ARC), St. Louis, MO, USA) emulsified with phosphatidylcholine/phosphatidylinositol (Sigma aldrich) was used as the

substrate. The cytosolic fractions, with or without a specific HSL inhibitor 76-0079 (NNC 0076-0000-0079, Novo Nordisk, Denmark) and with or without recombinant ABHD5 (CGI-58) protein, were incubated at 37 °C for 60 min under constant shaking. The reaction was terminated by the addition of methanol/chloroform/heptane (10:9:7), 0.1 M potassium carbonate and 0.1 M boric acid (pH 10.5). After centrifugation at 800 g for 20 min the radioactivity in the upper phase was determined.

Free fatty acid and Glycerol release assay

OP9 adipocytes were washed with PBS and incubated in Dulbecco's modified Eagle's medium (DMEM) (PAA) containing 2 % fatty acid-free bovine serum albumin (Sigma Aldrich) at 37 °C for 2 h. Aliquots of medium were analyzed for FFA and glycerol content (release) by commercial kits (Wako Chemicals, Neuss, Germany and Sigma-Aldrich respectively).

Intracellular TG measurement

Intracellular lipids were extracted with hexane:isopropanol (3:2; v/v), dried under N₂ gas and solubilized in 1% triton X-100 with sonication. TG concentration was determined using TG FS kit (Diasys, Holzheim, Germany). TG concentration was normalized by protein concentrations of cell extracts determined using Bio-Rad RCDC kit (Bio-Rad).

Co-immunoprecipitation (Co-IP) of mRNAs with anti-AGO2 antibody

Protein G agarose beads (Invitrogen) were rinsed with PBS, and incubated with anti-AGO2 antibody (2897S, Cell signalling) in PBS for at least 30 min. Protein G beads complexed with anti-AGO2 antibody were blocked with 0.5 mg/mL yeast RNA and 1 mg/mL BSA. Beads were washed with PBS and subsequently with lysis buffer (25 mM Tris-HCl at pH 8.0, 150 mM NaCl, 2 mM MgCl₂, 0.5% NP-40, and 5 mM DTT). Cells were washed with PBS, and lysed using lysis buffer with protease inhibitors (Roche, Porterville, CA, USA) and RNasin (250 U/mL;

Promega, Madison, WI, USA). The cell lysates were cleared with pre-blocked Protein G beads and subsequently incubated with protein G beads complexed with anti-AGO2 antibody. The beads were washed with lysis buffer containing 900 mM NaCl and 1% NP-40 and then with lysis buffer. Co-immunoprecipitated RNA was extracted using Trizol (Invitrogen) and cDNA was prepared from 1 µg of total RNA using high capacity reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA). PCR was performed using RXR- α specific primers and analyzed with 1% agarose gel.

Microscopic analysis of adipocytes

Isolated murine adipocytes were stained for neutral lipids using Bodipy 493/503 (Invitrogen) and analyzed by fluorescence microscope.

FACS analysis

The amount of intracellular bodipy staining (Invitrogen) was analysed by flow cytometry performed on a FACSAria flow cytometer using FACSDiva 6 software for data acquisition (BD Biosciences). Bodipy was excited with the blue (488 nm) laser and the emitted fluorescence was measured in the green (FITC) channel. Unstained, mock-treated cells served as negative control for instrument setting. A total of 50.000 events were recorded for each sample and a gate was set in the forward (FSC)-side scatter (SSC) plot for further analysis. The median intensity values were plotted for statistical analysis using FCS Express software (De Novo).

FA oxidation assay

C2C12 myotubes were washed with PBS and incubated with serum free DMEM containing 25 mM HEPES and 2% FA free BSA for 2 hours in 37 °C tissue culture incubator. The cells were washed with PBS and incubated with DMEM containing 25 mM HEPES, 2 % FA free bovine serum albumin complexed with 100 µM palmitate and 1 µCi/ml of [^{14} C] palmitic acid (ARC) for 2 h at 37 °C. The

wells were covered with a piece of filter paper (Whatman, Florham Park, NJ) soaked in 3M KOH. 100 µl of 70% perchloric acid (Sigma) was injected into the wells. The cells were put at room temperature for 1 h to capture $^{14}\text{CO}_2$. The filter paper was removed from the wells, dried and ^{14}C -radioactivity was determined in a LS 6500 Multi-Purpose Scintillation Counter from Beckman Coulter (Fullerton, CA, USA).

Luciferase assay

Firefly luciferase activity was measured by One-glo system (Promega) according to the company's protocol. The luciferase activity was normalized to β -gal activity measured by Beta-glo (Promega) according to company's protocol.

Protein extraction and Western blot analysis

Total protein was extracted from cells or mouse tissues by RIPA lysis buffer (Sigma) supplemented with protease inhibitor cocktail (Roche) using a conventional sonicator. Nuclear protein was isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, MA, USA) according to the manufacturer's protocol. Protein concentrations were determined in the cytosolic fraction using Bio-Rad RCDC kit (Bio-Rad). Protein was separated by SDS-PAGE and transferred to a nitrocellulose membrane (Millipore, Billerica, MA, USA). Nitrocellulose membranes were probed with anti-ATGL (2138, Cell Signalling, Danvers, MA, USA), anti-ABHD5 (H00051099-M01, Abnova, Taipei city, Taipei), anti-PPAR α (ab8934, Abcam, Cambridge, MA, USA), anti-RXR α (sc-553, Santa Cruz, CA, USA), anti-AGO2 (2897S, Cell Signalling) or anti- β -actin (sc-47778, Santa Cruz). The resultant bands were analyzed densitometrically.

qRT-PCR

Total RNA from cells or frozen mouse tissues was isolated using Trizol reagent (Invitrogen) according to the manufacturer's protocol. cDNA was prepared from 1

µg of total RNA using high capacity reverse transcription kit (Applied Biosystems) and qRT-PCRs were performed using primers for *Ppara*, *Rxr*, *Atgl*, *Cgi-58*, *Acs*, *Aco*, and *Cpt-1a* using Taqman Universal PCR master mix (Applied Biosystems) in triplicate. The relative expressions were calculated by ddCt method using *β-actin* as the housekeeping gene. Primer sequences are listed in Table S1.

For quantifying miR-124a expression, cDNA was prepared from the total RNA isolated from cells or frozen tissues using Universal cDNA Synthesis Kit (203300, Exiqon, Denmark) and qRT-PCR was performed using primers specific to miR-124 (LNA PCR primer set, Exiqon).

Immunohistochemistry

Primary murine adipocytes were probed for ATGL using ATGL specific antibody (10006409, Cayman, MI, USA) and detected using DAKO AEC detection system (DAKO, Denmark).

Immunostaining was performed by Silvia Schauer.

Statistical Methods

Results are presented as mean ± standard deviation (S.D.). Comparisons were performed using unpaired Students t-test and presented as *** for $p \leq 0.001$, ** for $p \leq 0.01$, * for $p \leq 0.05$.

Results...

miR-124a regulates ATGL

Since ATGL catalyzes the rate limiting first step of lipolysis, we examined its possible regulation by miRNAs. Out of 4584 miRNAs analyzed (miRBase 10.0 database), only miR-124a showed a potential match to the 3' untranslated region (UTR) of the murine *Atgl* mRNA (Figure 28 A, B). Theoretical free energy of the interaction was calculated to be -27.5 kcal/mol using RNAhybrid (Kruger *et al.*, 2006). Seed sequence in the 3'UTR of *Atgl* is conserved amongst various species as shown by global alignment of *Atgl* mRNA sequences by Clustalx (Thompson *et al.*, 2002) (Figure 28 C).

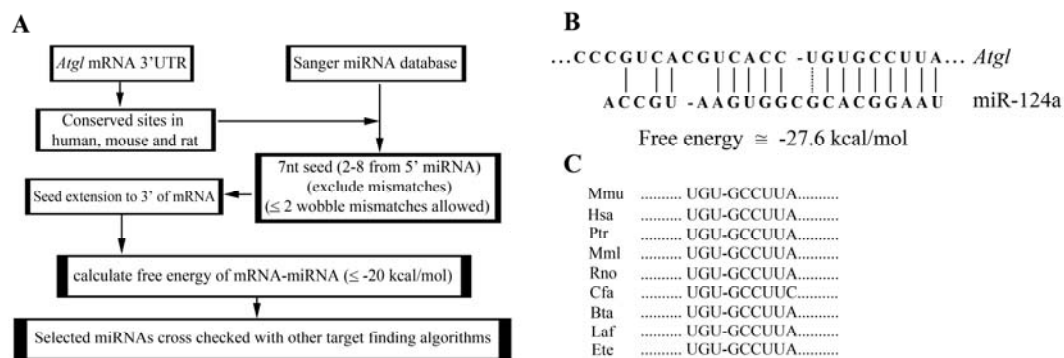


Figure 28. miR-124a, a possible miRNA regulating ATGL

(A) Algorithm used to search for a potential miRNA regulating the expression of ATGL. (B) miR-124a has a potential target region in the 3'UTR of ATGL. Free energy of binding was calculated by RNA-hybrid. (C) The seed sequence in the 3'UTR of ATGL is conserved across many species as shown by Clustalx global alignment analysis.

To test the regulation of ATGL by mir124a we differentiated OP9 cells into adipocytes and transfected them with either pre-miR-124a or scrambled control. miR-124a transfection resulted in a dose dependent downregulation of ATGL protein expression (Figure 29 A) that could be blocked by a specific miR-124a inhibitor as analyzed by Western blot (WB) (Figure 29 B). qRT-PCR using *Atgl* specific primers, after transfection of OP9 adipocytes with pre-miR-124a or scrambled control, showed that miR-124a downregulates *Atgl* mRNA by 44.6 % (Figure 29 C). This could be reversed using a miR-124a inhibitor (Figure 29 C),

demonstrating that the effect on the *Atgl* mRNA was specifically mediated by miR-124a.

To confirm this finding in a more physiological model, primary mouse white adipocytes were transduced with lentiviral constructs carrying either pre-miR-124a or scrambled control. miR-124a transduction resulted in a 72.7 % decrease in *Atgl* mRNA expression compared to scrambled control (Figure 29 D).

To check the regulation of presumably active lipid droplet bound ATGL by miR-124a, cultured primary mouse white adipocytes were transduced with pre-miR-124a or scrambled control and analyzed by immunohistochemistry. miR-124a transduction resulted in a significant reduction of active ATGL protein, localized on the surface of lipid droplets (Figure 29 E).

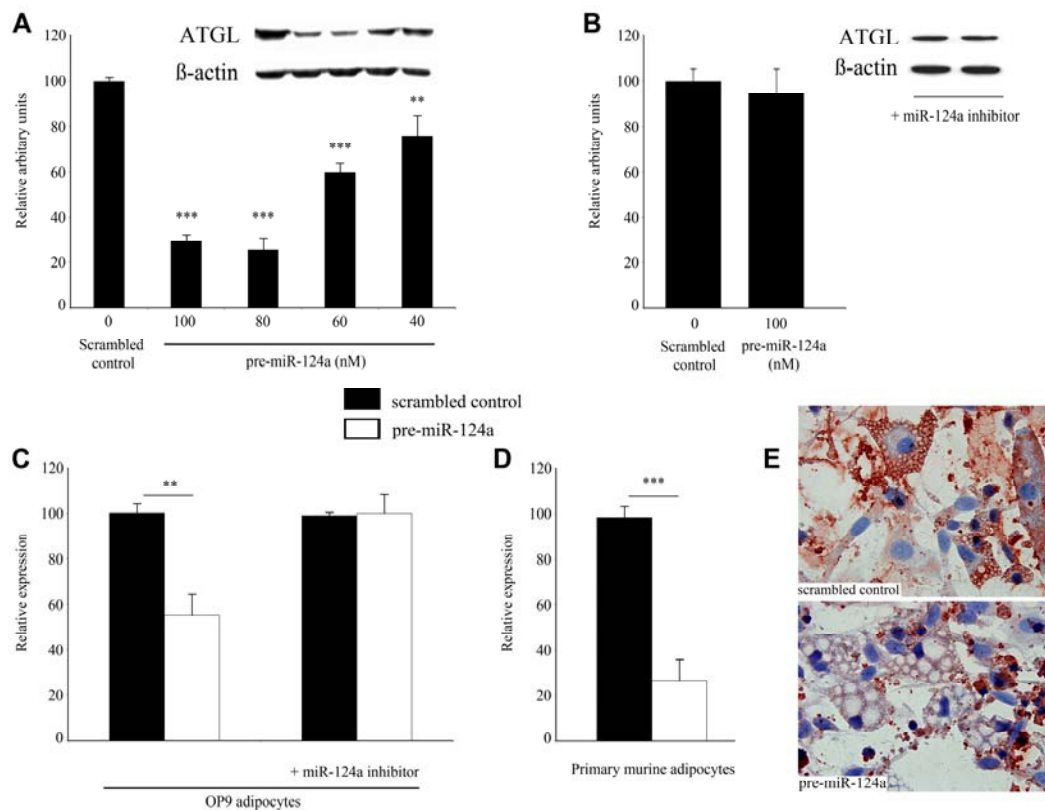


Figure 29. miRNA-124a regulates expression of ATGL.

(A) and (B). ATGL western blot analysis was performed from protein lysates of differentiated OP9 cells 60 hrs after transfection with various concentrations of pre-miR-124a or scrambled control. Cells were co-transfected with miR-124a inhibitor (B). Bar

graphs depict densitometric analysis of specific bands normalized to β -actin (loading control). (C) 100 nM pre-miR-124a or scrambled control was co-transfected with or without miR-124a inhibitor in OP9 adipocytes. mRNA expression of ATGL was analyzed by qRT-PCR and normalized to β -actin. (D) and (E) Lentiviral particles carrying either scrambled control or pre-miR-124a were transduced to primary cells isolated from mouse white adipose tissue and qRT-PCR was performed with ATGL specific primers and normalized to β -actin (D) Immunohistochemical analysis highlights the lipid bound “active pool” of ATGL (E). Data are shown as average $\pm$ standard deviation and represent 3 independent experiments. ***p<0.001, **p<0.01

Immunostaining was performed by Silvia Schauer.

miR-124a regulates ATGL by directly binding to the 3'UTR of its mRNA

miRNAs regulate gene expression by direct interaction with the respective mRNA or indirectly *via* regulators of the particular gene/protein. To understand miR-124a–*Atgl* interaction, we cloned the putative miR-124a target sequence from the 3'UTR of *Atgl* or a scrambled control sequence into the 3'UTR of a luciferase gene (LUC-MIR-ATGL and LUC-SCR-ATGL respectively, Figure 30 A). When LUC-MIR-ATGL was co-transfected with pre-miR-124a in Hela cells, it resulted in a 46.15% decrease in luciferase activity as compared to co-transfection with scrambled control miRNA (Figure 30 B). miR-124a inhibitor prevented the decrease in luciferase activity. Co-transfection of LUC-SCR-ATGL did not lead to any significant loss in luciferase activity. This demonstrates that miR-124a downregulates gene expression by direct interaction with the *Atgl* 3'UTR target sequence.

In order to verify direct regulation of ATGL by miR-124a, a CMV promoter controlled ATGL-YFP was constructed which including the putative miR-124a interaction site in its 3'UTR (YFP-ATGL-miR, Figure 30 C) was constructed. When YFP-ATGL-miR was co-transfected with pre-miR-124a, we observed a clear downregulation of YFP fluorescence compared to co-transfection with scrambled control pre-miRNA (Figure 30 D).

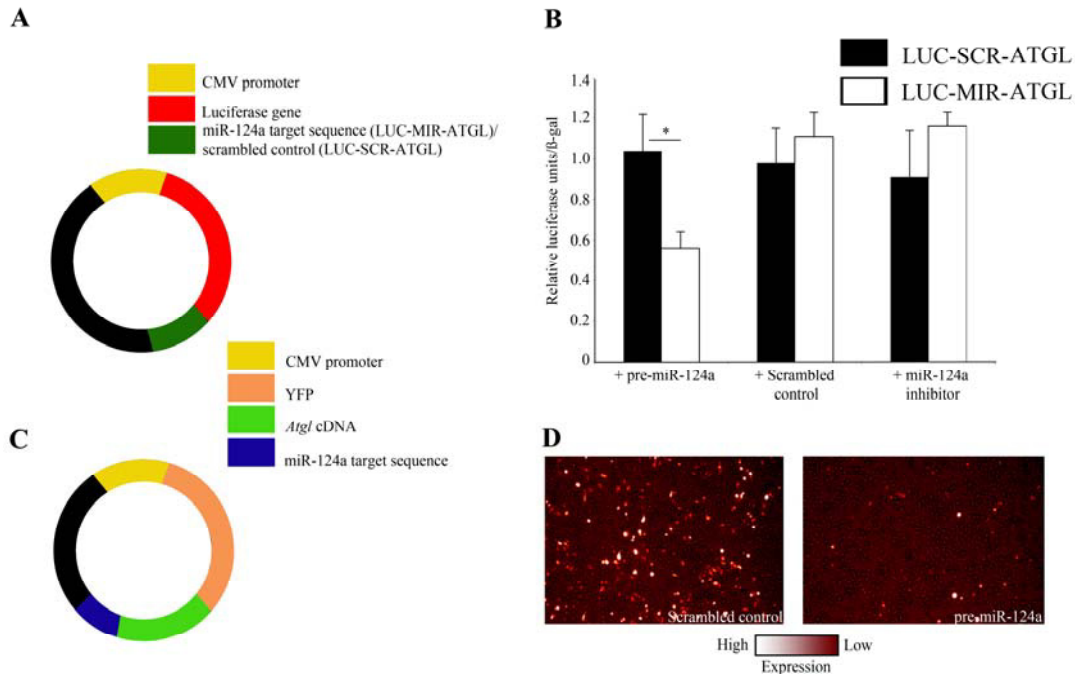


Figure 30. miR-124a regulates ATGL by directly binding to the 3'UTR of its mRNA

(A) Vector diagram representing the plasmid construct containing the putative miR-124a target region from the ATGL 3'UTR (LUC-MIR-ATGL) or scrambled control (LUC-SCR-ATGL) in the 3'UTR of a luciferase gene. (B) Luciferase activity (relative units, normalized to β -gal activity for transfection normalization) measured 60 h after co-transfection of HeLa cells with either LUC-MIR-ATGL or LUC-SCR-ATGL with scrambled control or pre-miR-124a and miR-124a inhibitor as indicated. (C) Vector diagram representin the plasmid construct containing the putative miR-124a target region from the ATGL 3'UTR in the 3'UTR of ATGL tagged with YFP (YFP-ATGL-miR). (D) HeLa cells were co-transfected with YFP-ATGL-miR and either 100nM pre-miR-124a or scrambled control. Images were captured with a fluorescence microscope after 48 hrs. Data are shown as average $\pm$ standard deviation and represent 3 independent experiments. * $p < 0.05$

miR-124a and ATGL expression are negatively correlated

ATGL expression varies considerably between different tissues/organs depending on its role in cell metabolism, necessitating intricate regulation. We hypothesized that miR-124a might at least in part govern differential expression of ATGL. Indeed mRNA expression of *Atgl* in various murine tissues (Figure 31

A) and protein levels (Figure 31 B) show a strong negative correlation with expression of mature miR-124a (Pearson $r = -0.77$, p -value = 0.03) (Figure 31 C, D).

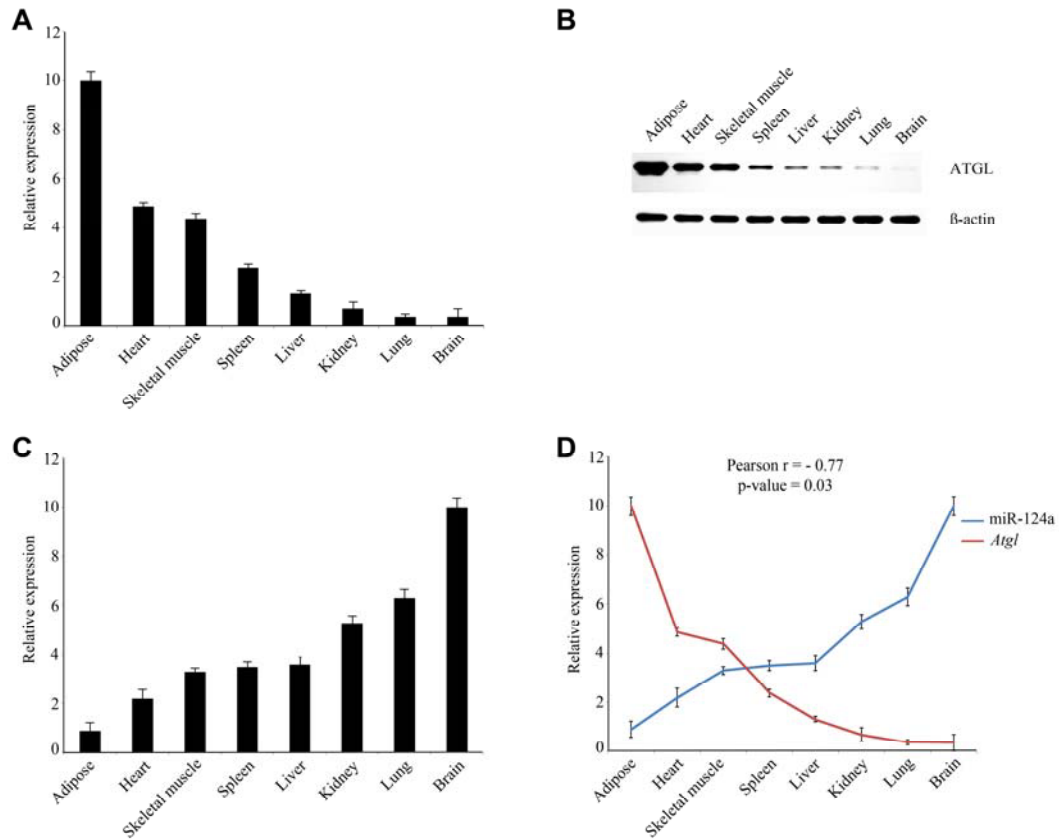


Figure 31. miR-124a expression negatively correlates to ATGL mRNA levels in various murine tissues. Relative ATGL mRNA (A) and protein (B) expression measured in various murine tissues extracted from C57Bl6 mice by qRT-PCR and immunoblot respectively. (C) Relative expression of mature miR-124a measured by qRT-PCR. (D) Correlation between ATGL mRNA and miR-124a expression in various murine tissues.

miR-124a regulates CGI-58 (ABDH5) by direct binding to the 3'UTR of *Abhd5* mRNA

Although ABHD5 itself does not show any lipase activity *in vitro*, it functions as an important regulator of lipid metabolism (Lass et al., 2006) by increasing ATGL activity ~20 fold. Interestingly, we identified a miR-124a target site in the 3'UTR

of *Abhd5* with a theoretical free energy of binding -22.36 kcal/mol (Figure 32 A). The putative interaction site is conserved amongst various species as shown by Clustalx sequence alignment (Figure 32 B).

Transfection of pre-miR-124a into OP9 adipocytes resulted in a downregulation of ABHD5 protein by 84.27 % and mRNA by 52 % compared to treatment with scrambled control miRNA (Figure 32 C, D). Downregulation of *Abhd5* by miR-124a could be prevented by a miR-124a inhibitor demonstrating the specificity of miR-124a. In addition miR-124a downregulated *Abhd5* mRNA in primary mouse white adipocytes as detected 24h after transduction of pre-miR-124a or scrambled control miRNA via lentiviral particles (Figure 32 E).

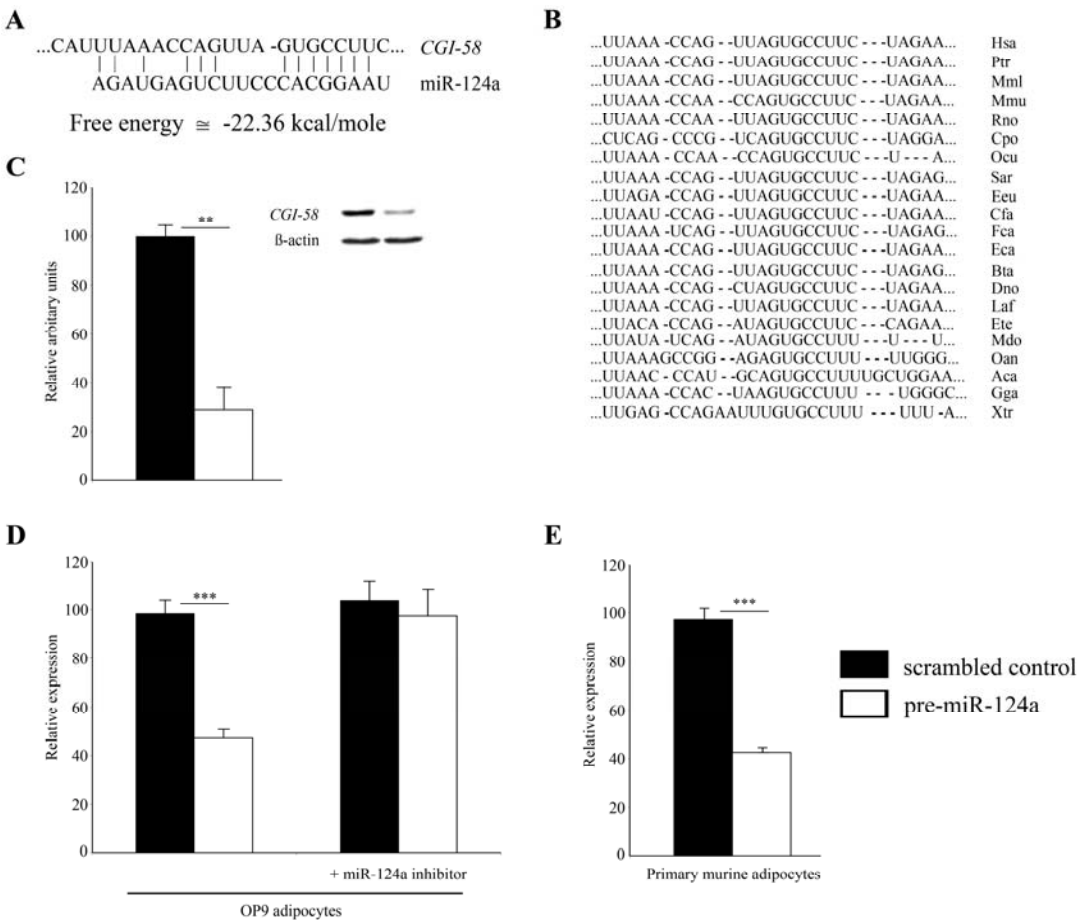


Figure 32. miR-124a regulates the expression of CGI-58.

(A) CGI-58 has a potential miR-124a target region at its 3'UTR. Theoretical free energy of binding was calculated by RNA-hybrid. (B) Multiple alignment analysis performed with Clustalx for the seed sequence in the 3'UTR of CGI-58. (C) Differentiated OP9 cells

were transfected with 100 nM pre-miR-124a or scrambled control and analyzed after 60h for protein expression of CGI-58 by immunoblot. β -actin was used as a loading control. Bar graph shows the densitometric analysis of specific bands normalized to β -actin. (D) Regulation of CGI-58 mRNA was analyzed by qRT-PCR 36h after co-transfecting pre-miR-124a or scrambled control with or without miR-124a inhibitor to OP9 adipocytes. Values are presented as relative expression after normalization to β -actin. (E) Regulation of CGI-58 mRNA expression analyzed by qRT-PCR from primary murine adipocytes transduced with lentiviral particles carrying either scrambled control or pre-miR-124a. Values are presented as relative expression after normalization to β -actin. Data are shown as average $\pm$ standard deviation and represent 3 independent experiments. *** $p < 0.001$, ** $p < 0.01$

To investigate the molecular regulation of *Abhd5* by miR-124a, we used a similar strategy as described above for ATGL. We cloned putative miR-124a target sequence from *Abhd5* 3'UTR or scrambled sequence in the 3'UTR of the luciferase gene (LUC-MIR-CGI and LUC-SCR-CGI respectively, Figure 33 A). LUC-MIR-CGI was down-regulated by 34.8 % when co-transfected with pre-miR-124a compared to co-transfection with scrambled control as detected by luciferase activity assay (Figure 33 B). The miR-124a inhibitor prevented the down-regulation of LUC-MIR-CGI, also no significant difference in the luciferase activity was observed when LUC-SCR-CGI was co-transfected with either pre-miR-124a or scrambled control. The results imply that miR-124a regulates the expression of *Abhd5* by directly binding to the target sequence present in the 3'UTR of *Abhd5* mRNA.

In order to confirm our observation, a CMV promoter controlled CGI58-CFP was constructed including the putative miR-124a interaction site in its 3'UTR (CFP-CGI-miR) (Figure 33 C). We observed a significant reduction in the expression of CFP-CGI-miR when co-transfected with pre-miR-124a as compared to scrambled control miRNA (Figure 33 D).

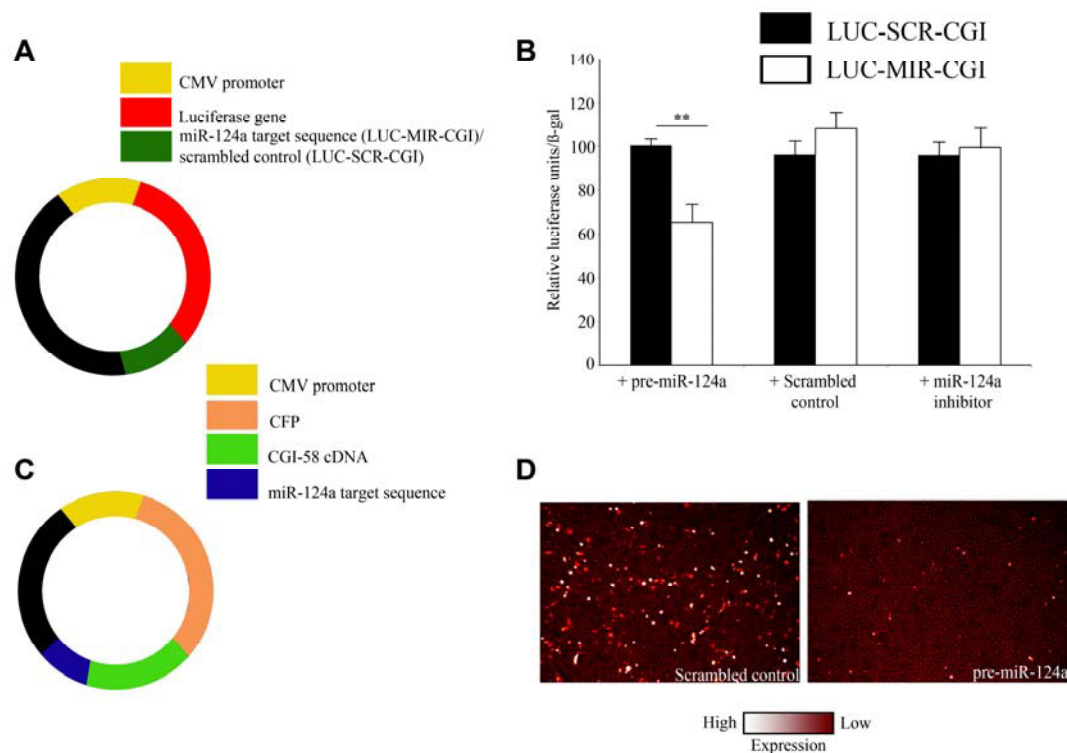


Figure 33. miR-124a regulates the expression of CGI-58 by binding to its 3'UTR.

(A) Vector diagram representing the plasmid construct consisting of the putative miR-124a target region from the 3'UTR of CGI-58 or scrambled control cloned in the 3'UTR of a luciferase gene. (B) LUC-MIR-CGI or LUC-SCR-CGI was co-transfected to HeLa cells with either miR-124a or scrambled control and miR-124a inhibitor. β -gal was transfected for transfection normalization. Luciferase assays were performed 60h after transfections. Values are presented as relative luciferase units normalized to β -gal activity. (C) Vector diagram representing the plasmid construct consisting of the putative miR-124a target region from 3'UTR of CGI-58 in the 3'UTR of CGI-58 tagged with CFP. (D) Fluorescent images of HeLa cells transfected with YFP-ATGL-miR and either 100nM pre-miR-124a or scrambled control. Data are shown as average $\pm$ standard deviation and represent 3 independent experiments. ** $p < 0.01$

miR-124a down-regulates lipolysis

Based on the observation, that miR-124a regulates the expression of two major players in the lipolytic pathway we speculated that miR-124a might influence lipolytic activity in general.

A significant increase in neutral lipid content was observed using fluorescence microscopy after Bodipy staining of primary murine adipocytes (isolated from gonadal WAT) lentivirally transduced with pre-miR-124a compared to scrambled control after 60 h (Figure 34 A). To confirm these findings, OP9 adipocytes were transfected with either pre-miR-124a or scrambled control and stained with BODIPY after 70 h. FACS analysis demonstrated an ~2 fold increase in cellular neutral lipids in pre-miR-124a were transfected adipocytes compared to scrambled control (Figure 34 B). Correspondingly, TG concentration in OP9 adipocytes transfected with pre-miR-124a was elevated by 56 % compared to the scrambled control as measured biochemically 36 h after transfection (Figure 34 C). When adipocytes were co-transfected with a miR-124a inhibitor, the TG concentrations were not affected by miR-124a.

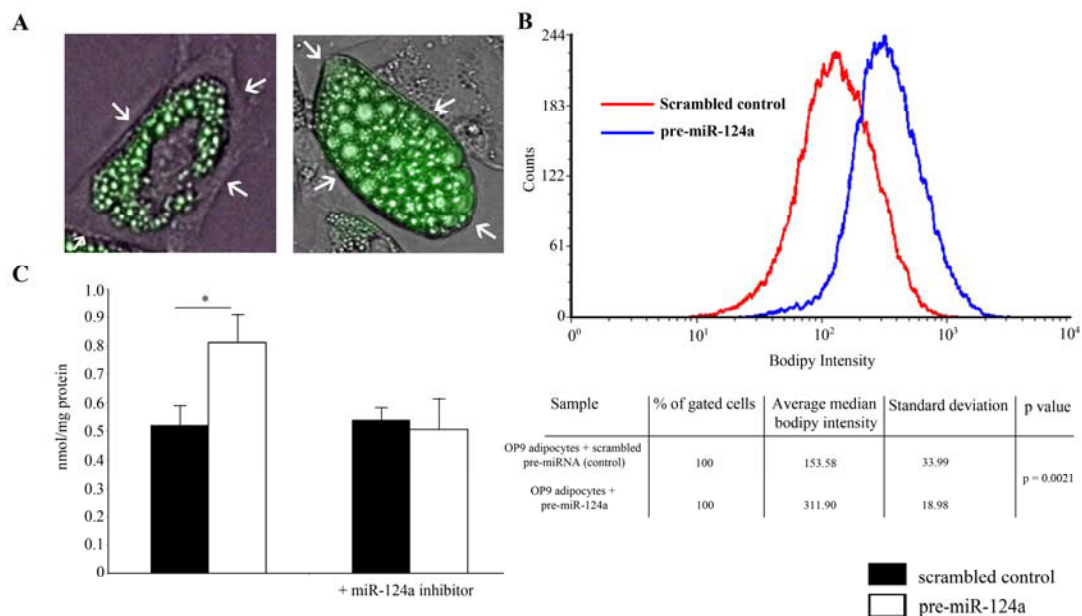


Figure 34. miR-124a regulates TG concentration of adipocytes.

(A) Primary murine adipocytes transduced with lentiviral particles carrying either scrambled control or pre-miR-124a were stained with BODIPY 493/503 after 60h and neutral lipid stores were analyzed using fluorescence microscopy. (B) OP9 adipocytes transfected with either pre-miR-124a or scrambled control were stained with BODIPY after 70h and neutral lipid stores were analyzed using FACS. (C) OP9 adipocytes were transfected with either pre-miR-124a or scrambled control in the absence or presence of a miR-124a inhibitor. TG was quantified biochemically after 30h. TG content was normalized to total protein in cell extracts. Data are shown as mean $\pm$ SD and represent 3 independent experiments. *p<0.05

Total TG hydrolase activity was significantly reduced by miR-124a (Figure 35 A). To differentiate the relative contributions of ATGL and HSL in total lipase activity, we performed lipase activity assay in the presence of the specific HSL inhibitor, 76-0079. We observed a 74 % reduction in HSL inhibited lipase activity in lysate of OP9 adipocytes transfected with pre-miR-124a compared to scrambled control (Figure 35 A) indicating that the effect is largely caused by modulation of ATGL activity. TG hydrolysis in presence of ABHD5, a positive regulator of ATGL, showed that miR-124a decreased the lipase pool which is able to respond to ABHD5 (Figure 35 C). Reduction in TG hydrolase activity was abrogated by anti-miR-124a demonstrating a miR-124a specific effect (Figure 35 B, D).

To verify the influence of miR-124a on lipolysis, we determined FA and glycerol release from OP9 adipocytes. FA and glycerol release was decreased by 33 % and 44 % respectively (Figure 35 E, F) after 36 h of transfection with pre-miR-124a compared to scrambled control. Decreased lipolysis could be rescued by anti-miR-124a.

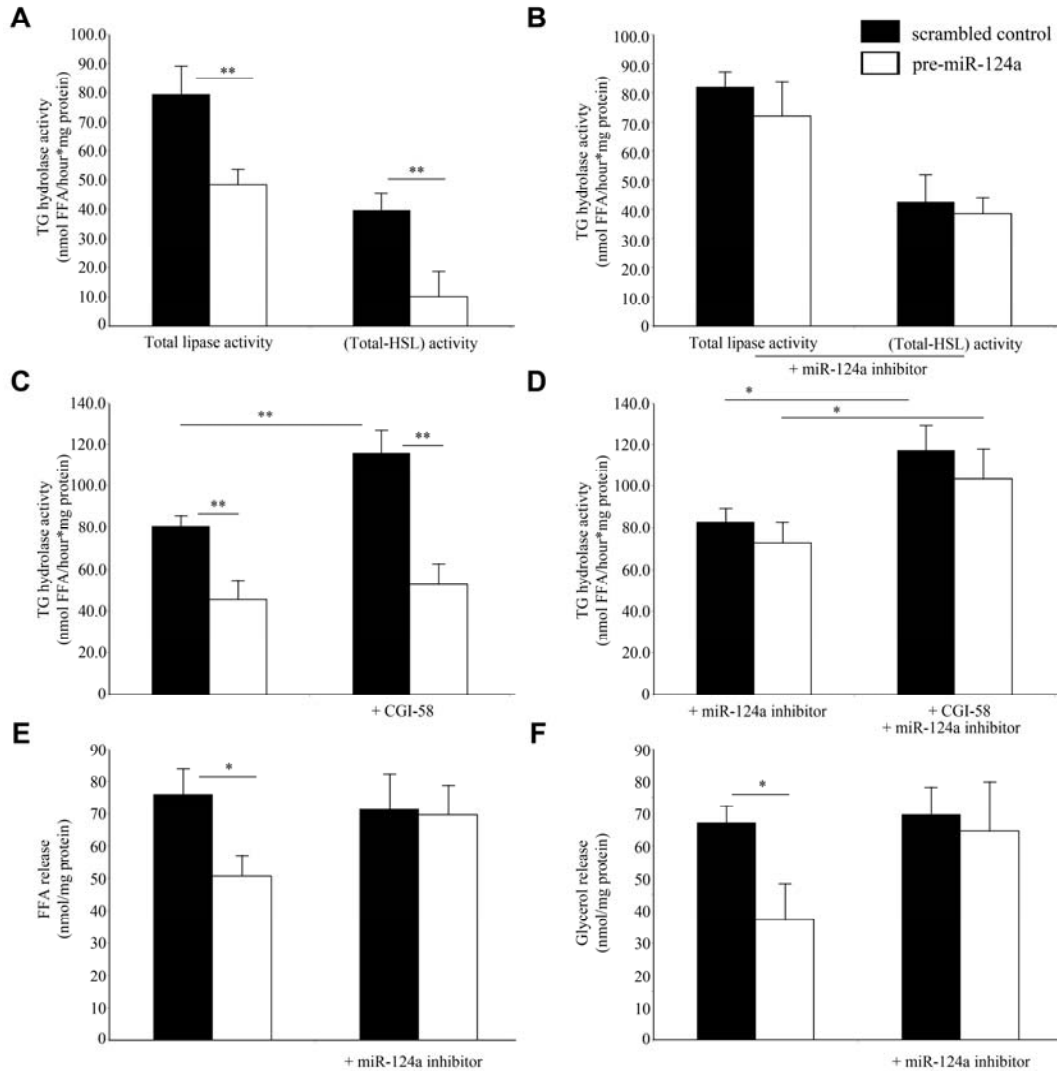


Figure 35. miR-124a regulates lipolysis

(A-F) OP9 adipocytes were transfected with either pre-miR-124a or scrambled control in the absence or presence of a miR-124a inhibitor. TG hydrolase activities in cytosolic protein fractions of adipocytes in the (A) absence or (B) presence of miR-124a inhibitor were analyzed in the presence or absence of 76-0079 (HSL inhibitor). TG hydrolase activities in cytosolic protein fractions of adipocytes in the (C) absence or (D) presence of miR-124a inhibitor were analyzed in the presence or the absence of recombinant CGI-58 protein. (E) FA and (F) glycerol release was quantified enzymatically after 40h and normalized to total protein in cell extracts. Data are shown as mean $\pm$ SD and represent 3 independent experiments. **p<0.01, *p<0.05

miR-124a regulates β -oxidation

Based on our results, we could establish that miR-124a is a potent regulator of the rate limiting step of lipolysis, ATGL expression and activation. Since lipolysis and β -oxidation are highly interdependent processes; we speculated that miR-124a might also influence β -oxidation. Intriguingly we identified a potential miR-124a target site in the 3'UTR of PPAR α , a central regulatory element of the β -oxidation pathway.

Since liver is the organ with the highest activity of PPAR α (Issemann *et al.*, 1990), we analyzed its potential regulation by miR-124a in the human hepatoma cell line, HepG2. Compared to scrambled control, miR-124a led to a significant downregulation of PPAR α mRNA and nuclear protein levels (Figure 36 A, C). Down-regulation of PPAR α mRNA was prevented by miR-124a inhibitor. To verify our findings, we investigated the effect of miR-124a on PPAR α target genes, acyl-CoA synthetase (ACS), acyl-CoA oxidase (ACO) and carnitine palmitoyltransferase 1 α (CPT-1 α). ACS, ACO as well as CPT-1 α were significantly downregulated at mRNA level after transfection of HepG2 cells with pre-miR-124a as compared to scrambled control pre-miRNA (Figure 36 B) which could be prevented by addition of miR-124a inhibitor.

To further investigate the regulation of the functionally active pool of PPAR α by miR-124a, we cloned the PPRE in front of a luciferase gene under the tyrosine kinase (tk) promoter (PPRE-LUC; Figure 36 D). PPAR α activity was analyzed by co-transfecting PPRE-LUC either with pre-miR-124a or scrambled control, and luciferase activity was determined after 60 h. miR-124a caused a 31.07 % decrease in luciferase activity as compared to the scrambled control. Again, decrease of luciferase activity triggered by miR-124a was abrogated by miR-124 inhibitor (Figure 36 E).

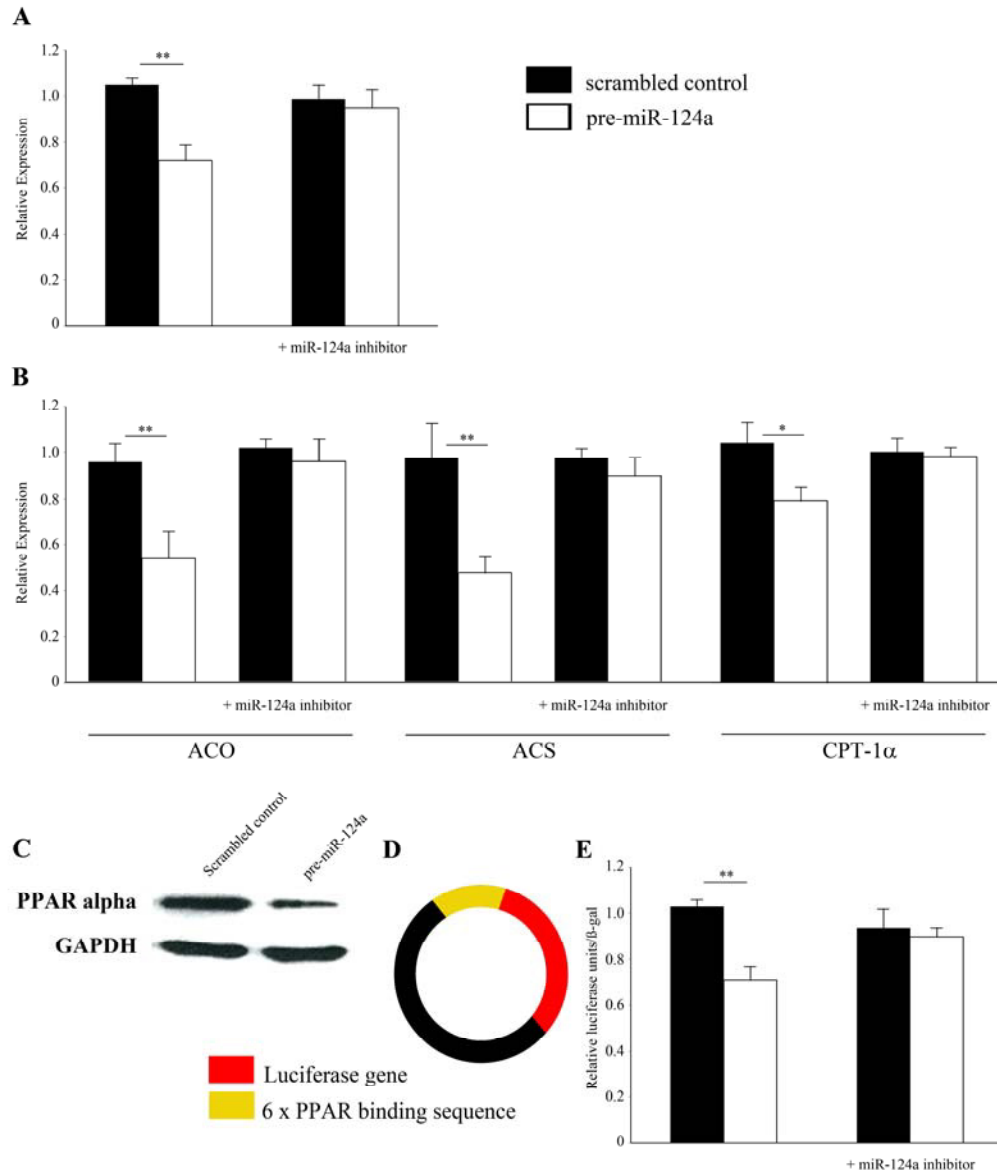


Figure 36. miR-124a regulates PPAR α expression and function.

(A, B) HepG2 cells were co-transfected with pre-miR-124a or scrambled control with or without miR-124a inhibitor and mRNA expression of (A) PPAR α , (B) ACO, ACS and CPT-1 α was analyzed by qRT-PCR. Values are presented as relative expression after normalization to β -actin. (C) Western blot from nuclear protein lysates of HepG2 cells transfected with 100 nM pre-miR-124a or scrambled control using a PPAR α specific antibody. GAPDH was used as a loading control. (D) A PPAR response element was cloned into the promoter of the luciferase gene (PPRE-LUC). (E) PPRE-LUC and β -gal was co-transfected with either pre-miR-124a or scrambled control along with or without miR-124a inhibitor. PPAR α activity was analyzed after 60h by luciferase assay and

values are presented after normalization to β -gal. Data are shown as mean $\pm$ SD and represent 3 independent experiments. ** $p < 0.01$, * $p < 0.05$

PPAR α activates its downstream target genes after heterodimerization with RXR α (Gearing et al., 1993; Issemann et al., 1993). Interestingly RXR α also contains a target site for miR-124a in its 3'UTR, which prompted us to investigate potential regulation by miR-124a. However, transfection of HepG2 cells with pre-miR-124a showed no significant difference on mRNA levels (Figure 37 A). In contrast, WB analysis of the nuclear protein extract showed 65% downregulation of RXR α by pre-miR-124a as compared to scrambled control (Figure 37 B). This effect was prevented by application of a specific miR-124a inhibitor. Thus we can conclude that miR-124a downregulates RXR α protein expression.

To investigate whether miR-124a downregulates RXR α *via* direct interaction with RXR α mRNA, we transfected HepG2 cells with either pre-miR-124a or scrambled control and analyzed the mRNAs pulled down using immobilized AGO2 by RT-PCR. Since we identified RXR α mRNA bound to AGO2 in HepG2 cells transfected with pre-miR-124a (Figure 37 C), we conclude that RXR α is downregulated by direct binding of miR-124a to its mRNA *via* AGO2.

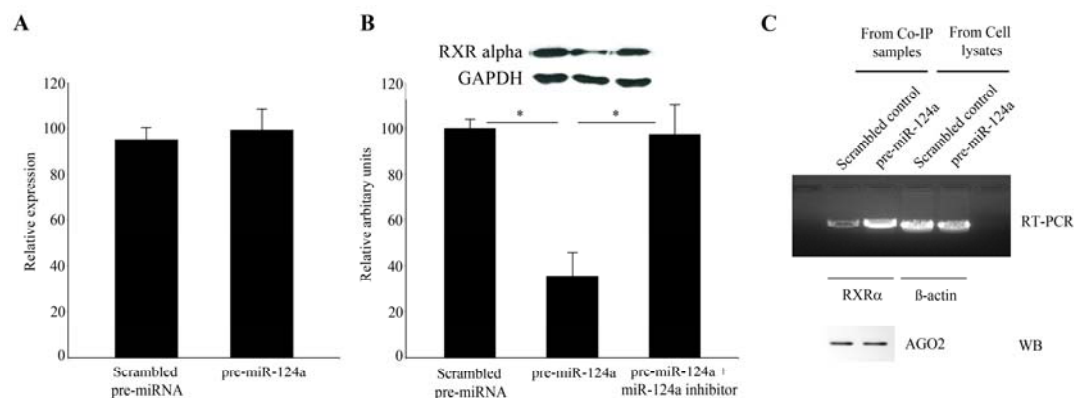


Figure 37. miR-124a regulates RXR α protein but not mRNA expression by binding to its 3'UTR.

(A) Pre-miR-124a or scrambled control was transfected to HepG2 cells and mRNA expression of RXR α was analyzed by qRT-PCR. Values are presented as relative expression after normalization to β -actin. (B) RXR α western blot analysis was

performed from nuclear protein lysates of HepG2 cells transfected with 100 nM pre-miR-124a or scrambled control. Cells were co-transfected with miR-124a inhibitor. GAPDH was used as a loading control. Bars show the densitometric analyses of the bands normalized to GAPDH. (C) mRNA bound with anti-AGO2 antibody complexed with protein G beads were analyzed for the presence of RXR α mRNA by RT-PCR. RT-PCR for β -actin from total cell extracts was used as a control. Data are shown as mean $\pm$ SD and represent 3 independent experiments. *p<0.05.

To investigate functional effects of miR-124a, we analyzed FA β -oxidation. As β -oxidation plays an important role in the energy metabolism of skeletal muscles, FA oxidation was determined in differentiated C2C12 myotubes. After transfection with pre-miR-124a we observed a 40.38 % decrease in palmitate oxidation as compared to transfection with scrambled control (Figure 38 A). Again, this reduction could be prevented by miR-124a inhibitor.

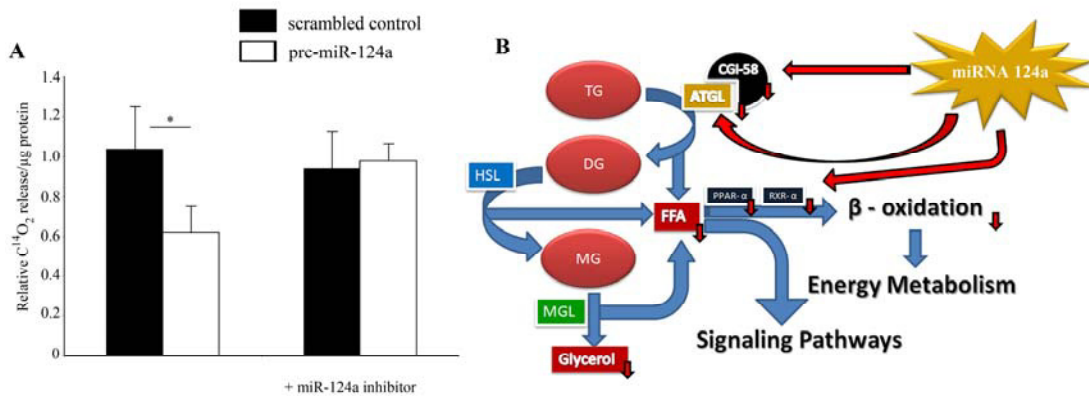


Figure 38. miR-124a: a master regulator of FA oxidation.

(A) ^{14}C -palmitate oxidation was determined in C2C12 myotubes 48h after transfection with pre-miR-124a or scrambled control with or without pre-miR-124a inhibitor. Values are presented after normalization to cellular protein. (B) Model how miR-124a binds directly to the putative target site in the 3'UTR of ATGL and CGI-58 and downregulates their expression. The FFA and glycerol release is reduced. PPAR α and RXR α are also direct targets of miR-124a and thus miR-124a negatively regulates β -oxidation. Data are shown as mean $\pm$ SD and represent 3 independent experiments. *p<0.05

Discussion...

Metabolism of FA is a highly co-ordinated process. Its deregulation can cause severe lipotoxic effects resulting in tissue and organ damage (Lelliott *et al.*, 2004). Therefore, in an attempt to understand the regulation of the process, we investigated the potential role of miRNAs in the control of lipid catabolism and β -oxidation. We identified miR-124a as a key component influencing the balance between TG mobilization and FA oxidation by regulating the expression of ATGL, ABHD5, PPAR α and RXR α .

By means of extensive bioinformatics analysis and database search we discovered a putative binding site of miR-124a in the 3'UTR of ATGL that has been shown to be the main enzyme responsible for catalyzing the first step of the TG catabolism pathway (Zimmermann *et al.*, 2004). Further studies have established ATGL activity as the rate limiting step of the process and therefore as an important factor in regulating lipid metabolism and energy metabolism of the heart, adipose tissue and muscle (Bezaire *et al.*, 2009; Olivecrona, 2010). It has also been identified as the major hepatic lipase regulating TG turnover and FA signaling (Ong *et al.*, 2011).

miRNA is known to regulate various factors in fat metabolism and adipocyte cell biology (Esau *et al.*, 2004; Kajimoto *et al.*, 2006; Krutzfeldt *et al.*, 2006; Xie *et al.*, 2009). Recently Parra *et al.* have suggested that miR-222 might target ATGL along with PPAR γ 2, Fasn, Scd1, HSL and a few other genes involved in adipogenesis and function (Parra *et al.*, 2010). However, the conclusions are based on correlational evidence and direct regulation of ATGL by miRNA has not been demonstrated so far. miRNAs are known to regulate the expression of their target genes by inhibiting ribosome function, decapping the 5' Cap structure, deadenylating the poly(A) tail or degrading target mRNA (Bartel, 2009). Our results clearly demonstrate that miR-124a negatively regulates ATGL at protein as well as mRNA levels in a differentiated adipocyte cell line and mouse primary adipocytes by directly binding to the target sequence present in the 3'UTR region of ATGL mRNA. This corresponds to the observation that some miRNAs regulate their target genes by promoting mRNA degradation (Bagga *et al.*, 2005). ATGL is located both in free form in the cytosol as well as bound to lipid

droplets. The catabolic cascade of lipids is mainly mediated by the lipid droplet bound “active pool” of ATGL (Yang *et al.*, 2010b). Indeed, we found that miR-124a significantly decreases total as well as active ATGL present on lipid droplets.

miR-124a is mostly but not only expressed in brain and regulates the expression of multiple genes involved in processes as diverse as the neural tube development, microglia function, pancreatic development, as well as cancer development (Baroukh *et al.*, 2007; Cao *et al.*, 2007; Lim *et al.*, 2005; Makeyev *et al.*, 2007; Papagiannakopoulos *et al.*, 2009; Ponomarev *et al.*, 2011; Rajasethupathy *et al.*, 2009; Visvanathan *et al.*, 2007). Accordingly, we also detected the expression of miR-124a in various murine tissues, however at much lower levels compared to the brain. Intriguingly, the expression of ATGL, both at mRNA and protein level, was found to be negatively correlated to the expression of mature miR-124a over a broad range of investigated mouse tissues. This suggests that miR-124a is co-expressed in various tissues and hence possibly regulates the expression of ATGL at basal level and/or during various metabolically active states. Negative correlation between miR-124a and ATGL expression might also indicate a developmental regulation of miR-124a and hence ATGL. The significance of the regulation of ATGL by miR-124a at various metabolic states remains to be investigated. ATGL protein expression has been recently found to be strongly correlated with BMI in obese patients and thus supports previous observations that overexpression of ATGL prevents diet-induced obesity in mice (Ahmadian *et al.*, 2009; Yao-Borengasser *et al.*, 2010). miR-124a might be instrumental towards the regulation of ATGL in diet-induced obesity.

The TG hydrolase activity of ATGL is enhanced by its cofactor, ABHD5 (Lass *et al.*, 2006; Yen *et al.*, 2006). Interestingly, *ABHD5* has a very long 3'UTR which is conserved among various species, pointing to a potential relevance in the regulation of ABHD5 and hence ATGL function. We were intrigued to identify the target sequence of miR-124a in the 3'UTR of ABHD5 as well. Using luciferase assays and microscopic analysis, we could show that miR-124a binds directly to

a putative site in the 3'UTR of *Abhd5* and downregulates ABHD5 expression both at the mRNA and protein levels. ABHD5 function is lost in Chanarin-Dorfman syndrome and neutral lipid storage disease (Lass et al., 2006; Lefevre et al., 2001). Therefore, it will be interesting to analyze a possible role of miR-124a in neutral lipid storage diseases.

Since we observed that miR-124a regulates the expression of ATGL and ABHD5, we explored the impact of miR-124a on TG metabolism. Intracellular neutral lipids as well as TG levels were significantly induced in cells transfected with miR-124a. Furthermore miR-124a significantly reduced total as well as ATGL-mediated TG hydrolase activity and hence FA and glycerol release. TG hydrolase assays performed in the presence of ABHD5 recombinant protein confirmed that the decrease in the lipolysis by miR-124a is mediated by the decrease in ATGL protein and not just by changes in ABHD5 protein levels.

Lipid catabolism is strongly coupled to mitochondrial β -oxidation. We investigated if the decrease in ATGL, ABHD5 and hence decreased lipolysis mediated by miR-124a has also an effect on β -oxidation. Indeed we found a miR-124a target site in 3'UTR of PPAR α and RXR α , two key nuclear receptors regulating the transcription of key enzymes involved in the uptake, transport, and oxidation of energy substrates (Ahmed *et al.*, 2007; Brown *et al.*, 2007).

miR-124a significantly reduced PPAR α mRNA as well as protein levels and downregulated PPAR α target genes ACO, ACS and CPT-1 α . Moreover, miR-124a also reduced RXR α protein levels by directly binding to the 3'UTR of its mRNA mediated by AGO2. However, miR-124a does not alter RXR α mRNA levels. Since PPAR α is known to control its own expression (Pineda Torra et al., 2002), it is tempting to speculate that miR-124a, by lowering PPAR α mRNA expression, is a modulator of this negative feedback loop that tightly controls PPAR α protein levels. Among various other factors, PPAR α in turn regulates the expression of miR-99, let-7c and miR-17-5p, a signaling cascade involved in the development of hepatocellular carcinoma (HCC) (Shah et al., 2007). In addition miR-124a has been shown to be downregulated in many tumors due to

methylation of its promoter (Furuta et al., 2010; Lim et al., 2005; Vazquez et al., 2010; Wilting et al., 2010). Hence, it is interesting to investigate the role of miR-124a in HCC as well as NAFLD *via* PPAR α . TG hydrolysis and ATGL activity influences β -oxidation by providing ligands for PPAR α (Sapiro et al., 2009). Since we found that miR-124a negatively regulates the expression and function of both ATGL and PPAR α it will be interesting to dissect the role of miR-124a in modulating the relative expression in health and disease.

Taken together our results show for the first time that miR-124a controls the mRNA and protein expression of key enzymes and regulatory proteins in TG hydrolysis and subsequent β -oxidation of FA in various cell culture systems representing liver, adipose tissue and skeletal muscle (Figure 38 B). HNF4 has also been reported to play a similar role, regulating lipid mobilization and β -oxidation in drosophila (Palanker et al., 2009). Therefore, along with HNF4, miR-124a is likely to exert a key regulatory function in metabolic conditions that require a coordinated regulation of lipolysis and potentially also pathological situations as diverse as lipotoxicity and cachexia that are associated with deregulation of lipolysis. Therefore, deciphering the molecular regulation of lipid and energy metabolism by miR-124a in tumor and syndromes involving metabolic de-regulation might open novel and promising therapeutic avenues.

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Original research articles published/communicated

1. **Das SK.**, Eder S., Schauer S., Diwoky C., Temmel H., Guertl B., Gorkiewicz G., Tamilarasan KP., Kumari P., Trauner M., Zimmermann R., Vesely PW., Haemmerle G., Zechner R., Höfler G. (2011) Adipose Triglyceride Lipase Contributes to Cancer-Associated Cachexia. *Science* 333:233-238.
2. Muehlfeld C.*, **Das SK.***, Heinzel FR., Schmidt A., Post H., Schauer S., Papadakis T., Kummer W., Hoefler G. (2011) Cancer Induces Cardiomyocyte Remodeling and Hypoinnervation in the Left Ventricle of the Mouse Heart. *PLoS ONE* 6(5): e20424. doi:10.1371/journal.pone.0020424. * **equal contribution**
3. Guertl B, Kratky D, Guelly C, Zheng L, Gorkiewicz G, **Das SK**, Tamilarasan KP, Hoefler G. (2009) Apoptosis and fibrosis are early features of heart failure in an animal model of metabolic cardiomyopathy. *Int J Exp Pathol.* 90 (3): 338-46.
4. **Das SK.**, Vesely PW., Schauer S., Schwarz A., Claudel T., Zechner R., Hoefler G. miR-124a: Master Regulator of Lipolysis and β -oxidation. (*Submitted*).
5. **Das SK.***, Pivec C.*, Yan S., Schauer S., Haemmerle G., Zechner R., Hoefler G., Gorkiewicz G. Olanzapine mediated disregulation of the lipid, glucose and cholesterol metabolism. (*Submitted*). ***equal contribution**.
6. Senanayake U., Koller K., Pichler M., Leuschner I., Strohmaier H., Hadler U., **Das SK.**, Hoefler G., Guertl B. The pluripotent stem cell regulator SIX2 is activated in renal neoplasms and influences cell proliferation and migration. (*Submitted*).
7. Tamilarasan KP*, Temmel H*, **Das SK.**, Vesely P., Schauer S., Gorkiewicz G., Hoefler G. Lipotoxic muscle atrophy in muscle specific LPL overexpressing mice. (*In preparation*). *equal contribution.
8. Graulich T., **Das SK.**, Krasteva G., Wessels L., Kummer W., Hoefler G., Muehlfeld C. Alveolar surface area and tracheal innervation in the cancer-cachectic mouse. (*In preparation*).
9. Kumari P., Claudel T., Knopf A., Koefeler H., Baghdasaryan A., **Das SK.**, Stojakovic T., Schittmayer M., Birner-Gruenberger R., Lass A., Zimmermann R., Haemmerle G., Zechner R., Hoefler G., Trauner M. Adipose triglyceride lipase (PNPLA2) as novel key player in protection from hepatic lipotoxicity and inflammation. (*In preparation*).