Lipases and Lipid Metabolism:
Functions, Mouse Models and Comparative Genomics

"Habilitationsschrift"

to obtain the

"Lehrbefugnis" – "Venia Docendi"

at the

Graz University of Technology

in the field

"Genomics and Molecular Biology"

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Graz, May 2007

List of publications selected for this „Habilitationsschrift“

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1 Introduction

Today, malnutrition and obesity are equally prevalent problems with 1 billion people suffering from extreme poverty and malnourishment and close to 1 billion being affected by significant overweight caused by environmental factors and/or inheritance.

Obesity in humans and mammals starts to develop when the intake of substrates repeatedly exceeds energy expenditure. Obesity can be defined as a condition where the accumulation of fat in various tissues is increased to a level at which it can be associated with specific health disorders and increased mortality. Excessive body weight has been shown to predispose individuals to various diseases, particularly cardiovascular diseases, such as atherosclerosis, diabetes mellitus type 2, sleep apnea, osteoarthritis, reduced fertility and many more. Importantly, obesity is not only an individual clinical condition but is increasingly becoming viewed as a serious public health problem.

However, just as obesity may lead to serious health problems, malnutrition and the loss of adipose tissue (lipodystrophy) can cause several illnesses, such as Syndrome X (also called metabolic syndrome) characterized by a combination of medical disorders that increase the risk for cardiovascular disease, diabetes, infertility and others.

In conclusion, a balanced adipose tissue homeostasis is of great importance, including a well-regulated synthesis of triacylglycerols (triglycerides, TG) in the postprandial state (lipogenic pathway) and a degradation of triacylglycerols during food deprivation (lipolytic pathway). Both processes should be in equilibrium.

2 Regulation of adipose tissue homeostasis

Adipose tissue homeostasis is regulated by several neuronal and hormonal signals, many of which have been identified during the last two decades. Some of these signals, known as adipocytokines, are produced and secreted by the adipose tissue and act in an autocrine and endocrine way to exert their regulatory effects.

Leptin is an adipocyte-derived cytokine that was first thought to have primarily an influence on preventing obesity. Therefore, it received its name from the Greek root

'leptos' for thin (1). Today, it is also known that leptin, when suppressed, signals the brain that the body is starving (2). Genetic leptin deficiency in humans, as well as in mice, leads to obesity by driving hunger, reduces energy expenditure and inhibits the initiation of puberty. A therapy with recombinant leptin can strongly reduce these abnormalities (3). In contrast, leptin levels in patients with normal obesity are positively correlated with the amount of adipose tissue (4) and thus, these subjects do not respond to a therapy with recombinant leptin, corresponding to a state of leptin resistance (5).

Adiponectin (also known as ACRP30, apM1 or adipoQ), is a 30 kDa protein produced only by adipose tissue (6). It is present in the plasma as a dimer-trimer or even larger oligomer and the number of complexed monomers has a great influence on the action of adiponectin (7). However, plasma adiponectin levels are reduced in obesity and correlated with insulin resistance and hyperinsulinemia (8) as well as with the development of cardiovascular disease and atherosclerosis (9). In healthy subjects adiponectin levels are negatively correlated with plasma TG and positively correlated with plasma HDL concentrations (10).

Resistin (also known as FIZZ3) - another adipocytokine - was discovered in 2001 (11) as the product of a gene that showed decreased expression in 3T3-L1 cells after treatment with antidiabetic thiazolidinediones (TZD). First studies about resistin have shown that it is induced in obesity and might lead to insulin resistance (12). Its cellular mechanism of action needs to be unraveled, though.

There are many more neural and hormonal regulators of adipose tissue homeostasis, such as visfatin, ghrelin, growth hormones, insulin, TNF alpha, interleucin-6 (IL-6), agouti-related protein (AgRP) and others (13-16). All of them serve one common purpose: to keep the adipose tissue, i.e. the body´s biggest energy store, in “a healthy state” by controlling triacylglyceride synthesis during energy excess and degradation of stored TG during energy demand.

3 Triglycerides: their route from intestine to peripheral tissues

3a Lipoprotein metabolism

Dietary lipids are first absorbed from the small intestine and emulsified by bile salts which are synthesized from cholesterol in the liver, stored in the gallbladder and secreted following the ingestion of fat. As an emulsion dietary fats are accessible to pancreatic lipase. The products of pancreatic lipase, i.e. free fatty acids (FFA) and a mixture of monoacylglycerols (MG) and diacylglycerols (DG) from dietary TG diffuse into the intestinal epithelial cells where the re-synthesis of triacyglycerols occurs.

Lipids are insoluble in plasma. Thus their transport is mediated by lipoproteins which differ in particle size, composition and density. These are chylomicrons (CYM), very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). All of them have a hydrophobic core containing TG and cholesteryl ester (CE) and a polar periphery with phospholipids (PL), cholesteryl (C) and apolipoproteins.

CYM are assembled in the intestinal mucosa from dietary absorbed lipids, mainly TG and the apolipoproteins apoB-48 and apoA-I, apoA-II and apoA-IV. From there the CYM leave the intestine via the lymphatic system and enter the general circulation where they acquire apoE and apoC-II from plasma HDL. ApoC-II activates lipoprotein lipase (LPL) which is bound to the capillary endothel of peripheral tissues such as adipose tissue (AT) and muscles. LPL hydrolyses the core TG of the chylomicrons and FFA are either taken up by the underlying tissues or transported in the bloodstream bound to albumin. The glycerol backbone is returned to the liver and kidneys. After TG lipolysis, the remaining particles are smaller (called CYM remnant, containing primarily cholesterol, apoE and apoB-48) and are immediately taken up by the liver which completes the exogenous lipoprotein pathway.

In the liver the endogenous lipoprotein pathway begins with the production of nascent VLDL from TG and ApoB-100. After hepatic secretion, ApoE, ApoC-II, ApoC-III and CE complete the mature VLDL particle which is, like CYM, transported via the bloodstream to the peripheral tissues where LPL, again activated by ApoC-II, hydrolyses the core TG. The produced FFA are mainly taken up by AT and muscles, whereas the

remaining particles, called VLDL remnants, are either taken up by the liver or processed to LDL particles (106).

HDL, which is mainly responsible for the reverse cholesterol transport from the peripheral tissues back to the liver, is produced in a multistep process. Lipid-poor or lipid-free HDL precursors (pre-β-HDL and apoA-I, respectively) are produced and secreted by hepatocytes or enterocytes. Alternatively, these particles are also produced by the LPL-mediated lipolysis of CYM and VLDL or the HDL modification by phospholipid transfer protein (PLTP) and cholesteryl ester transfer protein (CETP) (17, 18). HDL precursor particles accept phospholipids and cholesterol from extrahepatic tissues through an efflux mechanism that involves the ATP binding cassette transporter 1 (ABC1) (19-22). Subsequently, these particles are converted into mature, large, and spherical HDL-3 and HDL-2 by a process that involves the esterification of cholesterol by lecithin: cholesterol acyltransferase (23), the acceptance of surface remnants from TG-rich lipoproteins (24), and the fusion of HDL particles. The latter two processes are mediated by PLTP (25, 26).

During reverse cholesterol transport, cholesteryl esters of HDL-2 are taken up by the liver via a mechanism called selective uptake (27). It has been demonstrated that the scavenger receptor class B type I (SR-BI) mediates selective uptake of HDL-derived CE. Selective uptake of HDL-CE proceeds in two steps: an initial step involving incorporation of HDL-CE into the plasma membrane, followed by the transfer of CE into the cytosol in the second step. Silver et al. (28) provided evidence that implicates HDL recycling in the process of selective uptake. In addition to SR-BI, lipoprotein lipase (LPL) and endothelial (cell derived) lipase (EDL/EL) mediate selective HDL-CE uptake by hepatic cells *in vitro* (29-31).

3b Lipoprotein lipase and lipid metabolism

Within the lipoprotein metabolism, LPL is a key enzyme responsible for the clearance of plasma lipids and the concomitant uptake of FFA into various tissues. Additionally, LPL is required for the production of mature HDL-C, the so called “good cholesterol”.

LPL deficiency in humans (type I hyperlipoproteinemia) (32) is a rare autosomal, recessively inherited disease characterized by elevated plasma TG levels, low plasma

total cholesterol (TC) levels, and drastically decreased HDL cholesterol (HDL-C) concentrations. Besides these lipid abnormalities, this disorder is associated with the development of hepato- and splenomegaly, eruptive xanthomas, lipemia retinalis, and abdominal pain on a standard diet which leads to frequent attacks of pancreatitis.

Homozygous LPL knock-out mice (L0) die shortly after birth (33-35). At birth, these animals have elevated TG and TC levels compared to wild-type littermates. Upon suckling, they become pale, develop severe hypertriglyceridemia due to CYM and VLDL accumulation and die postnatally between 18 and 24 h. As mentioned above, LPL deficiency in humans (32) is not lethal. This species-specific difference and the actual cause of death in LPL-deficient mice have not been elucidated. Several hypotheses have been proposed: (i) the enormous accumulation of large TG-rich lipoproteins in plasma following suckling leads to a defective gas exchange in lung capillaries which causes insufficient oxygen supply, cyanosis, and premature death in the mouse because of the higher fat content in mouse milk (10% *versus* 4.5% in human milk) (ii) alternatively, the abnormally low glucose levels in newborn L0 pups due to the low carbohydrate content in mouse milk (15% *versus* 30% in human milk) might cause lethal hypoglycemia; (iii) the absence of HDL particles might be incompatible with survival because the majority of plasma lipids in mice are transported in the HDL fraction.

Previous studies, in which transgenic LPL expression in skeletal muscle, cardiac muscle, or liver (36-39) was achieved in otherwise LPL-deficient mice, revealed that these mice can be rescued independently of the site of LPL expression. This suggested that, for survival, the organ in which LPL is expressed is irrelevant as long as sufficient amounts of active enzyme are present in the vascular system. The expression of an enzymatically inactive protein on an L0 background is not sufficient for survival (40).

In order to investigate whether the presence of LPL was obligatory during suckling but dispensable after weaning, we used adenovirus-mediated gene transfer for transient expression of LPL [VIII]. Adenovirus producing LPL (AD-LPL) was injected into all animals of a litter immediately after birth. LPL expression reached a peak 7 days after injection. Subsequently, enzyme expression declined and was undetectable in weaned animals at 4 weeks of age. The transient expression of LPL after a single virus application resulted in a profound extension of viability in all L0 animals. However, only

a small percentage (3%) survived the entire suckling period. These animals were growth-retarded and severely hyperlipidemic.

To our best knowledge, data for newborn humans affected with type I hyperlipoproteinemia are not available. Apparently, the decreased availability of TG-derived FFA in suckling L0 mice is not adequately replaced by other substrates in muscle and AT, which might lead to the observed defects in body development. After weaning, when fed a chow diet with 2.5% fat, the animals recovered rapidly and exhibited similar body weight and body composition at 3 to 4 months of age compared with control mice. Thus, the complete absence of LPL in adult L0 animals did not affect growth. Similar results were obtained in a study of human adults affected with type I hyperlipoproteinemia. These patients were found to have normal AT and body weight (41). Additional evidence for normal fat mass development in the absence of LPL in AT was obtained from induced mutant mouse lines that expressed LPL exclusively in muscle but lacked the enzyme in AT (37). These animals had normal body weight and AT mass. However, their AT exhibited a profound change in fat composition. Essential fatty acids were drastically decreased and replaced by saturated and monounsaturated fatty acids which have been produced by *de novo* synthesis.

Weaned L0 animals on a chow diet (2.5% fat) live for over a year [VIII]. However, in the absence of LPL the animals are severely hypertriglyceridemic. Plasma cholesterol levels are also increased as a consequence of the drastic increase in the cholesterol content in the TG-rich lipoprotein fraction. In contrast, LDL-C and HDL-C were essentially absent in L0 mice. The absence of HDL-C is particularly remarkable, since in normal mice more than 75% of the plasma cholesterol moiety is found in HDL. HDL-C concentrations are also drastically reduced in human LPL deficiency (80-95%) (32).

In conclusion, our study showed that LPL expression is necessary during suckling but dispensable after weaning. In addition, LPL expression is needed for maturation of HDL-C, although lacking HDL-C is not the reason for premature death in LPL-ko mice [VIII].

3c Endothelial cell derived lipase and lipid metabolism

In 1999, a new lipase was cloned from endothelial cells and named endothelial cell derived lipase (EDL) (29, 42). Northern blot analysis of human tissues demonstrated high

expression of EDL in the placenta, thyroid, liver, lung, kidney, testis and ovary. In addition to that of endothelial cells, EDL expression has been detected in hepatocytes and macrophages (29, 42). EDL shows high sequence homology to LPL (44 percent identity) and is like LPL anchored, via the heparin binding site, to heparin sulphate proteoglycans on the luminal endothelial surface. But in contrast to LPL, EDL primarily exhibits phospholipase activity with relatively little TG lipase activity. Overexpression of EDL in mice by a recombinant adenovirus approach dramatically lowered HDL-C and apoA-I levels (29) and effectively increased hydrolysis of HDL-PL (43).

In our work we wanted to investigate which mechanism is responsible for the reduction of HDL-C levels observed *in vivo* following EDL overexpression [VI]. For this purpose, HepG2 cells were infected with an adenovirus coding for EDL (Ad-EDL) and with lacZ (Ad-LacZ) as a control. Following infection, EDL was detected in the cell lysate, on the cell surface bound to the heparin sulphate proteoglycans (HSPG) and in the cell medium. In these EDL-infected HepG2 cells HDL binding and holoparticle uptake were increased as was the selective HDL-CE uptake compared with controls. In order to clarify, if selective HDL-CE and HDL particle uptake depend on EDL activity, enzyme activity was inhibited with tetrahydrolipstatin (THL). Enzymatic inactive EDL was even more effective in holoparticle and selective HDL-CE uptake due to the fact that vastly more EDL protein remained bound to the cell surface in the inactive state which in turn could bind more HDL particles to the cell surface for particle and selective uptake. Furthermore, in cells expressing MUT-EDL (i.e. adenovirus expressing mutated and thus inactive EDL), binding and holoparticle uptake were markedly higher compared to cells expressing the active EDL. To make sure that the selective uptake in EDL overexpressing cells is not due to Cla-1 (Cla-1 is the human homologue of scavenger receptor class-B, type I (SR-BI)), experiments in CLA-1 deficient HEK-293 cells were performed. It was demonstrated that EDL alone has the ability to stimulate selective HDL-CE uptake independently of CLA-1. In summary, our results show that EDL mediates both HDL binding and uptake as well as the selective uptake of HDL-CE, independently of lipolysis and CLA-1.

As mentioned above, we and others have demonstrated that EDL very efficiently releases FFA from HDL-PL *in vitro* [VI, 43]. Hence, we wanted to evaluate if EDL is able to

supply FFA derived from HDL-PL to cells and to determine the effect of liberated FFA on the cellular lipid composition and FA synthesis [V]. For this purpose, HepG2 cells infected with EDL expressing or with control adenovirus were incubated with ^{14}C-HDL-PC (phosphatidylcholine). The analysis of the cellular lipids by thin layer chromatography (TLC) revealed that EDL overexpression led to an increase in the amount of cellular ^{14}C-lipids and the label was mainly incorporated into PL and TG. From this finding we concluded that ^{14}C-FFA derived from ^{14}C-HDL-PC by EDL phospholipase activity are taken up by cells and incorporated into newly synthesized lipids. To assess whether the effect of EDL is dependent only on its enzymatic function, we infected HepG2 cells with an adenovirus encoding the enzymatically inactive EDL. These cells accumulated considerably lower amounts of ^{14}C-PL and ^{14}C-TG than cells expressing enzymatically active EDL. However, the amount of ^{14}C-PL was significantly higher in cells expressing mutant EDL compared with control cells. This finding indicated that EDL has the ability to enhance the uptake of ^{14}C-HDL-PC by HepG2 cells independent of its enzymatic activity, but the accumulation of ^{14}C-lipids is much higher when EDL is active. One possible explanation for enzymatically independent uptake could be that inactive EDL anchors HDL to the cell surface [VI], thus facilitating the scavenger receptor class B Type I (SR-BI) mediated selective import of the lipoprotein-associated PL into cells (44).

Our results [V] showed that EDL supplies cells with FA, including polyunsaturated fatty acids (PUFA) (45). PUFA are known to suppress the expression of lipogenic genes, including FAS (46), a central enzyme in the *de novo* lipogenesis. Therefore, we assumed that EDL expression might have an impact on FA synthesis in HepG2 cells. Our assumption was confirmed by the finding that the amount of fatty acid synthase (FAS) mRNA as well as the rate of FA synthesis was decreased in EDL-expressing cells compared to controls.

In summary, EDL is able to supply cells with FFA that are incorporated into TG and PL and concomitantly *de novo* lipogenesis is down regulated [V].

As shown before, LPL deficiency in humans (32) and in mice [VIII], which have been rescued by an adenovirus approach, leads to massive accumulation of TG in the plasma,

but does not lead to reduced adipose tissue size as one might have suggested. However, the composition of AT differed markedly from that in control, showing a significant decrease in PUFA and a drastic increase in saturated FFA produced by *de novo* biosynthesis (39, 47). PUFA cannot be synthetized *de novo*. These fatty acids must be taken up by the AT either by absorption from plasma FFA/albumin complexes or by the involvement of another lipase. This led us to the assumption that EDL, which is able to supply at least HepG2 and HEK 293 cells with FFA [V], might be expressed in LPL-deficient adipose tissue and responsible for the uptake of PUFA, partially taking over the role of LPL.

When we analyzed AT lacking LPL, we could show that large amounts of EDL mRNA were found in LPL deficient adipose tissue, whereas no EDL mRNA was detected in LPL-expressing control AT [IV]. In addition, differentiated 3T3-L1 cells, which express high amounts of LPL, did not show any EDL expression. These observations suggested that the absence of LPL in AT is a prerequisite for the expression of EDL. Our findings indicate that adipocytes induce the transcription of the otherwise dormant EDL gene when LPL is absent. Interestingly, an opposite scenario - that is increased LPL mRNA expression in skeletal muscle (SM) in the absence of EDL - has been observed in EDL-ko mice (48). Our findings suggest that LPL and EDL expression are inversely regulated. However, the signals and mechanisms involved in these processes are unknown so far.

As expected, increased EDL mRNA levels in LPL-deficient AT were also associated with increased phospholipase activity in LPL-deficient fat pads and isolated adipocytes. Additionally, incubation of fat pads with ^{14}C-HDL-PC showed that AT-EDL was able to catabolize HDL-PL and the released FFA were taken up by the fat cells and subsequently incorporated into the TG droplets [IV].

In conclusion, we could show that in LPL-deficient AT, EDL activity is able to supply those essential fatty acids that are required to maintain essential adipocyte functions, such as accurate membrane fluidity and the biosynthesis of prostaglandins and leukotrienes. However, EDL can only partially take over the function of LPL, as PUFA levels are strongly reduced in LPL deficient AT compared to control AT, although EDL expression is highly induced [IV].

4 Adipose tissue: triglyceride synthesis

4a Introduction

The work of our group showed that LPL and, in its absence, EDL are essential for the uptake of FFA into adipose tissue [IV]. LPL mainly hydrolyses TG from CYM and VLDL whereas EDL lipase activity is directed towards HDL-PL. In any case, both lipases supply the AT with FFA, saturated as well as unsaturated. AT-LPL activity is high during periods of feeding and low during fasting. EDL activity can only be found in AT when LPL is missing to partially take over its function and it is not regulated by fasting-feeding. FFA enter the AT either by free diffusion or by uptake mediated by FFA transport proteins. Since adipose tissue, in the postprandial state, is responsible for the storage of excess of fatty acids within triglycerides, the FFA entering the AT are immediately reesterified. For that purpose, a whole cascade of enzymes is necessary (106, 107), as shown in *Figure 1*.

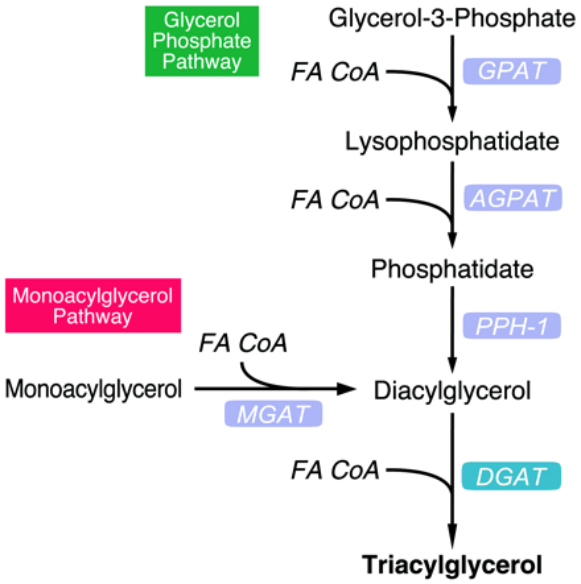

Figure 1. TG synthesis: Triacylglycerols are the end product of a multistep pathway. GPAT indicates glycerol-phosphate acyltransferase; AGPAT, acylglycerol-phosphate acyltransferase; PPH-1, phosphatidic acid phosphohyrdolase-1; MGAT, acyl CoA:monoacylglycerol acyltransferase; DGAT1 or DGAT2, diacylglycerol acyltransferase (51)

The major precursors for TG synthesis in AT are fatty acyl-CoAs and glycerol-3-phosphate (G-3-P). The fatty acids used to produce fatty acyl-CoAs are either provided

by the LPL mediated lipolysis as mentioned above or by *de novo* synthesis from pyruvat produced by glycolysis. In AT, G-3-P is mainly derived from the reduction of dihydroxyacetone phosphat (DHAP, an intermediate product of glycolysis), catalyzed by glycerol phosphate dehydrogenase. A second path for the production of G-3-P would be the ATP-dependent phosphorylation of glycerol by glycerol kinase but the activity of this enzyme is minimal in AT. Taking into account that G-3-P is mainly produced from DHAP, the production rate of TG is directly correlated to glycolysis. Independent of the method of synthesis, G-3-P undergoes two rounds of esterification with fatty acyl-CoA to form diacylglycerol-3-phosphate (also called phosphatidic acid), which is a precursor of both, TG and phospholipids. For TG synthesis, the phosphate is removed by phosphatidic acid phosphohydrolase-1 (PPH-1) followed by the esterification with a third fatty acyl-CoA (106, 107). Three different enzymes show responsibility for the three esterification steps. These are glycerol-phosphate acyltransferase (GPAT), acylglycerol-phosphate acyltransferase (AGPAT) and the last step is catalyzed by two known diacylglycerol acyltransferase enzymes: DGAT1 and DGAT2. This TG synthesis pathway is called the "Glycerol Phosphate Pathway" (106, 107).

A second way of TG synthesis is the "Monoacylglycerol Pathway", where DG are produced through the esterification of MG with fatty acyl-CoAs, catalyzed by the monoacylglycerol acyltransferase (MGAT), and further esterified by DGAT1 or DGAT2 to produce TG. But this pathway predominantly occurs in the small intestine and is therefore of less importance in the AT. Nevertheless, both pathways require DGAT for the last esterification step (106, 107).

4b Diacylglycerol acyltransferase (DGAT)

DGAT-deficient mice showed the importance of the last step in TG synthesis. Mice deficient in DGAT2 die shortly after birth (49). They are smaller than their wt littermates and their carcasses show 90% reduction in TG depots. Additionally, they lack essential fatty acids what leads to abnormalities in skin lipids with decreased epidermal barrier function and rapid dehydration of these mice. In contrast to DGAT2-deficient mice, DGAT1-ko mice are viable (50, 51). Compared to their wt littermates, they show a 50%

reduction of AT and concomitant with less adipose tissue TG content they have smaller lipid droplets. TG serum levels are normal, though.

When DGAT1-ko mice are challenged with a high-fat diet, they are resistant to diet-induced obesity and protected from hepatic steatosis. DGAT1-deficient mice show reduced weight gain despite increased food intake and correlating with the reduced adipose tissue they show higher insulin sensitivity than their wt littermates on a high-fat diet. The reason for resistance to diet-induced obesity in DAGT1-ko mice is the elevated energy expenditure, which is in part due to increased locomotor activity, elevated levels of uncoupling protein 1 (UCP-1) and leptin. What is still not really clear, though, is why DGAT1-deficiency increases energy expenditure. Additionally, post partum female ko mice do not lactate due to missing milk production (50, 51).

4c Glycerol-phosphate acyltransferase (GPAT)

For the glycerol phosphate pathway GPAT and AGPAT, as already mentioned above, are important for the first two esterification steps of G-3-P. At present, three different forms of GPAT are know, two mitochondrial and one microsomal isoform. The first ones are located in the outer mitochondrial membrane (mtGPAT1 and mtGPAT2) and responsible for around 10% of total GPAT activity in most tissues except the liver, where they contribute to around 40% of the total activity. The other one is located in the endoplasmatic reticulum (msGPAT) and seems to be responsible for the remaining GPAT activity.

In 2002, the first mtGPAT-deficient mouse has been produced by Hammond et al. (52). This initial report suggested that these mice gain less weight than their wt littermates, show decreased fat pad mass and have reduced plasma and hepatic TG levels, resulting in lower rate of VLDL secretion. In summary, these results suggested that mtGPAT plays an important role in normal TG synthesis in AT and liver. Three years later, Neschen et al. (53) could not really confirm these data concerning AT development since their mtGPAT-ko mice showed neither reduced weight gain nor less total fat pad mass on a high-fat diet. On a chow diet, weight of mtGPAT-deficient mice was comparable with the controls and whole body fat was marginally reduced. Consequently, mtGPAT seems to have little effect on TG synthesis in AT and the development of obesity.

Recently, the murine and the human microsomal GPAT have been cloned (54) and renamed as GPAT3. GPAT3 is mainly expressed in AT, kidney, small intestine and heart. In mammalian cells overexpression of GPAT3 elevates TG content but not PL content. As expected, GPAT3 is localized at the endoplasmatic reticulum when overexpressed in COS-7 cells. Additionally, this work has shown that during adipocyte differentiation GPAT3 is increasingly expressed and also significantly elevated in mice treated with a PPARy (peroxisome proliferator-activated receptor y) agonist (54). This means that GPAT3 could be the enzyme responsible for the first esterfication step in TG synthesis in AT. But more information will be obtained with the generation of GPAT3-ko mice.

4d Acylglycerol-phosphate acyltransferase (AGPAT)

Acetylation of the sn-2 position of the lysophosphatidate requires AGPAT. Up to now, 7 different AGPAT isoforms have been found, but their physiological functions are not well studied. Recently, Vergnes et al. (55) have generated an AGPAT6-deficient mouse. Normally, AGPAT6 is highly expressed in BAT, WAT and liver. AGPAT6-ko mice show significantly reduced body weight (around 25% reduction compared to wt littermates) and fat pad mass. When challenged with a high-fat diet, AGPAT6-ko mice were resistant to diet-induced obesity. The reduction of fat mass, both on chow and high-fat diet, was reflected by a strong reduction of leptin mRNA and leptin protein levels. When crossed to the genetically obese leptin-deficient OB/OB background, AGPAT6-deficiency also protected these mice from obesity. Additionally, AGPAT6-deficient mice show significantly reduced TG content in the subdermal regions and elevated energy expenditure. Food intake and activity are normal, core temperature at room temperature was also normal and, when challenged with exposure to 4°C, AGPAT6-deficient mice showed the same behavior in maintaining body temperature like their wt littermates. In addition, thermogenesis regulating genes in BAT, such as UCP-1 and PPARy coactivator 1 alpha, and genes for ß-oxidation were not changed in ko mice compared to wt mice. Thus, increased energy expenditure might only be due to reduced subdermal fat layer and concomitant decreased cold insulation to maintain body temperature (55).

In summary, these results show that msGPAT, AGPAT6 and DGAT1 and DGAT2 are very important for the production of TG in AT, whereas mtGPAT seems to be of minor importance.

5 Adipose tissue: hydrolysis of triglycerides

The most important organ for the storage of TG in mammals is the WAT. During fasting or in terms of energy demand, the hydrolysis and the concomitant release of FFA are essential processes for supplying non-adipose organs with substrates for energy conversion (56, 57). FFA can be taken up by skeletal and cardiac muscle, where they are predominantly used for oxidation and energy production. FFA are also delivered to the liver, either for oxidation or for storage as hepatic TG droplets, used afterwards for the synthesis of VLDL and ketone bodies (106).

5a Hormone sensitive lipase (HSL) mediated lipolysis

Until recently, hormone-sensitive lipase (HSL) was thought to be the master enzyme responsible for the hydrolysis of stored TG [II, VII]. HSL is a neutral lipase able to hydrolyze TG, DG, CE and retinyl esters (RE). However, it does not possess phospholipase activity (58-61). Additionally, the enzyme exhibits an about10-fold higher activity against DG, MG and CE than against TG. Although the highest levels of HSL expression are found in WAT and brown adipose tissue (BAT), the enzyme is also expressed in many other tissues, such as muscle (55), macrophages (62), testis (63) and pancreas (64). In WAT, HSL lipolysis is activated by lipolytic agonists, such as catecholamines (ß-adrenergic agonists, isoproterenol, forskolin). Stimulation of adenylyl cyclase activity (65-69), by binding at least one of these agonists to ß-adrenergic receptors, increases intracellular cAMP levels which further activate protein kinase A (PKA) (70). PKA phosphorylates HSL and perilipin, which leads to the translocation of HSL from the cytosol to the lipid droplet and induces a change in the lipid droplet surface allowing HSL to gain access to the lipid surface, where it can start its enzymatic activity. The important role of perilipin in PKA-stimulated hydrolysis has been shown with perilipin-knockout mice (71). These mice showed constitutively fat cell HSL activity that was 300% elevated in subcutaneous fat and even 650% increased in epididymal fat. In

addition, HSL activity in perilipin-ko mice was resistant to ß-adrenergic-stimulation. Perilipin-deficient mice eat more than their wt littermates, but they are leaner, which is reflected by higher muscle mass and about 60% smaller adipocytes. Furthermore, perilipin-knockout mice are resistant to diet-induced and genetic obesity. Thus, perilipin is essential for controlling the access of HSL to the lipid droplet and lipolysis. In contrast, overexpression of perilipin in 3T3-L1 adipocytes and Chinese hamster ovary cells leads to elevated TG storage (72-74).

Some years ago, we and others challenged the rate-limiting function of HSL in the catabolism of WAT triglycerides by studies in HSL-knockout mice (75, 76) [VII]. Surprisingly, HSL-deficient mice showed normal physical appearance. They were nonobese and showed normal WAT mass. Only BAT mass was strongly elevated and displayed increased cell size. Male HSL-deficient mice were infertile due to oligo- and azospermia. In addition, HSL-deficient mice were resistant to diet-induced and genetic obesity (77, 78).

Adipocytes of fasted HSL-deficient mice were isolated and *in vitro* lipolysis was studied, showing that catecholamine-stimulated glycerol release was totally blunted and FFA release was strongly reduced, whereas basal lipolysis was not affected in isolated fat pads of HSL-ko mice (75). Due to reduced lipolysis HSL-deficient mice exhibited strongly increased DG accumulation in WAT, BAT, testis, skeletal and cardiac muscle, which leads to the suggestion that HSL mainly functions as a DG-hydrolase. This suggestion was confirmed measuring TG hydrolase activity in HSL-knockout mice, which was only reduced in WAT (-50%) and in testis (-40%). In contrast, it was essentially unchanged in all other tissues, arguing at least for one other TG lipase. These tissues were analyzed for TG content as well and, as mentioned above, only BAT TG content was elevated, while it was identical to wt mice in WAT and skeletal muscle. Surprisingly, TG content was strongly reduced in cardiac muscle and liver. However, the main energy source for skeletal muscle is the glycolytic degradation of glucose, while the metabolic demand of cardiac muscle is mainly met by plasma derived FFA. With the reduced lipolytic activity in HSL-deficient mice one might expect reduced release of FFA from WAT into the plasma during the fasted state. Accordingly, decreased FFA are expected to result in decreased hepatic VLDL synthesis and other metabolic changes. To test this hypothesis,

we [VII] studied the role of HSL deficiency on the metabolism of plasma lipids and lipoproteins in HSL-ko mice. First, we could show that during fasting, when HSL activity is normally strongly induced, plasma TG and FFA levels were significantly reduced, while TC and HDL-C levels were strongly elevated. In the fed state, when HSL is not relevant, plasma levels of knockout mice were comparable with those of wt mice, except for HDL-C levels, which were again increased. The lipoprotein profile of HSL-ko mice showed that while HDL was elevated, VLDL was strongly reduced. Thus, the lack of HSL-mediated lipolysis leads to reduced FFA transport from the periphery to the liver and to a concomitant decrease in hepatic VLDL synthesis. This could, at least in part, explain the reduced plasma TG levels in HSL-ko mice. Furthermore, the decreased hepatic availability of FFA also explained the reduced liver TG storage and the strongly reduced ketone body concentration in the plasma. Additionally, we could show that tissue-specific LPL activity was strongly upregulated in cardiac and skeletal muscle of HSL-ko mice in the fasted state, whereas it was strongly reduced in BAT. The induction was highest in cardiac muscle, which can be explained by the concept that the heart prefers FFA to glucose, and lowest in BAT, where increased adipose tissue mass might inhibit LPL expression. Thus, the reduced plasma TG level was not only a result of reduced VLDL synthesis but was also caused by elevated LPL activity. This further explains the elevated HDL levels of HSL-deficient mice due to the general concept that LPL mediated lipolysis of CYM and VLDL provides the surface remnants as precursor particles for HDL synthesis (23).

In summary, our data showed that HSL is very important for the hydrolysis of DG. However, concerning the hydrolysis of TG there must be at least one additional TG lipase that can partly compensate for the lack of HSL [VII].

During the last years, apart from us, many other groups were also interested in finding the "triacylglycerol lipase" and therefore many different approaches were applied.

5b Adipose triglyceride lipase (ATGL) mediated lipolysis

We screened gene and protein databases for mammalian proteins that showed structural homologies to known lipases, i.e., the GXSXG motif for serine esterases and for α/β hydrolase folds. We found a couple of fitting candidates that were controlled for TG

hydrolase activity and expression in AT. One of them fulfilled the requirements. We named it “adipose triglyceride lipase” (ATGL) [III].

The murine gene for ATGL (also known as PNPLA2: patatin-like phospholipase domain containing 2) encodes a 486–amino acid protein with a molecular weight of 54 kD. There are two very closely related genes, namely adiponutrin (PNPLA3) and PNPLA5. The human ATGL gene, also designated TTS-2.2, encodes a 504–amino acid protein with 86% identity to the mouse enzyme. The N-terminal regions of both the murine and the human enzyme contain a predicted “esterase of the α/ß hydrolase fold" domain as well as a GXSXG site with a putative active serine (amino acid 47). Moreover, a "patatin" domain (Pfam01734) can be detected in the same region (79). Patatin domain-containing proteins are commonly found in plant storage proteins such as the prototype patatin, an abundant protein of potato tubers (80). These proteins have been shown to have acyl-hydrolase activity on PL, MG, and DG substrates. Patatin-domains are also present in TGL3, a TG-lipase of Saccharomyces cerevisiae (81), and human cytosolic phospholipase A2 (82).

First of all, we studied the mRNA expression profile of ATGL [III]. It was not only highly expressed in WAT but also in BAT. Additionally, ATGL was expressed in cardiac muscle, skeletal muscle, testes and in differentiated 3T3-L1 cells, reaching the highest expression around day 6 after induction of differentiation. Next, ATGL hydrolase activity towards neutral TG, DG and CE was assayed *in vitro* by using cell lysates of COS-7 and HepG2 cells overexpressing ATGL. ATGL showed high TG lipase activity, whereas - in contrast to HSL - ATGL was not able to hydrolyze DG and CE. This was true for murine and human ATGL. These results suggest that ATGL and HSL act coordinately in the breakdown of stored TG, ATGL being mainly responsible for hydrolyzing the first ester bond of the TG to produce FFA and DG, while HSL catalyzes the release of a further FFA from DG [III]. In the last step of TG breakdown, MGL hydrolyzes monoglycerides by producing glycerol and FFA. It is well known that HSL is distributed in the cytosol until hormonal stimuli activate lipolysis, which requires the phosphorylation of HSL, followed by the translocation of the enzyme from the cytosol to the lipid droplet. In order to study the localization of ATGL in the cell, an adenovirus producing histidin-tagged ATGL was produced and 3T3-L1 cells were infected at day 8 of differentiation.

Localization of ATGL was determined using an antibody against the histidin tag. Our results showed that around 50% of ATGL were distributed in the cytoplasma. However, about 10% could be found associated to the lipid droplet. Furthermore, the distribution of ATGL within the cell did not change after hormonal stimulation [III].

To evaluate if ATGL is also able to hydrolyze TG from lipid droplets of differentiated 3T3-L1 cells, we infected the adipocytes with adenoviral constructs transiently expressing ATGL. The overexpression of ATGL led to an increased FFA and glycerol efflux under basal as well as under isoproterenol-stimulated conditions. In contrast, silencing of ATGL by siRNA led to decreased stimulated and unstimulated lipolysis in differentiated 3T3-L1 adipocytes. These results could be confirmed by incubating adipose tissue extracts from mice with a polyclonal antibody against ATGL, which led to a drastic reduction in TG hydrolase activity reflected by reduced glycerol and FFA release. Finally, we could show that ATGL can be phosphorylated, but in contrast to HSL, this post-translational modification is not mediated by PKA [III].

In summary, our findings clearly show that ATGL is responsible for the first step in TG catabolism (III), while HSL, as already suggested in our earlier published work (VII), seems to be much more a DG than a TG hydrolase.

At the same time as we published our findings about ATGL, two other groups (83, 84) demonstrated the importance of this lipase and added some important insights. Villena et al. (83) named the protein "desnutrin" due to its nutritional response. Desnutrin is upregulated by fasting and reduced again after refeeding. Furthermore, they showed that desnutrin mRNA levels are increased in 3T3-L1 adipocytes upon glucocorticoid treatment and strongly reduced in genetically obese mice like the OB/OB (deficient for the "satiety factor" leptin) and the DB/DB mice (deficient for the leptin receptor). Jenkins (84) found the TG lipase in the course of a general analysis of patatin domain-containing proteins and named it calcium-independent phospholipase A2ζ.

Meanwhile, in the human genome, 10 putative, patatin domain-containing proteins are found in databases, four of them closely related to ATGL. Therefore they are systematically named PNPLA1-5 (patatin-like phospholipase domain containing proteins 1-5). Only 4 of the human proteins have murine orthologs. Today, PLPLA4 is unknown

in the mouse (II) (Table 1 of our review exactly shows the list of ATGL related sequences in mouse and human).

ATGL (PNPLA2) shows the greatest - around 40% - sequence homology to adiponutrin (PNPLA3). However, adiponutrin is exclusively expressed in WAT and BAT. Furthermore, Kershaw (85) et al. showed that in contrast to ATGL, adiponutrin is strongly downregulated in fasted mice (and humans), whereas it is significantly upregulated in genetically obese rats. In addition, insulin levels have a strong influence on ATGL (86) and adiponutrin expression. While insulin deficiency led to upregulation of ATGL and insulin replacement reversed this effect in streptozotocin-induced diabetic mice, adiponutrin showed the opposite pattern. Finally, adiponutrin is a transmembrane protein (87) and ATGL is, as reported in our first work, mainly found in the cytosol and attached to the lipid droplet [III]. These results, despite the high sequence homology of these two proteins, support existence of different functions. However, Jenkins et al. (84) found TG lipase activity for the human adiponutrin, a finding that could not be confirmed in our lab for murine adiponutrin. Lake et al. (88) could also show TG lipase activity for human ATGL and adiponutrin, as well as for two other family members, namely GS2 (PNPLA4) and GS2-like protein (PNPLA5). Consistent with our results, they showed that ATGL overexpression led to reduced TG content in cells by increasing FFA and glycerol release. TG content was also significantly decreased in cells transiently expressing GS2 and GS2-like protein but this was not true for adiponutrin. Taken together, these results suggest that there are different functions for ATGL and adiponutrin.

5c Regulation of ATGL

As mentioned above, HSL is tightly regulated by post translational modification. Upon phosphorylation mediated by PKA, HSL translocates from the cytosol to the lipid droplet, gaining its lipase activity. For ATGL we found that it is phosphorylated. However, until now it is not known which kinase is responsibel for this effect. Additionally, ATGL is also located in the cytosol, but a good part of it can be found constitutively attached to the lipid droplet. That implies that the translocation-based activation pathway is unlikely. Therefore, there might be alternative mechanisms that control ATGL activity, such as the

action of co-factors. For some lipases it has been shown that they require co-factors for optimal function. For example, HSL interacts with adipocyte lipid-binding protein and this interaction is necessary for optimal activity (89). LPL requires ApoC-II for activation (90) - otherwise LPL only shows marginal TG lipase activity.

In fact, we recently discovered that CGI-58 is required for efficient ATGL lipase activity [I]. It is important to mention that to that date no human genetic disorders that affect the lipolysis due to mutations in HSL and ATGL had been found. However, a defect in the lipolytic catabolism of stored TG has been discussed in association with a rare autosomal recessive inborn error of neutral lipid metabolism: Chanarin-Dorfman Syndrome (CDS [MIM 27630]) (91, 92). CDS is characterized by ichthyosis, often associated with mild myopathy, liver steatosis, hearing loss, short stature and mild mental retardation. Affected individuals accumulate neutral lipids in multiple tissues, leading to the alternative name "neutral lipid storage disease". Lipids do not accumulate due to increased cellular FFA uptake or TG synthesis rates, though (93). Instead, it was suggested that lipolysis of stored TG might be affected (94). In 2001, Lefevre et al. identified the gene for comparative gene identification 58 (CGI-58, identical to α/β-hydrolase domain-containing protein 5, ABHD5) as causative for CDS, although its role in CDS could not be explained (95). CGI-58 contains α/β-hydrolase folds, making it a member of the esterase /thioesterase/lipase subfamily of proteins. It also contains the esterase/lipase motif GXSXG, where the putative serine is replaced by asparagine (96). Adipose-tissue CGI-58 binds to lipid droplets by interaction with perilipin (97). The gene defects presently known for CDS include truncations, deletions, and point mutations of the CGI-58 gene implying a loss of its function. Our work was based on the premise that CGI-58 acts either as a TG hydrolase or as a cofactor for TG hydrolysis. Our first attempt was the measurement of TG activity in cells overexpressing CGI-58 [I]. But compared to ATGL and HSL, CGI-58 only showed very low lipase activity. The addition of purified CGI-58 to tissue lysates from testes, liver, skeletal muscle, cardiac muscle, BAT and WAT, however, strongly increased their TG lipase activity. This suggested that CGI-58 might be rate limiting for lipolytic activity. Subsequently the question arose, if CGI-58 is required for activation of TG lipases. For that purpose we mixed ATGL or HSL cell lysates with CGI-58 expressing cell extracts and performed TG lipase activity assays.

CGI-58 was able to enhance ATGL activity 20-fold, while it had no influence on HSL mediated hydrolysis.

It is known from CDS that either a truncation or a deletion or a mutation of human CGI-58 is responsible for the disease. To address this question, we generated three different CGI-58 mutations which all failed to activate ATGL. This showed that mutations in the human CGI-58 gene with known associations with CDS result in a complete loss of ATGL-activating function. Finally, the consequences of dysfunctional activation of ATGL by CGI-58 were assessed by comparing the lipolytic process in healthy human skin fibroblasts (HSF) and fibroblasts from a CDS patient (CDS-HSF) whose CGI-58 gene contains a point mutation, and thus, encodes a truncated protein with complete loss of function. ATGL and CGI-58 mRNA levels were found to be comparable in both cell lines. In an attempt to restore lipolysis, HSF from healthy donors and CNS patients were infected with an adenovirus expressing either a control gene, functional CGI-58 or ATGL. Control infected CDS-HSF contained about 5-fold more TG than HSF. Expression of functional CGI-58 resulted in a 51% reduction of the cellular TG content in CDS-HSF, whereas overexpression of ATGL had no effect. Thus, the abnormal accumulation of TG in CDS-HSF can be reversed by the expression of functional CGI-58.

In conclusion, our study showed that ATGL requires CGI-58 as a cofactor/coactivator for breakdown of stored TG. Mutations in CGI-58, as observed in patients affected with CDS, result in dysfunctional lipolysis and cellular lipid accumulation. This suggests that CGI-58 activation of ATGL seems to control cellular TG catabolism and may represent the dominant genetic defect in CDS [I].

No human genetic disorder that affects lipolysis due to mutations of ATGL or HSL had been found before we published this work [I]. At the same time, we found out that CGI-58 is required for optimal ATGL activity, Schoenborn et al. (97) showed an influence of a genetic variation within the human *ATGL* gene on fasting FFA and glucose levels, as well as a risk factor for type2 diabetes. In these patients, the associations with TG levels were less-pronounced. Recently, Fischer et al. (98) could report from a NLSD subgroup that is, in addition to lipid droplet accumulation, characterized by the mutation in both ATGL alleles concomitant with mild myopathy but without ichtyosis.

Together, these results show that ATGL and its co-activator CGI-58 are involved in disorders like NLSD and in pathways of the metabolic syndrome.

5d ATGL-deficient mice

In 2006, Haemmerle et al. generated the ATGL-deficient mouse model (99). These mice had only slightly elevated body mass, whereas measurement of body mass composition showed that fat mass was significantly elevated in these mice. Many tissues of ATGL-ko mice had strongly elevated TG stores, i.e., BAT, heart, skeletal muscle, liver, kidney and testes. Unexpectedly, in WAT the increase in TG storage was significant, but less pronounced than in other tissues. However, consistent with the increased AT mass, the leptin levels were higher in ATGL-ko mice compared to wt littermates. Concerning the cardiac muscle, the TG accumulation in myocytes was around 20-fold increased compared to wt mice and led to cardiac insufficiency and premature death around the age of 4 months. Comparison of basal and isoproterenol stimulated lipolysis of ATGL-ko and wt mice showed that basal release of FFA and glycerol was not affected in WAT, whereas isoproterenol-stimulated lipolysis was reduced by about 75%. Furthermore, the TG hydrolase activities were reduced by more than 80% in WAT and BAT. In several tissues, such as CM, SM, testis and liver, TG lipase activity was decreased between 30-70%. The impaired TG catabolism in BAT caused a severe defect in thermogenesis concomitant with a reduction of UCP-1. Compared to wt mice, plasma levels of fasted ATGL-ko mice showed significantly reduced FFA, ketone bodies, TG and cholesterol levels. The latter two were due to reduced plasma VLDL and HDL levels. Glucose tolerance tests showed an increased glucose tolerance in SM, CM and liver in ATGL-ko mice compared to control mice, which would explain the reduced plasma glucose levels. This might be due to the reduced capacity to mobilize FFA and the concomitant low plasma FFA levels in fasted, as well as fed state. Due to the reduced availability of FFA, in the fasted state ATGL-ko mice showed strongly reduced oxygen consumption indicating reduced energy expenditure. After prolonged fasting, body temperature also dropped compared to their wt littermates. Hence, the results of ATGL-deficient mice confirmed the assumptions made after the *in vitro* experiments: that ATGL is the master enzyme for TG lipase activity. In summary, these mice have strongly elevated TG stores

in several tissues, reduced energy expenditure and oxygen consumption due to reduced mobilization of FFA and die from cardiac insufficiency (99).

6 Functional genomics

6a Introduction

At the beginning of this decade the complete human and mouse genomes were published (100, 101) but their functional annotation is far from complete. Today, only about 50 % of the human genes are annotated and even less for the mouse genome. Over the last decade, the field of functional genomics has emerged rapidly. High throughput techniques for studying the expression and regulation of genes were developed. One of these techniques, namely microarrays, was established in 1995. This technique can be used to study protein expression, alternative splicing, posttranslational modification, such as methylation, single nucleotide polymorphism (SNP), binding of transcription factors to DNA and gene expression, which is the most common field of application of microarrays.

There are two major forms of microarrays for gene expression studies, the single-channel and the dual-channel systems. In our lab, we use the latter, based on the following procedure: DNA probes, either as oligonucleotides or as PCR-products, are spotted onto glass slides with chemically modified surfaces. Each spot contains DNA from one single gene. Our inhouse produced mouse cDNA-microarray slide consists of 27,648 spots representing 16,016 genes. To determine differentially expressed/regulated genes, each slide must be hybridized with DNA from two different samples, i.e. treated and untreated cells, or wt and ko mice.

We used ATGL-deficient mice (99) and their wt littermates at the age of 3 months to perform the microarray experiments. 6 male ATGL-ko mice and 6 wt controls were sacrificed in the fed state and WAT, BAT, cardiac muscle (CM), skeletal muscle (SM), liver and kidney were collected for the isolation of total RNA. RNAs from each two mice were pooled leading to a final number of 3 ATGL-ko and 3 wt samples. 20 µg of total RNA from each sample were reverse transcribed into cDNA and ATGL-ko and wt samples were indirectly labeled with Cy5 and Cy3, respectively. This procedure was repeated with reversed dye assignment. Therefore, we used biological (three pooled

samples) and technical replicates (dye swap) for this experiment. The biological ones are necessary for minimizing the influence of individual variations and the technical ones are important for minimizing the influence of sample preparation, labeling, hybridization and quantification.

Slides were hybridized with pairwise labeled cDNA samples at 42°C over night. Following washing, slides were scanned with a GenePix 4000B microarray scanner (Axon Instruments) at 10 µm resolution. The resulting TIFF images were analyzed with GenePix Pro 4.1 software (Axon Instruments). Data were filtered for low quality spots reflected by low intensity, inhomogeneity and saturated spots. Global median and dye swap normalization using ArrayNorm (102) were performed. Only the genes that were at least two-fold up- or downregulated in at least one of the tissues were chosen for further analysis. Differentially expressed genes were classified according to the GO (gene ontology) (103) considering the biological description for each gene including molecular function, biological process and cellular components. Additionally, using the Pathway Explorer (104) the differentially expressed genes were mapped on pathways from the KEGG database (105). The corresponding protein sequences were annotated *de novo* using 40 academic prediction tools integrated in the ANNOTATOR sequence analysis system. The structure and function were annotated on a sequence segment/domain-wise basis. After extensive literature search and curation using the sequence architecture, gene products were mapped onto known pathways, possible cellular roles, and subcellular localizations (*Figure 3 and 4*), using the PathwayExplorer web service (104) as well as manual literature and domain-based assignment.

In all analyzed tissues, we found 1566 genes (unique ESTs) that were at least two-fold up- or downregulated. Most of them could be found in BAT (1027), followed by the CM with 358 and the WAT with 92 genes. There were still around 60 regulated genes in the SM, whereas only a couple of them could be found in liver and kidney (*Figure 2*).

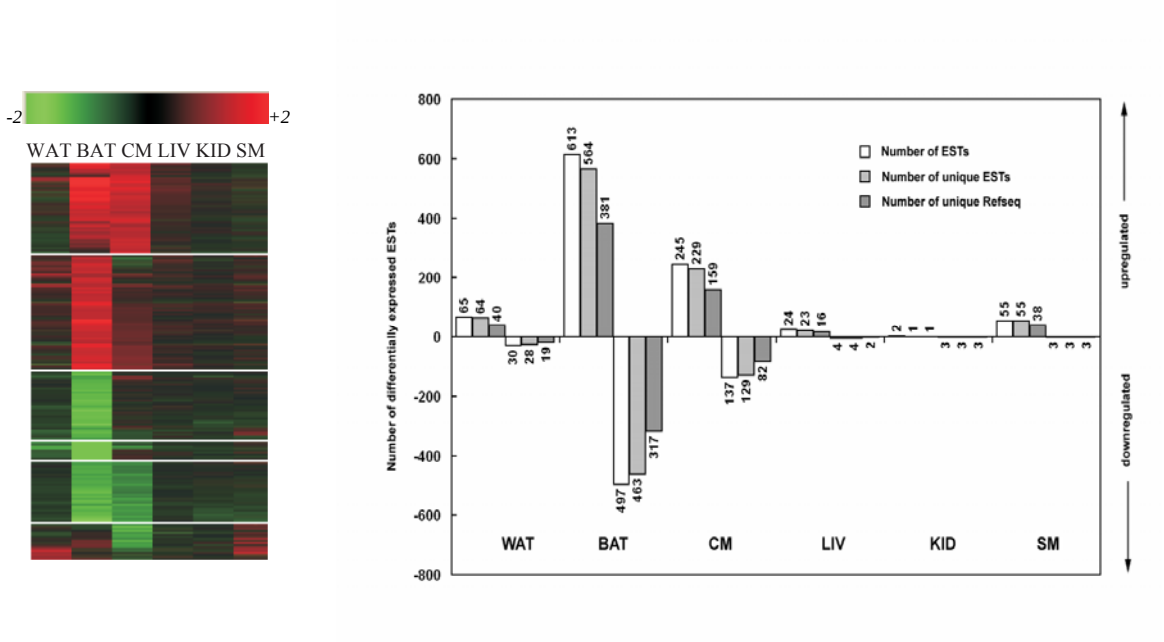

Figure 2. Left: Compact view of clustered gene expression profiles in different tissues of ATGL-ko versus wild-type mice (color coding according to legend at the top).
Right: Number of differentially expressed ESTs and genes (Refseq) in each analyzed tissue based on a two-fold cut-off.

6b Genes regulated in BAT of ATGL-deficient mice

By looking in detail at the differentially expressed genes detected in ATGL-ko BAT (*Figure 3*), we found that many genes involved in ß-oxidation of saturated and unsaturated FFA were significantly down regulated, i.e. fatty-acyl-CoA dehydrogenase, enoyl-CoA hydratase and hydroxyl-acyl-CoA dehydrogenase for the saturated pathway, as well as 2,4-dienoyl-CoA reductase and trans-enoyl-CoA isomerase for the unsaturated pathway. Furthermore, many genes involved in the Krebs cycle were down regulated, such as isocitrate dehydrogenase, succinate-CoA ligase GDP-forming alpha, succinate dehydrogenase and malate dehydrogenase, which catalyze reactions that produce reducing equivalents (NADH, NADPH) and GTP. Thus, these results reflect the reduced acetyl-CoA production due to reduced FFA-oxidation, which could not be compensated by acetyl-CoA production from glucose or amino acids. Following reduced ß-oxidation and decreased flux through the Krebs cycle, one might expect a concomitant down regulation of genes necessary for the respiratory chain/electron transport. *Figure 3* (right upper corner) shows that many genes responsible for the electron transport chain were significantly down regulated, namely NADH dehydrogenase (ubiquinone-Ndu), succinate

dehydroginase (Sdh), ubiquinol cytochrome-c reductase (uqcr), cytochrome-c oxidase (Cox), ATP-synthase (Atp). Due to the reduction in genes involved in the electron transport chain, one might argue for a reduced consumption of molecular oxygen, which is in agreement with the reduced O_2 consumption found in ATGL-deficient mice (99). For these mice it was also shown that the insufficient amount of FFA released as energy substrate for uncoupled mitochondrial respiration led to a severe defect in thermoregulation (99). We did not find any differentially regulated genes involved in thermogenesis and thus could not confirm their data. Taken together, these results show that the reduced lipolysis due to the lack of ATGL leads to reduced ß-oxidation and subsequently to a decreased flux in the Krebs cycle and a strongly reduced electron transport chain action.

Additionally, the BAT of ATGL-ko mice showed reduced expression of the solute carrier family 2, member 8 and member 4 genes (facilitated glucose transporter), which are responsible for glucose uptake. Furthermore, some genes involved in glycolysis were significantly down regulated, such as phosphoglycerate kinase 1 and pyruvate kinase, followed by the significant reduction in the expression of pyruvate dehydrogenase. Therefore, the decreased amount of acetyl-CoA in BAT caused by a reduced production and oxidation of FFA could not be compensated by increased glucose uptake, glycolysis and oxidation of pyruvate to produce acetyl-CoA.

Furthermore, the down regulation of DGAT2, one of the two master enzymes for the last step in TG-synthesis, was very obvious in BAT. DGAT1 and DGAT2 are responsible for esterification of DG to form TG. While mice deficient in DGAT2 die due to missing fat depots, DGAT1-deficient mice survive into adulthood, but show strongly reduced TG storage (49-51). One might expect now that ATGL-ko mice show reduced TG synthesis due to decreased expression of DGAT2. Since in our dataset ATGL-deficient mice showed no reduction of genes involved in the synthesis of DG, one can hypothesize that the produced DG are used for phospholipid synthesis. However, we could not find any differentially regulated genes in PL synthesis. Additionally, acetylCoA carboxylase, the rate limiting enzyme and first step in FFA synthesis to form malonyl CoA, was down regulated in BAT. Taken together, these results suggest that ATGL-ko mice try to

counteract the accumulation of TG in BAT by reducing the expression of lipogenic genes.

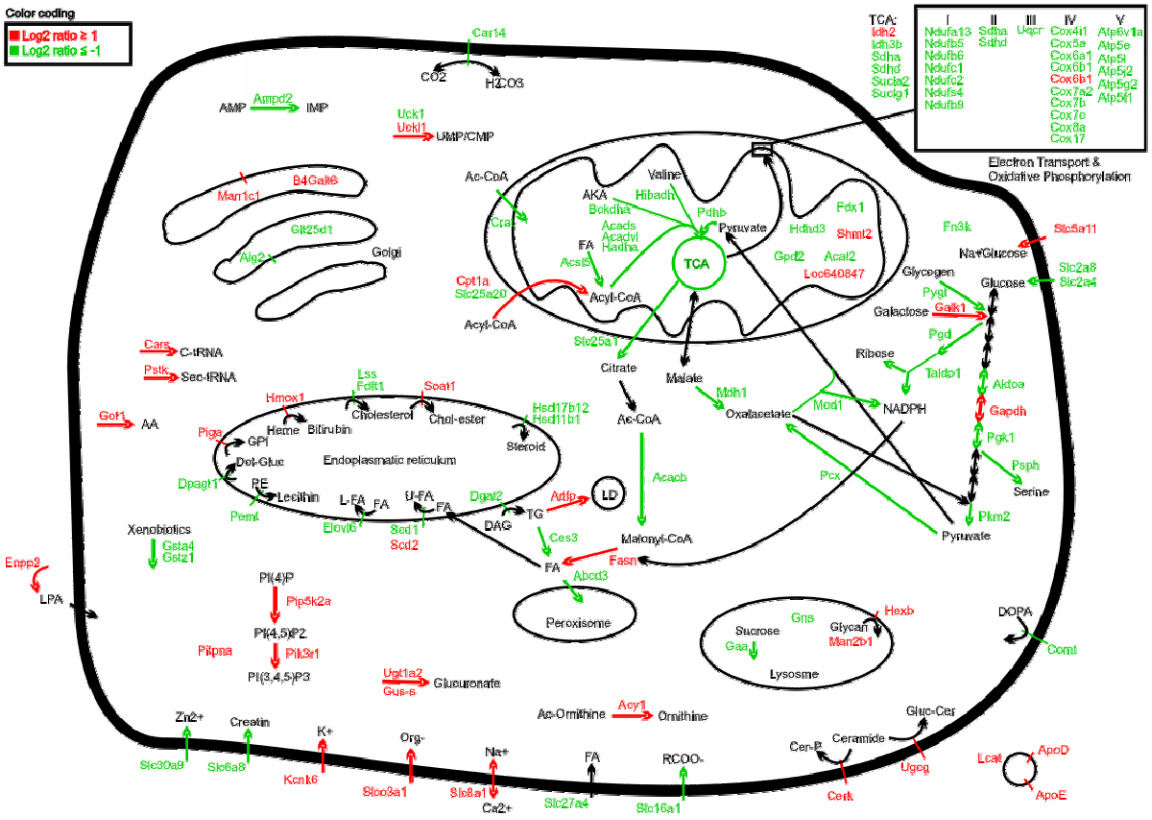

Figure 3. Cellular role and localization of genes up (red) and down (green) regulated in BAT of ATGL-knockout mice compared to wt-mice.
For additional information go to http://genome.tugraz.at/ATGL/

6c Genes regulated in cardiac muscle of ATGL-deficient mice

As mentioned before, around 400 genes were up or down regulated in CM of ATGL-ko mice in the fed state. With reference to *Figure 4* it is obvious that most genes differentially expressed in the CM are regulated the same way as in the BAT (*Figure 3*) of ATGL-deficient mice compared to wt mice. For example, genes involved in ß-oxidation of saturated as well as unsaturated FFA are significantly down regulated in CM. Moreover, many genes of the Krebs cycle and the electron chain transport are also significantly decreased in the cardiac muscle. Glucose transport and pyruvate production are reduced as well in the heart of these mice. Despite the fact that DGAT2 expression is strongly reduced in this tissue, TG accumulation is quite high in ATGL-ko mice and has been described as the cause of premature death in these mice due to cardiac insufficiency

caused by a mechanical contraction defect (99). This shows that a significant reduction in the gene responsible for the last step of TG synthesis is not enough to compensate the missing TG hydrolase activity caused by ATGL deficiency.

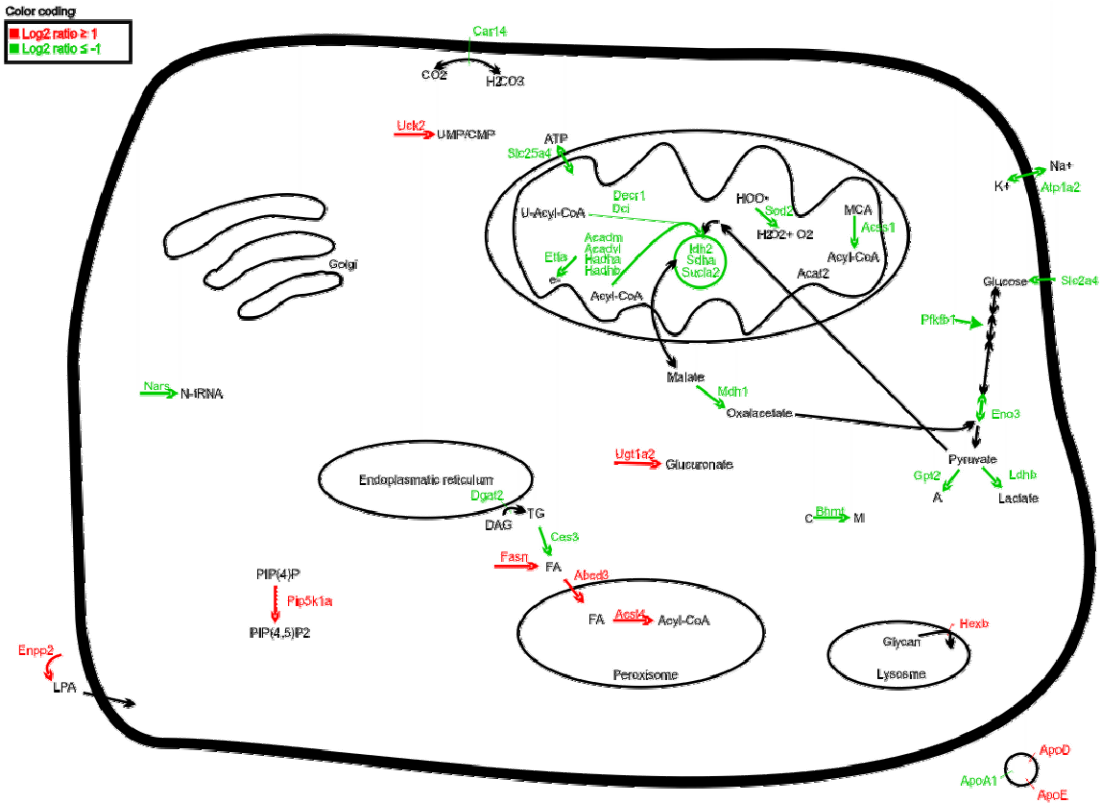

Figure 4. Cellular role and localization of genes up (red) and down (green) regulated in CM of ATGL-knockout mice compared to wt-mice.
For additional information go to http://genome.tugraz.at/ATGL/

6d Genes regulated in WAT of ATGL-deficient mice

When the phenotype of the ATGL-deficient mouse was published in 2006, the finding that these mice were visually hardly distinguishable from their littermates and their WAT mass was only slightly increased was quite unexpected. Looking at the differentially expressed genes of the WAT of ATGL-ko mice compared to their littermates, only a couple of genes were significantly up or down regulated, reflecting the poor phenotype of ATGL-deficient WAT. For example, some genes for ß-oxidation and DGAT2 were significantly down regulated, whereas Krebs cycle and electron chain transport were not significantly regulated. This might suggest that other lipases compensate for the lack of

ATGL activity. But neither HSL nor Ces3 (carboxylesterase 3/TGH) were up regulated. On the contrary, both were down regulated. The reason for this might either be further unknown lipases with TG hydrolase activity in WAT or this tissue has found different compensatory mechanisms to counteract the TG accumulation.

6e Conclusion

In summary, the genes regulated in different tissues of ATGL-deficient mice reflect their tissue-specific phenotype. In BAT and CM, where TG storage was strongly elevated and many physiological defects took place, such as improper thermoregulation and cardiac insufficiency, many genes were deregulated. In contrast only a couple of genes were significantly regulated in WAT, SM, liver and kidney where the tissue specific phenotype was more or less weak.

The next step in my work will be to study the microarray results of ATGL-ko mice in more detail and to compare them with microarray data from HSL-deficient mice. Although both enzymes have TG lipase activity, the phenotypes of the ko mice are quite different. Consequently, genes differentially regulated in these two mouse lines might have a yet unknown key function in the regulatory pathway/metabolism of these lipases.
In addition, genes significantly regulated in ATGL-ko and/or HSL-ko mice with an unknown function will be subjected to further investigation, such as BLAST search, promoter studies, expression in cultured cells and many more to elucidate their functions.

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