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Regulation of Sterol Regulatory Element-Binding Protein-1 by lipolysis products from Adipose Triglyceride Lipase.

by

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1. Abstract

Sterol Regulatory Element Binding Protein (SREBP) transcription factors are master regulators of lipid homeostasis. SREBP-1c is strongly expressed in hepatocytes during high carbohydrate feeding. There, it is part of a fatty acid (FA) sensory complex. When unsaturated (u) FAs are scarce, SREBP-1c is transported to the Golgi apparatus, where it is proteolytically activated. Subsequently, the N-terminal portion is transported to the nucleus where it activates a lipogenic transcriptional program. Hence, it helps to re-establish the lipid equilibrium. During fasting however, FA biosynthesis is stalled. At the same time, plasma FA levels are enhanced through Adipose Triglyceride Lipase (ATGL) activity. ATGL is the rate limiting enzyme of triacylglycerol (TG) hydrolysis from white adipose tissue (WAT). Because ATGL knockout (AKO) mice show a fatal cardiac phenotype we use “heart rescued” AKO mice with cardiac specific transgenic ATGL expression or tissue specific AKO mice.

The finding that ATGL preferentially liberates uFAs from WAT, proposes a link between ATGL activity and SREBP-1c regulation. Therefore, we hypothesized that WAT and liver communicate through lipolytic factors, *i.e.* uFAs, to regulate lipid homeostasis. Remarkably, the potential interplay between ATGL and SREBP-1c has not been studied in detail so far.

Thus, we set out to explore SREBP-1c regulation in livers of three AKO mouse variants. When (i) AKO or (ii) adipose tissue specific AKO mice (AAKO) were starved and subsequently refed a carbohydrate rich diet, we observed enhanced proteolytic SREBP-1c activation and lipogenic target gene expression compared to controls. Because both models showed reduced uFA levels in the fasting plasma we suspected that uFA from ATGL lipolysis commonly blunt proteolytic SREBP-1c activation. In addition to its strong role in WAT, ATGL is important in liver. Mice lacking ATGL only in the liver (ALKO), rapidly develop steatosis. (iii) To test if ALKO mice also show abnormal SREBP-1c regulation we exposed them to a similar fasting / refeeding regimen. Again, we recorded SREBP-1c hyper activation in the knockout group compared to controls however, to a slightly lesser extent. To confirm that intracellular ATGL mediated lipolysis is needed to suppress SREBP-1c processing we employed primary mouse hepatocytes. When control hepatocytes were treated with the ATGL inhibitor Atglistatin, SREBP-1c processing was blunted. ALKO hepatocytes however, showed strong SREBP-1c processing under all circumstances.

Therefore, we propose the following scenario. During fasting ATGL driven lipolysis liberates uFAs from WAT that accumulate in the liver. There, they directly blunt SREBP-1c processing at the ER membrane. Furthermore, they are re-esterified and bound in lipid droplets (LD). These LD are subsequently degraded by liver ATGL when needed. This resulting uFAs additionally suppress SREBP-1c proteolytic activation. Currently, we use primary hepatocytes to investigate molecular details of this interesting facet of SREBP regulation. Overall, our findings have the potential to explain an important aspect of the interplay between lipolysis and lipogenesis.

2. Introduction

Cholesterol is an essential component of the plasma membrane in animals. In the plasma membrane sterols maintain the barrier function between cells. Furthermore, the amount of cholesterol modulates the membrane fluidity. Moreover, cholesterol is converted into steroid hormones and bile salts [1, 2]. Cholesterol originates from two main sources, the biosynthesis in the liver and the fat in the food. In mammalians cholesterol is packed together with proteins into particles which are called lipoproteins. These lipoproteins are distributed in the body to fuel cells [1, 3]. The cholesterol biosynthetic pathway was the first discovered anabolic pathway which is regulated by end-product feedback suppression. The initial experiments showed that mice which had cholesterol in their diet synthesized less cholesterol then the control mice [4]. The importance of the cholesterol in belong to human health was shown later, cholesterol rich diets raise blood cholesterol levels in patients and cause heart attacks [5]. In the 1950's and 1960's, two kinds of lipoproteins were described: Low-density lipoprotein (LDL) and high-density lipoprotein (HDL). Elevated concentrations of LDL lead to heart attacks; however elevated amounts of HDL have a protective function. These lipoproteins and therefore the cholesterol can be taken up by the cells via LDL receptor. To protect the cells, high cholesterol diets reduce the liver uptake of cholesterol by suppressing production of receptors for LDL [6]. This uptake of cholesterol via the LDL receptor was discovered by Brown and Goldstein 1973. Brown and Goldstein demonstrated the receptor mediated internalization of LDL in lysosomes and the following suppression of the HMG CoA reductase activity. Since this discovery new drugs could be developed to prevent heart attacks and Brown and Goldstein got the Nobel Prize in physiology or medicine in 1985 [1, 7].

To maintained cholesterol levels at constant amount, a strict regulation is necessary. Therefore the level of cholesterol in cell membranes has to be sensed and modulated. This modulation occurs by the transcription of genes encoding enzymes of cholesterol biosynthesis and uptake from plasma lipoproteins. This factors which maintain lipid homeostasis are called Sterol Regulatory Element Binding Proteins (SREBPs) [2, 8]. They are translated as inactive precursors into the ER membrane (P-SREBP) where they are localized within a multi enzyme lipid sensory complex including Insulin Induced Gene 1 (INSIG-1) and SREBP Cleavage Activating Protein (SCAP). The NH2-terminal and the COOH-terminal domains of SREBPs project into the cytosol. The central domain is anchored to ER membranes by a two membrane-spanning sequence, separated by a short loop that projects into the lumen of the ER (Figure 1). The NH2-terminus domains belongs to the basic-loop-helix-leucine zipper family and act as a transcription factors. This part of the SREBP protein binds to enhancer sequences in the promoters of lipogenic genes, called sterol response elements or (SREs) [9-11].

Subsequent to the transcription of SREBP, the protein is bound to SCAP. In the presence of unsaturated fatty acids (uFA) and sterols, SCAP interacts with INSIG-1 and therefore the INSIG-1-SCAP-SREBP complex is stabilized in the ER membrane [12, 13]. In the presence of saturated fatty acids (sFA) or in the absence of uFA and sterols, SCAP undergoes a conformational change and both, SCAP and SREBP are packed into Coat Protein Complex II (COPII) vesicles [14]. For COPII binding Sar1, a small GTP binding protein that attaches to ER membranes upon exchanging GTP for GDP is required. When Sar1 is bound to the membrane, Sec23/24 is recruited and Sec24 component can bind to SCAP. Next Sec23 recruits other proteins to form the coat of the budding vesicle. Sec24 binds to a hexapeptide sequence from the SCAP protein, MELADL, located in the cytoplasmic loop. The crucial MELADL sequence is located in the transmembrane area between helices 6 and 7. Mutations in this region abolish Sec23/24 binding. Therefore, SCAP cannot exit the ER [15]. If sterols are present they bind to SCAP, which leads to a confirmation change of the protein

and therefore Sec24 cannot recognize and bind the MELADL sequence. Under these high sterol conditions SCAP is bound to INSIG [14, 16]. Interestingly, INSIGs does not directly bind to cholesterol, instead it interacts with oxysterols such as 25-hydroxycholesterol (25-HC). 25-HC also potently inhibits cleavage of SREBPs [17]. However, oxysterol do not bind SCAP, or induce the conformational change. Nevertheless, both molecules lead to the same result, the ER retention of the INSIG-SCAP-SREBP complex [18-20]. There are two INSIG isoforms, which are both extremely hydrophobic. Through topology studies we get to know that the protein consists of six transmembrane helices separated by short hydrophilic loops [21]. At the NH2 and COOH terminus INSIG project to the cytoplasm. These cytoplasmic sequences contain the major differences between the INSIG isoforms. Both, INSIG-1 and INSIG-2 bind to SCAP if cholesterol is available. This binding always leads to a stabilization of the INSIG-SCAP-SREBP complex in the ER membrane [22, 23]. The major difference between INSIG-1 and INSIG-2 is there expression. INSIG-1 is a SREBP target gene and therefore INSIG-1 transcription just takes place when cleaved SREBP is present in the nucleus. Therefore, INSIG-1 mRNA rises at low cholesterol levels but the protein is immediately ubiquitinated and degraded. The INSIG-1 SCAP binding inhibits the ubiquitination and increases its half-life from 30 min up to 2 h [24, 25]. INSIG-2 is always expressed at a low but constitutive level and not regulated by SREBPs [23]. INSIG-1 stability is not only dependent on sterol levels in the cell. Unsaturated fatty acids also inhibit INSIG-1 degradation. INSIG-1 binds to the ubiquitin like domain containing protein 8 (UBXD8), a protein that recruits the p97 complex. Recruitment of p97 to INSIG-1 protein and gp78 binding, an E3 ubiquitin ligase that attaches polyubiquitin chains to proteins, leads to recognition and subsequent degradation of INSIG-1 by proteasomes. When sterols are present INSIG-1-SCAP binding prevents the binding of gp78. Interestingly Under these conditions UBXD8/p97 complex can bind but due to the lack of ubiquitination via gp78 INSIG-1 is not degraded. Unsaturated fatty acids do not block the ubiquitination, but the recruitment of p97. Inasmuch both processes are necessary either sterols or uFA can block the degradation of INSIG-1 [10, 12, 26].

At low cholesterol and uFA levels, when INSIG-1 is degraded the SCAP-SREBP-1 complex is transported to the Golgi apparatus, where the SREBP protein is processed from side 1 protease (S1P) and the side 2 protease (S2P). Cleavage by S1P separates SREBPs into two membrane bound parts. Just the NH2 terminal part is then cleaved by S2P [14, 16]. This cleavage does not occur until S1P mediated proteolysis removes the luminal portion of SREBP [27]. This processing set free the N-terminal part of the protein (N-SREBP). This as a transcription factor active part is subsequently imported into the nucleus where it activates the transcription of its target genes. Target genes include all important fatty acid synthesis genes like Fatty Acid Synthase (FASN) or Acetyl CoA Carboxylase (ACC-1) but also genes which regulate SREBPs, like INSIG-1 and SREBP itself. Proteolytic SREBP regulation helps to keep the cellular lipid equilibrium as described in Figure 1 [8].

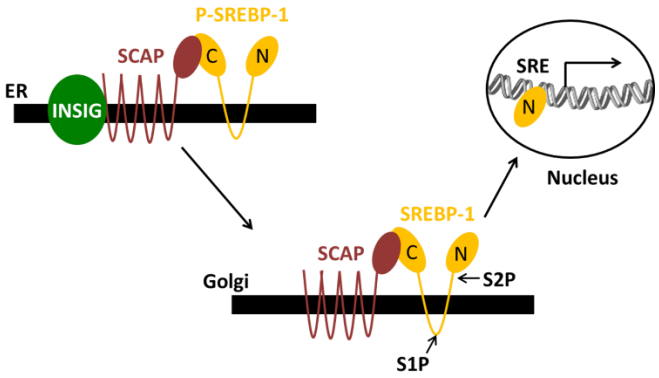

Figure 1: SREBP regulation. When cellular lipids are low, the ER anchor protein INSIG-1 is degraded. As a result, SREBPs are transported to the Golgi apparatus by their chaperon SCAP, where proteolytic processing by S1P and S2P occurs. The active N-SREBP fragments subsequently enter the nucleus to activate sterol regulatory element (SRE) sequence containing promoters that drive genes for lipid synthesis. If lipids are abundant, INSIG-1 is stabilized and restrains SREBPs in their inactive state in the ER. This convergent feedback regulates cellular lipid equilibrium [8, 13, 14, 16].

The mammalian genome encodes three SREBP isoforms; SREBP-1a is a potent activator of all SREBP target genes, including synthesis of sterols, FA, and triglycerides (TG). The function of SREBP-1c and SREBP-2 are more specific. SREBP-1c preferentially enhances transcription of genes required for FA synthesis. SREBP-2 favourably activates cholesterol synthesis and uptake genes. In cell culture SREBP-1a and SREBP-2 are the predominant isoforms, whereas in liver SREBP-1c and SREBP-2 are leading. In general, all the three SREBP isoforms can activate all known cholesterol and FA synthetic SREBP target genes [28].

We are especially interested in the effects of the lipid metabolism in liver and it´s crosstalk with adipose tissue. Therefore, we focus on SREBP-1c in our mouse and hepatocyte study. A lot of studies dissected the function and regulation of SREBPs and their different isoforms. So far there is no study which analyses where the FAs which regulate SREBP come from. Do understand the function of our metabolism in a new way we want to address this question. One way to generate FAs is lipolysis which describes the hydrolysis of TGs. This process from TGs to FAs and glycerol, requires three steps that involve three different enzymes: Adipose Triglyceride Lipase (ATGL) which catalyses the initial step of lipolysis, converting TGs to diacylglycerols (DGs), Hormone Sensitive Lipase (HSL), is mainly responsible for the hydrolysis of DGs to monoacylglycerols (MGs) and MonoacylGlycerol Lipase (MGL) which hydrolyses MGs (Figure 2) and yields FA and glycerol [29].

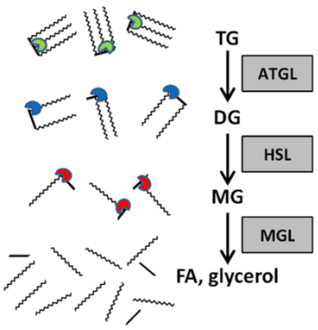

Figure 2: ATGL, the key enzyme in lipolysis. ATGL hydrolyses the first FA from TGs and therefore generated DGs. DGs are further processed by HSL. The last step in lipolysis is the release of FA by MGL. Biochemically, ATGL prefers hydrolysis of uFA from the sn-2 position of the glycerol backbone [29-31].

ATGL was identified as key enzyme for intracellular lipolysis in 2004 by three independent groups [30, 32, 33]. ATGL hydrolyses TGs preferentially at the sn-2 position of the glycerol backbone. The catalytic site of ATGL consists of serine 47 and aspartate 166 located within the patatin domain at the N-terminus [7]. The C-terminal part contains a hydrophobic lipid droplet (LD) binding region [8]. This binding of ATGL to TG-rich intracellular LDs is influenced by mutations within the C-terminus [34].

ATGL is highly expressed in white and brown adipose tissue but also at lower levels in several non-adipose tissues like skeletal muscle, liver, heart, testes, lung, retina immune cells, pancreas and small intestine [35]. Transcriptional regulation of ATGL occurs via peroxisome proliferator-activated receptor gamma (PPAR-γ). ATGL mRNA expression is induced by specificity protein 1 which interacts with the ATGL promoter [36]. ATGL transcription levels are reduced by fat specific protein 27 and insulin. These two factors modulate the binding of early growth response protein 1 to the promoter [37, 38]. During fasting conditions forkhead box protein O1 (FoxO1) and interferon regulatory factor 4 bind to the ATGL promotor and therefore, increase the mRNA and protein levels of ATGL [37, 39]. In contrast to these findings, β-adrenergic stimulation reduces transcript levels of ATGL, but its activity is massively increased. This highlights the significance of post-transcriptional regulation of lipolysis [30].

Post-transcriptionally, ATGL is mainly regulated by phosphorylation and protein interactions. There are several phosphorylation sites at, Thr101, Thr210, Thr372, Tyr378, Ser87, Ser393, Ser406, and Ser430 [40, 41]. ATGL phosphorylation at Ser406 by AMP-activated protein kinase (AMPK) or by protein kinase A (PKA) leads to increased ATGL activity [42, 43]. For full hydrolase activity ATGL requires a coactivator protein, comparative gene identification-58 (CGI-58). When nutrients are available perilipin-1 interacts with CGI-58 and thus prevents binding to ATGL in white and brown adipocytes. During fasting upon β-adrenergic stimulation, PKA phosphorylates perilipin-1 at multiple sites, causing the release of CGI-58 and a significant increase of ATGL activity [44]. Furthermore, ATGL promotor activity and therefore mRNA levels are increased during fasting [39]. In non-adipose tissues with high FA oxidation rates, like liver or muscle, ATGL activation is different. There, perilipin-5 recruits ATGL and CGI-58 to lipid droplets by direct binding [45]. During feeding, when CGI-58 levels are low, G0G1 switch protein 2 (G0S2) increases. This protein was originally identified to act at the G0 to G1 transition of the cell cycle. Later it was recognized that G0S2 is also a specific inhibitor for ATGL [46]. G0S2 expression is very low in adipose tissue during fasting but increases after feeding and thereby inhibits ATGL activity. Expression of G0S2 is low in adipose during fasting but interestingly it is high in oxidative tissues like liver [47]. Furthermore, ATGL targeted for proteasomal degradation by ubiquitination and therefore also regulated by protein stability [48, 49].

The balance between energy uptake and expenditure is important for all organisms. If an energy surplus exists, the energy is converted into fatty acids which are esterified to glycerol and furthermore stored as triglycerides. When energy expenditure is higher than the caloric intake this stored TGs are hydrolysed and FA are released, in a process called lipolysis [35].

The most FAs which are set free during this process are unsaturated ones [50]. It was recently published that uFA block the transport from P-SREBP to the nucleus [13].

One factor which plays an important role in the regulation of SREBP-1c and ATGL is insulin. Insulin activates SREBP-1c transcription via liver X receptor (LXR) and increases therefore the amount of P-SREBP-1c. High insulin levels occur especially when mammalians eat a lot of sugar. Under these conditions the body wants to store the excess energy as TGs. Therefore SREBP-1c activates the FA pathway target genes to produce TGs in the liver [51]. Under this condition ATGL is inactive in WAT because of the high insulin levels. Furthermore, it would be a big waste of energy if stored TGs would be used under these conditions. Under fasting conditions however, insulin levels are very low and therefore SREBP-1c transcription is reduced but ATGL activity is increased [35, 52]. We are interested in physiological conditions which are often a mixture between fasting and feeding. In this state there should be an activation of both SREBP-1c and ATGL. Therefore, we used fasting and refeeding protocols to study the transitional state between high ATGL activity in the WAT and high SREBP-1c activity in the liver.

3. Own results acquired before the research stay

Mice are mainly active during the night. During this time they have the highest food intake. Through daytime they show an intermediate state between fasting and feeding. Both, night-time and daytime with high- and relatively low- food intake, respectively, contribute to the overall metabolism of the animals. The situation in humans is of course vice versa but apart from that it is quite comparable. ATGL is activated under fasting conditions when it fuels the organism with energy by cleaving off FAs from TGs at LD of the WAT. At the same time however, liver SREBP-1c levels are relatively low. The first meal after a fasting period decreases ATGL levels and increases SREBP-1c [35, 51].

To understand the interplay between ATGL and SREBP-1c we established a fasting refeeding protocol that was originally established in the Brown and Goldstein laboratories [51]. Therefore, we used three groups of mice that were treated in different ways. The first group was fed a normal chow diet. This yields relatively high insulin levels over the time. These mice were sacrificed in the morning and livers were resected. The second group of mice was fasted overnight, sacrificed in the morning and livers were resected. During fasting insulin levels are very low and therefore SREBP-1c signalling is reduced [51]. The third group of mice was fasted overnight and then refed a high carbohydrate diet the next morning. Fasting is necessary to synchronize the metabolism and increase ATGL activity during night time. This increases the release of FAs to a high extent. Refeeding a high carbohydrate diet leads to a massive increase in insulin levels and therefore to a huge amount of Srebp-1c transcription and subsequently to high P-SREBP-1c and N-SREBP-1c abundance in the liver (Figure 3) [51].

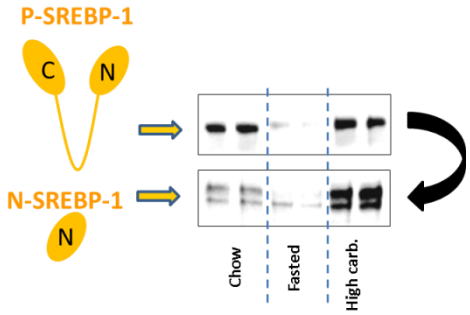

Figure 3: SREBP-1c regulation under differed feeding conditions. C57Bl6 mice were either fed a normal chow diet, fasted over night or fasted overnight and then refeed in the morning with a high carbohydrate diet. Mice were sacrificed and membrane or nuclear extracts were prepared from the livers. 2 mice per condition are shown.

We believe that FAs that are set free by ATGL during fasting dampen the proteolytic activation step of P-SREBP-1c during the refeeding period. This means that the already high abundance of active nuclear N-SREBP-1c should even be higher during re-feeding when ATGL is not present. After establishing this feeding / fasting / refeeding protocol we therefore used genetically engineered mice to analyse the crosstalk between ATGL and SREBP-1c. During fasting, ATGL hydrolyses fat for continuous energy supply of the body. Consequently, FAs are released into the bloodstream and are available for the liver [30, 53]. To test if these FAs regulate SREBP-1c we used heart rescued ATGL knockout mice (AKO) because the complete knockout of ATGL in the whole body leads to a severe cardiac phenotype. With this AKO mice we performed the established feeding / fasting / refeeding regimen that we described in figure 3, above, to analyse SREBP-1c activation [3, 51]. Indeed, during the refeeding process we observed strongly increased N-SREBP-1c signals as compared to the fasted state. Interestingly, the increase in AKO mice was significantly higher than the

increase in control mice. As P-SREBP-1c levels in the control and knockout groups were fairly comparable during re-feeding, this seemed to confirm our theory that ATGL lipolysis dampens the generation of active nuclear N-SREBP-1c from P-SREBP-1c (Figure 4, A). Furthermore, we could observe a significantly higher expression of SREBP-1c target genes in livers of the animals from the knockout group compared to the controls, during refeeding. In Figure 4, B the qPCR analysis from Acetyl-CoA Carboxylase-1 (ACC-1) and Fatty Acid Synthase (FASN) are shown.

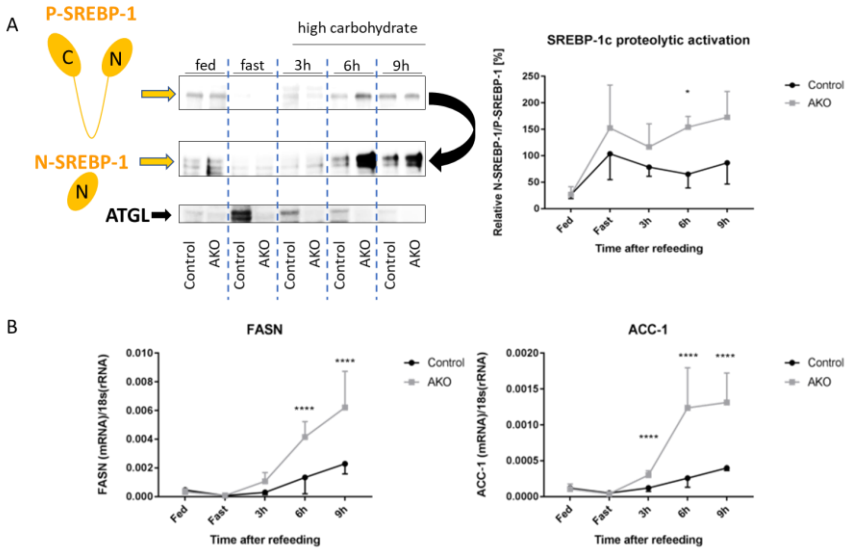

Figure 4: ATGL knockout mice show an increased SREBP-1c expression after carbohydrate refeeding. AKO mice and control mice were fed a normal chow diet, fasted overnight or fasted overnight and refeed with a high carbohydrate diet. Mice were sacrificed at indicated time points after refeeding. (**A**) Immunoblot of SREBP-1c liver extracts. Membrane or nuclear extracts were isolated. For that western blot extracts from 3 mice were pooled for each time point. (**B**) qPCR analysis was performed independently from livers of each animal. N=3/group.

After observing this interesting effect on SREBP-1c levels upon refeeding we decided to investigate if this effect was dependent on ATGL activity in white adipose tissue. Therefore, we applied our established feeding, fasting and refeeding regimen (see figure 4) with adipose-tissue specific ATGL knockout mice (AAKO). Hence, we set out to analyse if lipolysis products liberated by ATGL in adipose tissue are essential to dampen an otherwise hyper activated P-SREBP-1c proteolytic activation as seen in AKO animals in figure 4. Remarkably, we could observe a relatively similar P-SREBP-1c cleavage phenotype under refeeding in AAKO mice (Figure 5, A) when compared to AKO. As expected, plasma non esterified fatty acids (NEFAs) were elevated in control but not in AAKO mice upon fasting (Figure 5, B). Therefore, we hypothesized that SREBP-1c is hyper activated by the lack of FAs in the plasma of AAKO after fasting. During this experiment we furthermore analysed plasma FA levels. Interestingly, our analyses show that plasma NEFAs are high after starving and then slowly return to normal levels during high carbohydrate diet (HCD) refeeding (Figure 5, B). It was therefore, not clear why the strongest effect on P-SREBP-1c cleavage in mice lacking ATGL in most parts of the body (AKO) or in WAT (AAKO) is seen around six hours post HCD re-feeding on protein levels. Also mRNAs of SREBP-1c target genes increase over time (Figure 5, C). One explanation is that FAs from the circulation are deposited as TG in LDs of hepatocytes during fasting [35].

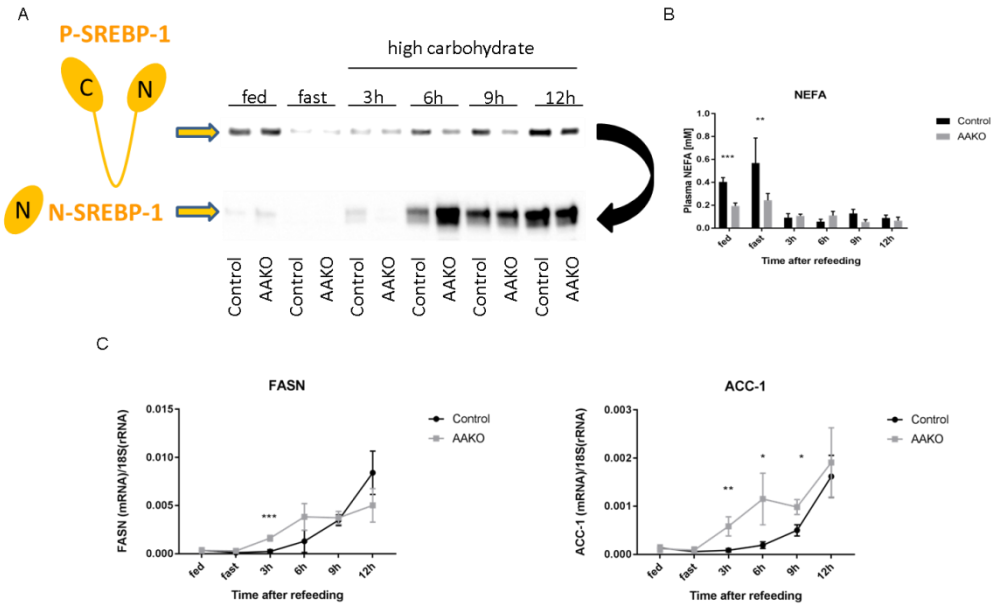

Figure 5: ATGL adipose tissue knockout mice show an increased SREBP-1c expression after carbohydrate refeeding. Mice were fed a normal chow diet, fasted overnight or fasted overnight and refeed with a high carbohydrate diet. Mice were sacrificed at indicated time points after refeeding. (**A**) Immunoblot of SREBP-1c liver extracts. Membrane or nuclear extracts were isolated. For that western blot extracts from 3 mice were pooled for each time point. (**B**) NEFA levels were analysed from the animals used in (A). (**C**) qPCR from AAKO mice after feeding, fasting and HCD refeeding. N=3/group.

Interestingly, ATGL is not just an important lipase in fat but also in liver [54]. We, hence, believe that liver LDs that accumulate during fasting are later hydrolysed during refeeding by ATGL in the liver. This would theoretically yield a cellular pool of FAs that should be capable of triggering the observed P-SREBP-1c cleavage effects [50, 54]. To test this hypothesis we therefore, decided to analyse liver specific ATGL knockout animals (ALKOs) [55]. Interestingly though, we recorded mildly enhanced amounts of N-SREBP-1c in liver nuclei of ALKO mice post carbohydrate refeeding (Figure 6, A). We analysed plasma NEFA levels of ALKO mice during our established feeding / fasting / refeeding protocol. As expected, we could not observe any difference in plasma NEFA levels between genotypes here because ATGL is solely lacking in the liver (Figure 6, B). This finding suggests that lipolytic activity of ATGL in liver is also important during HCD refeeding. We suggest that there are less uFAs liberated under such conditions in hepatocytes lacking ATGL just in the liver. As a result, lower uFA levels suppress P-SREBP-1c cleavage in a milder way then in AKOs or AAKOs. This is reflected in the SREBP-1c target gene activation (Figure 6, C).

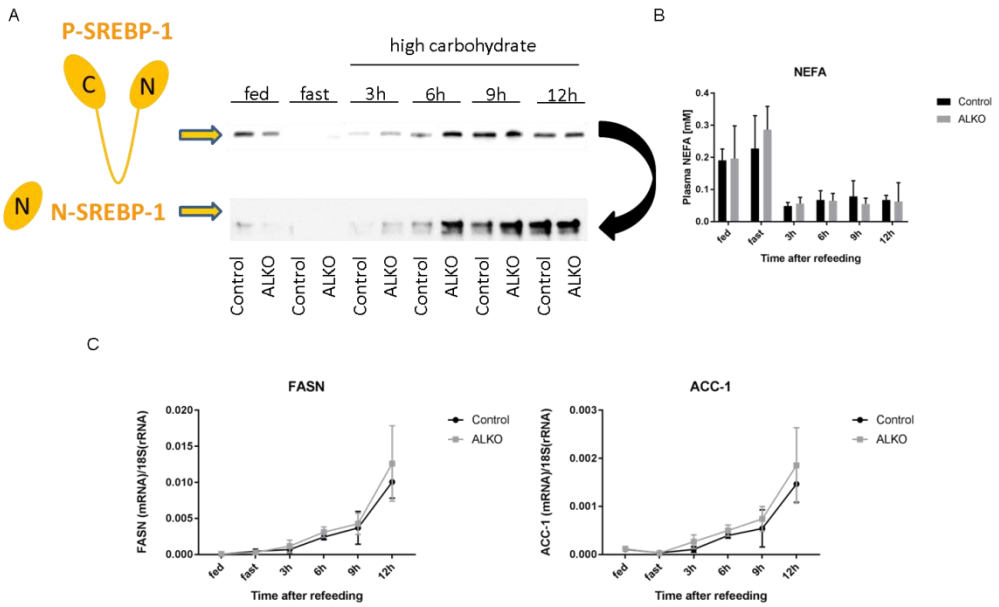

Figure 6: ATGL liver tissue knockout mice show an increased SREBP-1c expression after carbohydrate refeeding. Mice were fed a normal chow diet, fasted overnight or fasted overnight and refeed with a high carbohydrate diet. Mice were sacrificed at indicated time points after refeeding. (**A**) Immunoblot of SREBP-1c liver extracts. Membrane or nuclear extracts were isolated. For that western blot extracts from 3 mice were pooled for each time point. (**B**) NEFA levels were analysed from the animals used in (A) for the western blot. **C**) qPCR from SREBP-1c target genes in ALKO mice after feeding, fasting and HCD refeeding. N=3/group.

To investigate this proposed mechanism directly in liver cells, we isolated primary hepatocytes. To stimulate intracellular lipolysis we used Forskolin that activates HSL and ATGL by increasing cyclic AMP (cAMP) levels in the cell [31, 35, 56]. To specifically block the activity of ATGL we used the ATGL inhibitor Atglistatin. As previously reported Atglistatin inhibits lipolysis in mice for 8 h after admission. Therefore, Atglistatin treatment significantly reduced plasma FA, cholesterol and TG levels in mice [56, 57]. It is also well known that Atglistatin inhibits ATGL activity in mouse cell lines [31, 56, 57]. Consequently, primary mouse hepatocytes where either treated with Forskolin or Forskolin and Atglistatin at the same time to either activate lipolysis or activate lipolysis while specifically blocking ATGL. As a result, we observed hyper activation of SREBP-1c in primary hepatocytes when lipolysis was activated but ATGL was suppressed as compared to activated lipolysis only. Moreover, genetic abrogation of ATGL, as in primary hepatocytes isolated from ALKO mice, similarly led to SREBP-1c hyper activation (Figure 7). Consistently, and in contrast to control hepatocytes, ALKO hepatocytes did not show any change of active N-SREBP-1c levels after Atglistatin treatment, see figure 7. This strengthens the hypothesis that ATGL mediated hydrolysis of TG stored in hepatocyte lipid droplets is instrumental for SREBP-1c regulation.

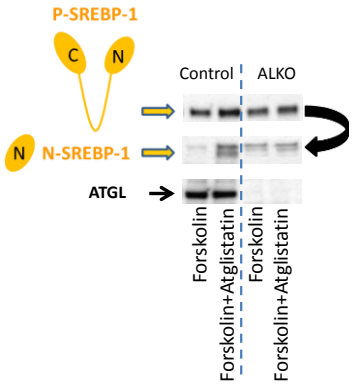

Figure 7: Primary hepatocytes show SREBP-1c hyper activation post ATGL inhibition. Control or ALKO primary hepatocytes were isolated and cultured in 20% fetal bovine serum medium overnight. Subsequently, they were treated with Forskolin or Forskolin and Atglistatin in medium containing 5% lipoprotein deficient serum. 16 h post treatment nuclear and membrane fractions were prepared, and immunoblotting was performed.

On the basis of these findings we defined our further working hypothesis:

SREBP-1c is proteolytic activated via FAs generated by ATGL mediated lipolysis.

We have found that knockout of ATGL in mice and inhibition or knockout of ATGL in isolated mouse hepatocytes suppresses accumulation of N-SREBP-1c. We believe that this happens because the proteolytic activation of P-SREBP-1c is blunted. The classical proteolytic activation of P-SREBP-1c was described in the introduction in great detail. To analyse if ATGL activity affects the classical SREBP cleavage pathway, that was described by the Goldstein and Brown laboratories [8], I joined the lab from Prof. Dr. Peter Espenshade at the Johns Hopkins University in Baltimore, USA. I was able to perform this laboratory exchange visit through the great support of the Marshall Plan scholarship. Dr. Espenshade worked as a post-doctoral fellow in the Brown and Goldstein laboratory. Profs. Brown and Goldstein won the Nobel Prize for Physiology or Medicine in 1985 for their discoveries concerning the regulation of cholesterol metabolism. The Brown and Goldstein laboratory deciphered the mechanism of SREBP regulation during the last few decades in great detail. Dr. Espenshade specifically investigated the mechanism through which SREBP is transported from the ER to the Golgi and furthermore, how the recycling and processing of SREBP works [14, 16]. Therefore, he accumulated great knowledge on the regulatory mechanism of SREBP. To improve the scientific impact of my PhD project, we decided to start a collaboration with him and I eventually got the opportunity to join is great research laboratory at the Johns Hopkins University School of Medicine in Baltimore, USA. Thus, not only my PhD project greatly benefitted from the research stay, but we could additionally start research collaboration on “the regulation of SREBP factors by lipolytic enzymes” between Dr. Espenshade’s laboratory and our research group of Prof. Hoefler at the Medical University in Graz, Austria. Prof. Dr. Espenshade helped me a lot with my project and supported me with his great ideas. Also, all lab members shared their knowledge and their hands-on laboratory experience with me. This enabled me to learn several new techniques and skills. I am very thankful that I could

perform my research stay in such a great environment. The following results describe my research work during this research stay.

4. Materials and Methods

a. Cell culture media

D10F: DMEM containing 100 units/ml penicillin and 100 mg/ml streptomycin supplemented with 10% (v/v) fetal bovine serum (FBS)
Hepatocyte medium: DMEM containing 100 units/ml penicillin and 100 mg/ml streptomycin, 20% FBS, 100 nM Dexamethason, 1x ITS-Supplement
Medium A: DMEM containing 100 units/ml penicillin and 100 mg/ml streptomycin and 5 % Lipoprotein deficient serum (LPDS)
Medium B: Medium A with 50 µM compactin and 50 µM sodium mevalonate

b. Hepatocyte isolation

Primary hepatocytes were isolated by perfusion of the livers from mice with 40 ml perfusion buffer (5.5 mM KCl, 0.1% Glucose, 2.1 g/L $NaHCO_3$, 700 µM EDTA, 10 mM Hepes and 150 mM NaCl). After 20 minutes the buffer is changed to 50 ml collagenase buffer (5.5 mM KCl, 0.1% Glucose, 2.1 g/L $NaHCO_3$, 10 mM Hepes and 150 mM NaCl, 3.5 mM $CaCl_2$, 1% BSA, 500 µg/mL Collagenase Type I about 300 U/mg). Livers were perfused with a perfusion rate of 2 ml/min. Afterwards livers are dissociated with the plunger part of a syringe in 10 mL of D10F. This homogenate was pipetted onto 100 µM strainer and flow through was collected. Hepatocytes were centrifuged at 100 g for 2 min and afterwards washed twice with DMEM containing 100 units/ml penicillin and 100 mg/ml streptomycin. Cells were resuspended in hepatocyte medium. Cells were counted and stained with trypan blue to check the viability. Primary hepatocytes were seeded in rat tail collagen I coated 6 wells at a density of $6*10^5$ cells per well.

c. Hepatocyte transfection

Primary hepatocytes were transfected one day after isolation. Therefore 1,5 µg of the plasmid was used and 3 µl of Lipofectamine 2000. Standard Lipofectamine protocol was used. Mixture was incubated for 30 min. Transfection was performed in D10F, 6-8h later medium was changed. Used plasmids were GFP-SCAP [58] and a human CMV-INSIG-1-6x Myc construct.

d. Membrane and nuclear fractionation

Membrane and nuclear fractions were isolated from hepatocytes as described by Goldstein, Brown and co-workers with some modifications [13]. In brief, cells were washed with PBS and Buffer B (10 mM Hepes-KOH pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 25 µg/ml ALLN (N-acetyl-Leu-Leu-Norleu-al; Calpain Inhibitor I) and protease inhibitor cocktail). Cells were incubated 10 min on ice in buffer B. Cell suspension was passed through a 23 gauge needle 7 times and centrifuged at 1000 g at 4 °C for 5 min.
The resulting 1000 g pellet was used to prepare a nuclear extract. The pellet was resuspended in buffer C (10 mM Hepes-KOH at pH 7.4, 0.42 M NaCl, 2.5% (v/v) glycerol, 1.5 mM $MgCl_2$, 0.5 mM sodium EDTA, 0.5 mM EGTA, 1 mM dithiothreitol, and the

protease inhibitor mixture) followed by rotation of the suspension at 4 °C for 45 min and centrifugation at top speed in a micro centrifuge at 4 °C for 30 min.
The resulting 1000 g supernatant was used to prepare the membrane fraction by centrifugation at 10^5 g in a Beckman TLA 100.4 rotor for 30 min at 4 °C, followed by resuspension of the 10^5 g pellet in final sample buffer (FSB) (60 mM Tris-HCl at pH 7,4, 2% (w/v) SDS, 10% glycerol).

e. Mouse experiments

We performed mice experiments as described elsewhere [51]. Briefly, mice were fed, fasted, and refed. The nonfasted group was fed ad libitum with a standard chow diet, the fasted group was fasted for 12 hours, and the refed group was fasted for 12 hours and then refed a high-carbohydrate/ low-fat diet (equivalent to TD 88122; Harlan Teklad) for 12 hours.

f. SREBP liver assay

Factions of livers were prepared as described previously with small modifications [51, 59]. Liver was excised and washed in PBS Buffer (PBS, 1x Protease Inhibitors, 50 µg/ml ALLN). To isolate the active SREBPs in the nuclear fraction 1 g of the frozen liver was mixed with 6 ml Buffer A (10 mM Hepes at pH 7.6, 25 mM KCl, 1 mM sodium EDTA, 2 M sucrose, 10% vol/vol glycerol, 0.15 mM spermine, 2 mM spermidine, 1x Protease Inhibitor cocktail, 50 µg/ml ALLN). Three strokes of a Teflon pestle were done in a Potter homogenizer at low speed and the homogenate was filter through a cell strainer. Samples were over layered with 2 ml Buffer A in SW 41Ti tube and filled up with Buffer 1 (10 mM Hepes at pH 7.6, 25 mM KCl, 1 mM sodium EDTA). The samples were then centrifuged at 25,000 rpm (75,000g) for 1 h at 4°C in the SW 41 Ti Rotor. After that the tube was turned over and the part with the resulting nuclear pellet was cut off and resuspended in 1 ml Buffer B (10 mM Hepes pH 7.6, 100 mM KCl, 2 mM MgCl2, 1 mM sodium EDTA, 10% (vol/vol) glycerol, 1 mM DTT, 1x Protease Inhibitor cocktail, 50 µg/ml ALLN). Then about 140 µl of Buffer AS (3.3 M ammonium sulfate (pH 7.9) were added to the solution. This suspension was agitated gently for 40 min at 4°C on wheel in cold room and afterwards centrifuge at 78,000 rpm in Beckman TLA-100.4 rotor for 45 min at 4°C. The supernatant was mixed with FSB.
For the membrane fraction (P-SREBPs) 50 mg frozen liver were homogenised with three strokes of a Teflon pestle in a Potter at low speed homogenized in 1 ml buffer M (20 mM Tris-HCl pH 7.4, 2 mM $MgCl_2$, 0.25 mM sucrose, 10 mM sodium EDTA, 10 mM sodium EGTA, 1x Protease Inhibitor cocktail, 50 µg/ml ALLN). The suspension was centrifuged at 6,800 g for 5 minutes 4°C in a micro centrifuge. The resulting supernatant was then centrifuged at 48600 rpm for 30 minutes at 4°C in the Beckman TLA-100.4 rotor. The pellet was dissolved in FSB.

g. NEFA levels

Plasma NEFA levels were analysed using the NEFA kit HR Series NEFA-HR(2) from Fujifilm according to manufactures instructions.

h. Cloning and stable cell lines

SREBP-1c and SREBP-2 vector were made as previously described [60]. In brief SREBP-1c or SREBP-2 full length cDNA was cloned into pCDNA3.1 vector after a CMV promotor. The used primers are shown in table 1. Amino acids 1-13 from SREBP-2 and amino acid 1 from SREBP-1c are missing. These vectors were transfected into U2OS cells and selected with G418. Clones were picked after some days and so stable SREBP cell lines were generated.

i. Western Blot

Western blotting was performed using standard protocols for SDS-PAGE and wet transfer onto nitrocellulose membranes (Bio-Rad). Antibodies used: 2A4 (Santa Cruz, cat No. sc-13551), SREBP-1c (Merck, Cat No. MABS1987), Anti-Myc Tag Antibody (Millipore, Cat No. 06-549).

j. RNA isolation, reverse transcription and qPCR

Cells were harvested with TRIzol™ and RNA was isolated using standard protocols. In short: Samples are homogenized/suspended and incubated for 5 min at room temperature. 0,2 mL of chloroform per 1 mL of TRIzol™ Reagent were used for lysis. Tubes are agitated vigorously by hand for 15sec and incubated at RT for 3min. Followed by a 15 minutes 12000 g centrifugation at 4°C. The upper phase was transferred to a fresh tube containing 0,5ml Isopropylalcohol. Samples were homogenized by vortexing for 15sec and incubated for 10 min. In the next step samples awerere centrifuged at 12000g for 15 min at 4°C. Supernatants are removed and the RNA pellets were washed once with 1ml 75% Ethanol per 1 ml TRIzol™. Samples were mixed by vortexing and centrifuged at 12000g for 15min at 4°C. The RNA pellet was dried completely by air, on ice. In the end RNA was dissolved in RNase free water and incubated for 10 min at 56°C and subsequently stored at -70°C.

After RNA isolation revers transcription was performed using iScript™ Reverse Transcription Supermix for RT-qPCR kit.

Next qPCR was performed using PowerUp™SYBR™Green Master Mix from Thermo Fischer Scientific.

Relative mRNA levels were quantified using the comparative ΔCT method, normalized to 18S rRNA. Primers used for qPCR are listed in the table below.

Primer	Forward	Reverse
SREBP-1c	GGA GCC ATG GAT TGC ACA TT	GGC CCG GGA AGT CAC TGT
FASN	GGC CCC TCT GTT AAT TGG CT	GGA TCT CAG GGT TGG GGT TG
ACC-1	GGC CAG TGC TAT GCT GAG AT	CCA GGT CGT TTG ACA TAA TGG
18sRNA	GTA ACC CGT TGA ACC CCA TT	CCA TCC AAT CGG TAG TAG CG

k. Immunofluorescence (IF)

IF was done as described before from the Espenshade lab [58]. Hepatocytes were transfected one day after isolation with Lipofectamine 2000 with 1 µg GFP-SCAP plasmid.
48 h later cells were treated with 1% hydroxypropyl-beta-cyclodextrin (HPCD) to deplete sterols for 2 h. After that cells were refed in medium B or medium B supplemented with 10 µg/ml 25-Hydroxycholesterol (25-HC) for 4 h. Cells were fixed in 3% paraformaldehyde in PBS at room temperature for 10 min and then permeabilized by 0.5% Triton X-100/PBS/glycine for 3 min at room temperature. Primary antibodies (anti-GFP, 1:500 or anti-GM130, 1:250) and secondary antibodies (Alexa-488 goat anti-rabbit IgG or Alexa-594 goat anti-mouse IgG, 1:250) were incubated for 30 min, respectively. Coverslips were mounted to slides and dried in the dark overnight before visualization by an Olympus IX81 inverted microscope.

Antibodies used: Rabbit polyclonal anti-green fluorescent protein (GFP) (ab290) from Abcam; GM130, mouse monoclonal anti-GM130 (clone 35, 610822) from BD Biosciences; Alexa 488-conjugated goat anti-rabbit (A11034) and Alexa 594-conjugated goat anti-mouse (A11005) IgG from Invitrogen.

l. Statistical analyses

Statistical significance was determined with Graph Pad prism 7 using the Student's t test. Differences were considered significant for $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

5. Results acquired during the research stay

a. Regulation of SREBP-1c by 25-Hydroxycholesterol

As described in figures 4 to 7 we found that lack of ATGL leads to enhanced nuclear N-SREBP-1c abundance. To investigate if ATGL activity suppresses nuclear accumulation of N-SREBP-1c through canonical P-SREBP-1c activation I established several experimental cell systems in Dr. Espenshade's laboratory.

My primary goal was to modify the well-known SREBP proteolytic activation- and SREBP transport- test systems that were previously established by the Goldstein Brown laboratories and by Dr. Espenshade [14, 16, 58, 61, 62] so that I could use them in primary hepatocytes. Here I had to deal with several difficulties. First I had to establish the isolation of the primary hepatocytes in the Espenshade laboratory. This was possible and the protocol is described in the methods section. Second, it turned out that primary hepatocytes, a non-dividing cell species, react differently to known SREBP regulatory stimuli that dividing cells. Nevertheless, I was eventually able to establish SREBP cleavage activation and SREBP transport assays as follows.

First I developed a protocol for the treatment of primary hepatocytes with 25-Hydroxycholesterol (25-HC) and various relevant FAs. These different conditions should serve us as a control for all following experiments. Therefore, I compared the effects of the following mentioned compounds in primary hepatocytes with those in a cultured cell system. In cultured dividing cells it is established that 25-HC leads to a stabilization of the INSIG-1-SCAP-SREBP-1c complex in the ER membrane already at a concentration of 1 µg/ml 25-HC [20, 26, 62]. Therefore, I exposed a U2OS cell line with stable CMV promoter driven expression of a N-terminally flag tagged P-SREBP-1c construct (UF1c) to 1 µg/ml 25-HC as compared to lipid depleted conditions. As a result, I could recapitulate the P-SREBP-1c to N-SREBP-1c cleavage suppressing effect of 25-HC in our UF1c cell line (Figure 8, A).

In primary C57bl6 hepatocytes however, 1 µg/ml 25-HC showed no proteolytic cleavage suppressing effect on P-SREBP-1c (data not shown). I had to increase the amount of 25-HC to 10 µg/ml to see a clear transcriptional feed forward effect on target gene transcription in hepatocytes (Figure 8, B). To directly assess P-SREBP-1 cleavage to N-SREBP-1c and its cleavage kinetics in hepatocytes post lipid depletion or lipid depletion and 25-HC repletion I performed a time course experiment. Figure 8, C, shows my results how lipid depletion versus 25-HC repletion influence P-SREBP-1c cleavage over time in hepatocytes.

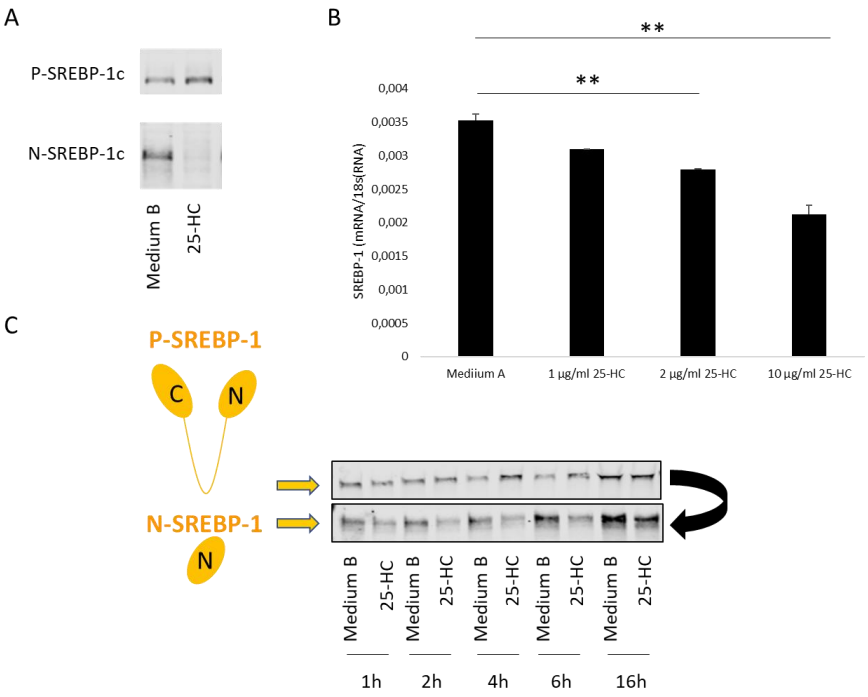

Figure 8: Regulation of SREBP-1c by 25-HC. (A) Stable overexpression SREBP-1c cell line was treated with medium B (5% LPDS with Mevastatin and Mevalonate) +/- 1 µg/ml 25-HC for 16 h. (B) qPCR from wild type primary hepatocytes 16 h after treatment with different 25-HC concentrations. (C) Time course from primary hepatocytes treated in medium B +/- 10 µg/ml 25-HC.

b. Treatment of primary hepatocytes with fatty acids

Previous reports from actively dividing cultured cell systems showed that unsaturated fatty acids at culture medium concentrations around 100 µM rather had a repressive effect on P-SREBP-1 cleavage and the subsequent shift of N-SREBP-1c to the nucleus, while 100 µM sFA increased the amount of N-SREBP-1 in nuclear extracts significantly [13]. When we repeated this experiment in our stable overexpressing SREBP-1c cell line, UF1c, we observed the quite similar effects (Figure 9, A). Nevertheless, to our knowledge no publication exists that shows the effects of sFA or uFA on primary mouse hepatocytes. Interestingly, our own tests show that P-SREBP-1c cleavage is not influenced by the abundance of various different FA species at 100 µM in the cell culture medium of primary hepatocytes. Not even an increase of the FA concentration to 1 mM showed any effect on P-SREBP-1c cleavage or N-SREBP-1c target gene expression (Figure 9, B).

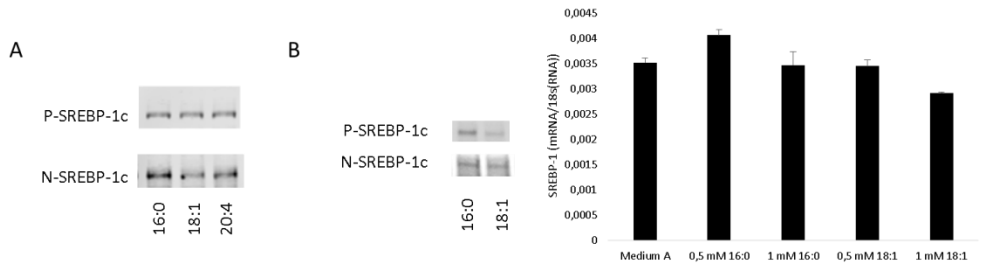

Figure 9: Effect from FA on SREBP-1c nuclear transport. (A) Stable overexpressing SREBP-1c cell line reacts on 100 µM FA in 5% LPDS medium after 16 h. (B) Western Blot and qPCR from wild type C57bl6 primary hepatocytes 16 h after FA treatment. For Western Blot primary hepatocytes were treated with 1 mM FA.

c. INSIG-1-MYC cycloheximide assay

Fatty acid supplemented in the cell culture medium did not show any effect on primary hepatocytes (Figure 9). We have however, previously found that activation versus blockage of intracellular lipolysis by treatment with Forskolin or Atglistatin, respectively shows strong effects on SREBP-1c cleavage (Figure 7). Therefore, we wanted to analyse if endogenously generated FAs in primary hepatocytes blunt SREBP-1c cleavage via the canonical SREBP activation pathway that includes SCAP and INSIG-1 interactions. Generally, when cellular lipid levels are low, the SCAP-SREBP complex in the ER membrane can dissociate from its ER anchor INSIG-1 and hence it is transported to the Golgi apparatus for proteolytic SREBP activation and downstream nuclear transport and SREBP target gene activation occurs. As a first step toward analysis of the canonical SREBP activation we therefore, needed to establish an assay that allows for INSIG-1 analysis. Interestingly, dissociation of the SCAP-SREBP complex from INSIG-1 can be best traced by INSIG-1 stability analysis. INSIG-1 protein stability decreases from 1 h down to 15 min in cultured dividing cells when SCAP dissociates from INSIG-1 [12, 24, 26]. To specifically investigate INSIG-1 protein half-life inside living hepatocytes we used a cycloheximide (CHX) chase assay. CHX is a toxin that inhibits transcription and therefore gives us the possibility to investigate INSIG-1 protein stability. To our knowledge there is unfortunately no INSIG-1 antibody available that can be used to reliably detect INSIG-1 in cultured primary hepatocytes. Therefore, we decided to transfect an INSIG-1-MYC construct into our hepatocytes. I first needed to establish a specific transfection protocol for primary hepatocytes. Therefore, I tested several concentrations of Lipofectamine 2000 and several plasmid DNA to Lipofectamine ratios. The final protocol is described in the methods section. For the CHX assay, primary hepatocytes were isolated and transfected with INSIG-1-MYC cDNA plasmid on the next day. One day later 1% hydroxypropyl-beta-cyclodextrin was added in DMEM for 2 h. HPCD removes cholesterol from cellular membranes and therefore, the SCAP-SREBP complex detaches from INSIG-1, that in turn leads to INSIG-1 destabilisation. After that 2 h HPCD treatment, medium B (DMEM with lipid depleted serum supplemented with Mevalonate and Mevastatine) or medium B with 25-HC containing 50 µM CHX was added. Mevalonate is important that cells can generate farnesylated proteins under the presents of statins which further increase the N-SREBP-1c amount by blocking cholesterol synthesis. As a result, we found that 25-HC could stabilize our surrogate INSIG-1-MYC protein stability reporter in primary hepatocytes. Its half-life was about half an hour in lipid depleted medium B as compared to roughly 2 to 3 hours in medium B with 25-HC repletion (Figure 10).

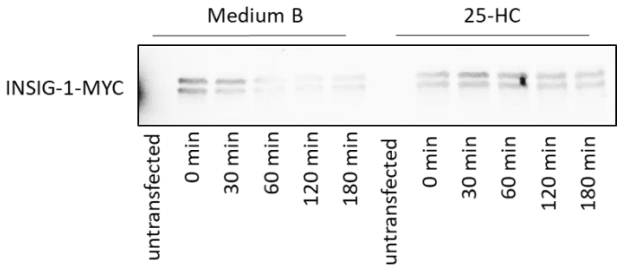

Figure 10: INSIG-1-MYC stability. Primary hepatocytes were transfected with 1 µg INSIG-1-Myc Plasmid. One day later 1% HPCD was added in DMEM medium. 2 h later cells were refed using medium B or medium B with 25-HC with 50 µM CHX. Cells were harvested at indicated time points. Western Blot was performed afterwards using Anti Myc-Tag antibody.

d. GFP-SCAP Immunofluorescence

When lipids are scarce and SCAP-SREBP detaches from INSIG-1 then INSIG-1 is degraded. At the same time the SCAP-SREBP complex migrates to the Golgi apparatus where proteolytic P-SREBP activation takes place. Up until this point I had established assays that could trace INSG-1 stability and P-SREBP-1c to N-SREBP-1c cleavage and, their respective subcellular localizations. Importantly, I also needed to establish an assay that lets me directly trace the shift of SCAP from the ER to the Golgi apparatus upon lipid depletion. Therefore, I made use of the GFP-SCAP reporter-system that was previously established in Dr. Espenshades laboratory [58]. To establish this system in primary hepatocytes I tested several transfection conditions. Eventually I came up with a protocol that worked in hepatocytes. This protocol is described in the methods section. Essentially, I first transfected primary hepatocytes with the GFP-SCAP construct. 48 h later cells were treated for 2 h with 1% HPCD in plain DMEM medium. Next, hepatocytes were refed with the lipid depleted serum medium B or medium B containing 10 µg/ml 25-HC. After that immunofluorescence (IF) was performed as described in the method section. On the next day slides were analyzed via an Olympus IX81 inverted microscope. As a result I found that there was a co-localization (yellow) of the Golgi marker GM130 (red) and SCAP-GFP (green) in lipid depleted cells (Figure 13, left panel) while cells with 25-HC lipid repletion (Figure 13, right panel,) did not show such a co- staining.

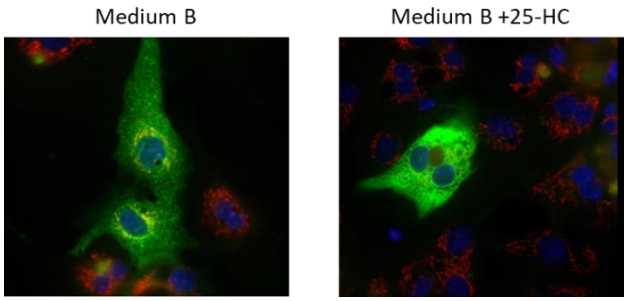

Figure 13: GFP-SCAP Immunofluorescence**:** Primary hepatocytes were isolated and transfected on the next day with 1,5 µg of SCAP-GFP plasmid with 3 µl Lipofectamine 2000. 48 h later cells were treated for 2 h with 1% HPCD in DMEM. After that time cells were refed for 2 h with medium B or medium B and 25-HC. Then IF was performed using Anti GFP antibody to stain GFP SCAP plasmid (shown in green) and Anti GM130 antibody for Golgi staining (shown in red).

Procedures established in Dr. Espenshades laboratory will be instrumental to understand if our findings of SREBP-1c regulation by ATGL do follow the canonical SREBP activation pathway [14, 16, 58, 63] or, if an alternative regulatory pathway is at work here.

6. Discussion

SREBP is an important protein for the control of the lipid metabolism of the cell. It does not only control the synthesis and uptake of sterols and therefore the fluidity of the cellular membrane but also the synthesis of fatty acids. Therefore, it is important to better understand the processes which influence this lipid equilibrium in the cell. Proteolytic processing of P-SREBP-1c to active nuclear N-SREBP-1c is regulated through the ratio between saturated and unsaturated FAs [13]. However, the source(s) of FAs that regulate SREBP-1c are not fully understood. One potential source is lipolysis. To analyse the impact from lipolysis on lipogenesis the interplay of FA released by ATGL is interesting. ATGL is the key enzyme in lipolysis and sets free the first FAs from the TGs under fasting conditions. As seen in ATGL knockout mice, ATGL primarily hydrolyses uFAs in adipose tissues that are subsequently excreted into the bloodstream in albumin bound form [16]. Unsaturated fatty acids stop P-SREBP-1c processing and lead to a stabilization of the P-SREBP-1c form. Once they reach the liver, those uFA are potentially important for the regulation of SREBP-1c. To investigate the hypothesis that uFA hydrolysed by ATGL regulate SREBP-1c we used different ATGL knockout mouse models. AKO that are completely lacking ATGL except for the heart, AAKO, that are lacking ATGL in the adipose tissues and ALKO, that are lacking ATGL in the liver.

This knockout and control animals were fasted overnight and then refeed with a high carbohydrate diet. Thereafter livers were collected, and membrane and nuclear fractions were prepared. Insulin promotes the transcription of SREBP-1c precursor form. After the high carbohydrate diet SREBP-1c (mRNA) and therefore P-SREBP-1c levels at the ER membrane were increased in control as well as in knockout animals. In AKO mice we saw a significant increase of nuclear N-SREBP-1c in the knockout group compared to controls at 6 and 9 hours after carbohydrate refeeding. Also, SREBP-1c target genes, FASN and ACC-1 were increased there and then.

To figure out which influence FAs from the adipose tissue have on SREBP-1c signalling in the liver we exposed AAKO mice to the same fasting and feeding regime then the AKO mice before. The observed results were quite similar to that in the AKO mice. These results lead us to the next experiment where we analysed ATGL liver knockout mice under this feeding protocol. The response of SREBP-1c was weaker in these animals but still visible. This leads us to the assumption that ATGL at both sites, adipose tissue as well as in the liver is important for SREBP-1c regulation.

To analyse the effects of ATGL just in the liver without the complex organ crosstalk in vivo, we decided to isolate primary hepatocytes. To investigate the role of ATGL in these hepatocytes we decided to use Atglistatin. Atglistatin is a selective inhibitor for ATGL in mouse. Moreover, it does not work in cancer cells. Therefore, it is even more important that we work with freshly isolated hepatocytes and not with liver cell lines to avoid side effects. Basal release of FA and glycerol was reduced up to 64% and 43%, respectively in organ cultures. In the presence of Forskolin, Atglistatin reduced FA release up to 72% and glycerol release up to 62% in experiments with fat pads. When ATGL knockout mice where treated with Atglistatin they showed no effects. In vivo a dose-dependent decrease in FA and glycerol levels up to 50% and 62% was observed [56]. When we treated wildtype C57/Bl6J hepatocytes with Forskolin and Atglistatin, N-SREBP-1c levels were increased in comparison to Forskolin controls. This suggests that intracellular ATGL mediated lipolysis can indeed suppress proteolytic P-SREBP-1c to N-SREBP-1c activation and nuclear transport of the latter one. Furthermore, we also isolated primary hepatocytes from ALKO mice. Western

blotting of the membrane and nuclear fractions of these cells did not show an increase with Atglistatin. Interestingly the amount of N-SREBP-1c was higher in comparison to control mice. On account of these results in Wildtype hepatocytes and hepatocytes lacking ATGL (ALKO), we suggest that blocking of ATGL by Atglistatin shows clear and ATGL specific hyper activation of SREBP-1c regulation in liver cells.

To study the underlying molecular mechanisms, we tested the reaction from primary wildtype hepatocytes on 25-HC. In immortalized cultured cell systems 25-HC is a component that is routinely used to analyse regulation of SREBP-1c [8, 64]. 25-HC stabilizes the INSIG-SCAP-SREBP complex in the ER membrane and therefore, SREBP is unable to re-locate to the Golgi apparatus where its proteolytic processing could take place. As a result 25-HC treatment of lipid depleted cells commonly leads to enrichment of P-SREBP in the ER membrane fraction and loss of N-SREBP in nuclear extracts can be observed by doing so [20]. We want to analyse changes on SREBP-1c transport in control mice in comparison to knockout mice. Therefore, we developed a protocol for 25-HC treatment in primary hepatocytes. Cultured cells are always mutated cells and have a completely different mechanism in comparison to primary cells. They do not take up everything from the culture medium and need another environment to grow. This is due to the function of the body. If small changes in the body composition would lead to huge effects on the whole metabolism we could not survive. Therefore, we figured out that we have to increase the concentration of 25-HC 10-fold in these primary hepatocytes to observe the same effects then in cultured cells.

Additionally, we also wanted to establish a protocol for FA treatment in the primary culture. In cancer cells we and others observed the regulation from FA on SREBP-1c. Unsaturated fatty acids block P-SREBP-1 processing while sFA promote the transport to the nucleus [13]. Increasing the concentration from FAs did not work in hepatocytes. Furthermore, we used different medium and insulin conditions but also this test did not show effects. We used M199 medium to avoid influences from fetal bovine serum on the system and have a more controlled environment in the culture. Furthermore, we tested low glucose DMEM because the standard concentration of 450 mg/dl is not physiological for liver cells. In mouse livers concentrations around 140 mg/dl in the fed and 82 mg/dl in the fasted animal were measured [54]. Nevertheless, we could not observe changes in SREBP-1c processing with all tested conditions. Recently it was shown that the uptake of primary WT hepatocytes of FA is around 600 times lower than in liver culture cells like AML 12 or IHH cells. This effect comes from a changed transport of lipid receptors to the surface of the cells [65].

We therefore, propose that uFAs liberated from ATGL, possess the ability to regulate SREBP-1c in vivo and in vitro. Further tests will involve analysis of hepatocytes genetically lacking ATGL as well as pharmacological inhibition of ATGL in mice. Most importantly we plan to dissect the regulation of the SREBP-1c system by lipolysis products from ATGL on molecular level. Hence, we will analyse the integrity of the INSIG-SCAP-SREBP complex in our model systems.

7. References

1. Goldstein, J.L. and M.S. Brown, *The LDL receptor.* Arterioscler Thromb Vasc Biol, 2009. **29**(4): p. 431-8.
2. Brown, M.S. and J.L. Goldstein, *A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood.* Proc Natl Acad Sci U S A, 1999. **96**(20): p. 11041-8.
3. Horton, J.D., et al., *Regulation of sterol regulatory element binding proteins in livers of fasted and refed mice.* 1998.
4. Schoenheimer, R. and F. Breusch, *Synthesis and destruction of cholesterol in the organism.* J. Biol. Chem, 1993. **103**: p. 439-448.
5. Keys, A., *Coronary heart disease--the global picture.* Atherosclerosis, 1975. **22**(2): p. 149-92.
6. Brown, M.S. and J.L. Goldstein, *Familial hypercholesterolemia: defective binding of lipoproteins to cultured fibroblasts associated with impaired regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity.* Proc Natl Acad Sci U S A, 1974. **71**(3): p. 788-92.
7. Brown, M.S. and J.L. Goldstein, *The SREBP Pathway: Regulation of Cholesterol Metabolism by Proteolysis of a Membrane-Bound Transcription Factor.* Cell, 1997. **89**(3): p. 331-340.
8. Goldstein, J.L., DeBose-Boyd, R.A., and Brown, M.S., *Protein sensors for membrane sterols.* Cell, 2006. **124**: p. 35–46.
9. Horton, J.D., et al., *Combined analysis of oligonucleotide microarray data from transgenic and knockout mice identifies direct SREBP target genes.* Proc Natl Acad Sci U S A, 2003. **100**(21): p. 12027-32.
10. Ye, J. and R.A. DeBose-Boyd, *Regulation of cholesterol and fatty acid synthesis.* Cold Spring Harb Perspect Biol, 2011. **3**(7).
11. Smith, J.R., et al., *Identification of nucleotides responsible for enhancer activity of sterol regulatory element in low density lipoprotein receptor gene.* J Biol Chem, 1990. **265**(4): p. 2306-10.
12. Lee, J.N., et al., *Unsaturated fatty acids inhibit proteasomal degradation of Insig-1 at a postubiquitination step.* J Biol Chem, 2008. **283**(48): p. 33772-83.
13. Hannah, V.C., et al., *Unsaturated fatty acids down-regulate SREBP isoforms 1a and 1c by two mechanisms in HEK-293 cells.* J Biol Chem, 2001. **276**(6): p. 4365-72.
14. Espenshade, P.J., W.P. Li, and D. Yabe, *Sterols block binding of COPII proteins to SCAP, thereby controlling SCAP sorting in ER.* Proc Natl Acad Sci U S A, 2002. **99**(18): p. 11694-9.
15. Sun, L.P., et al., *Insig required for sterol-mediated inhibition of Scap/SREBP binding to COPII proteins in vitro.* J Biol Chem, 2005. **280**(28): p. 26483-90.
16. Espenshade, P.J., et al., *Autocatalytic Processing of Site-1 Protease Removes Propeptide and Permits Cleavage of Sterol Regulatory Element-binding Proteins.* Journal of Biological Chemistry, 1999. **274**(32): p. 22795-22804.
17. Hua, X., et al., *Regulated cleavage of sterol regulatory element binding proteins requires sequences on both sides of the endoplasmic reticulum membrane.* J Biol Chem, 1996. **271**(17): p. 10379-84.
18. Radhakrishnan, A., et al., *Sterol-regulated transport of SREBPs from endoplasmic reticulum to Golgi: oxysterols block transport by binding to Insig.* Proc Natl Acad Sci U S A, 2007. **104**(16): p. 6511-8.
19. Radhakrishnan, A., et al., *Direct binding of cholesterol to the purified membrane region of SCAP: mechanism for a sterol-sensing domain.* Mol Cell, 2004. **15**(2): p. 259-68.
20. Adams, C.M., et al., *Cholesterol and 25-hydroxycholesterol inhibit activation of SREBPs by different mechanisms, both involving SCAP and Insigs.* J Biol Chem, 2004. **279**(50): p. 52772-80.
21. Feramisco, J.D., J.L. Goldstein, and M.S. Brown, *Membrane topology of human Insig-1, a protein regulator of lipid synthesis.* J Biol Chem, 2004. **279**(9): p. 8487-96.

22. Adams, C.M., J.L. Goldstein, and M.S. Brown, *Cholesterol-induced conformational change in SCAP enhanced by Insig proteins and mimicked by cationic amphiphiles.* Proc Natl Acad Sci U S A, 2003. **100**(19): p. 10647-52.
23. Yabe, D., M.S. Brown, and J.L. Goldstein, *Insig-2, a second endoplasmic reticulum protein that binds SCAP and blocks export of sterol regulatory element-binding proteins.* Proc Natl Acad Sci U S A, 2002. **99**(20): p. 12753-8.
24. Gong, Y., et al., *Sterol-regulated ubiquitination and degradation of Insig-1 creates a convergent mechanism for feedback control of cholesterol synthesis and uptake.* Cell Metab, 2006. **3**(1): p. 15-24.
25. Goldstein, J.L., R.A. DeBose-Boyd, and M.S. Brown, *Protein sensors for membrane sterols.* Cell, 2006. **124**(1): p. 35-46.
26. Lee, J.N., et al., *Sterol-regulated Degradation of Insig-1 Mediated by the Membrane-bound Ubiquitin Ligase gp78.* Journal of Biological Chemistry, 2006. **281**(51): p. 39308-39315.
27. Sakai, J., et al., *Molecular identification of the sterol-regulated luminal protease that cleaves SREBPs and controls lipid composition of animal cells.* Mol Cell, 1998. **2**(4): p. 505-14.
28. Horton, J.D., J.L. Goldstein, and M.S. Brown, *SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver.* J Clin Invest, 2002. **109**(9): p. 1125-31.
29. Zechner, R., et al., *FAT SIGNALS--lipases and lipolysis in lipid metabolism and signaling.* Cell Metab, 2012. **15**(3): p. 279-91.
30. Zimmermann, R., et al., *Fat Mobilization in Adipose Tissue Is Promoted by Adipose Triglyceride Lipase.* Sience, 2004. **306**: p. 1383-1386.
31. Schweiger, M., et al., *Adipose triglyceride lipase and hormone-sensitive lipase are the major enzymes in adipose tissue triacylglycerol catabolism.* J Biol Chem, 2006. **281**(52): p. 40236-41.
32. Jenkins, C.M., et al., *Identification, cloning, expression, and purification of three novel human calcium-independent phospholipase A2 family members possessing triacylglycerol lipase and acylglycerol transacylase activities.* J Biol Chem, 2004. **279**(47): p. 48968-75.
33. Villena, J.A., et al., *Desnutrin, an adipocyte gene encoding a novel patatin domain-containing protein, is induced by fasting and glucocorticoids: ectopic expression of desnutrin increases triglyceride hydrolysis.* J Biol Chem, 2004. **279**(45): p. 47066-75.
34. Schweiger, M., et al., *The C-terminal region of human adipose triglyceride lipase affects enzyme activity and lipid droplet binding.* J Biol Chem, 2008. **283**(25): p. 17211-20.
35. Schreiber, R., H. Xie, and M. Schweiger, *Of mice and men: The physiological role of adipose triglyceride lipase (ATGL).* Biochim Biophys Acta Mol Cell Biol Lipids, 2019. **1864**(6): p. 880-899.
36. Roy, D., et al., *Coordinated transcriptional control of adipocyte triglyceride lipase (Atgl) by transcription factors Sp1 and peroxisome proliferator-activated receptor gamma (PPARgamma) during adipocyte differentiation.* J Biol Chem, 2017. **292**(36): p. 14827-14835.
37. Chakrabarti, P., et al., *Insulin inhibits lipolysis in adipocytes via the evolutionarily conserved mTORC1-Egr1-ATGL-mediated pathway.* Mol Cell Biol, 2013. **33**(18): p. 3659-66.
38. Singh, M., et al., *Fat-specific protein 27 inhibits lipolysis by facilitating the inhibitory effect of transcription factor Egr1 on transcription of adipose triglyceride lipase.* J Biol Chem, 2014. **289**(21): p. 14481-7.
39. Nielsen, T.S., et al., *Fasting, but not exercise, increases adipose triglyceride lipase (ATGL) protein and reduces G(0)/G(1) switch gene 2 (G0S2) protein and mRNA content in human adipose tissue.* J Clin Endocrinol Metab, 2011. **96**(8): p. E1293-7.
40. Xie, X., et al., *Identification of a novel phosphorylation site in adipose triglyceride lipase as a regulator of lipid droplet localization.* Am J Physiol Endocrinol Metab, 2014. **306**(12): p. E1449-59.
41. Bartz, R., et al., *Lipidomics reveals that adiposomes store ether lipids and mediate phospholipid traffic.* J Lipid Res, 2007. **48**(4): p. 837-47.
42. Kim, S.J., et al., *AMPK Phosphorylates Desnutrin/ATGL and Hormone-Sensitive Lipase To Regulate Lipolysis and Fatty Acid Oxidation within Adipose Tissue.* Mol Cell Biol, 2016. **36**(14): p. 1961-76.

43. Pagnon, J., et al., *Identification and functional characterization of protein kinase A phosphorylation sites in the major lipolytic protein, adipose triglyceride lipase.* Endocrinology, 2012. **153**(9): p. 4278-89.
44. Lass, A., et al., *Adipose triglyceride lipase-mediated lipolysis of cellular fat stores is activated by CGI-58 and defective in Chanarin-Dorfman Syndrome.* Cell Metab, 2006. **3**(5): p. 309-19.
45. Granneman, J.G., et al., *Interactions of perilipin-5 (Plin5) with adipose triglyceride lipase.* J Biol Chem, 2011. **286**(7): p. 5126-35.
46. Yang, X., et al., *The G(0)/G(1) switch gene 2 regulates adipose lipolysis through association with adipose triglyceride lipase.* Cell Metab, 2010. **11**(3): p. 194-205.
47. Zechner, R., et al., *FAT SIGNALS- Lipases and lipolysis in lipid metabolism and signaling.* Cell Metab, 2012. **15**(3): p. 279-91.
48. Ghosh, M., et al., *Ubiquitin Ligase COP1 Controls Hepatic Fat Metabolism by Targeting ATGL for Degradation.* Diabetes, 2016. **65**(12): p. 3561-3572.
49. Dai, Z., et al., *Dual regulation of adipose triglyceride lipase by pigment epithelium-derived factor: a novel mechanistic Insight into progressive obesity.* Mol Cell Endocrinol, 2013. **377**(1-2): p. 123-34.
50. Eichmann, T.O., et al., *Studies on the substrate and stereo/regioselectivity of adipose triglyceride lipase, hormone-sensitive lipase, and diacylglycerol-O-acyltransferases.* J Biol Chem, 2012. **287**(49): p. 41446-57.
51. Engelking, L.J., et al., *Overexpression of Insig-1 in the livers of transgenic mice inhibits SREBP processing and reduces insulin-stimulated lipogenesis.* Journal of Clinical Investigation, 2004. **113**(8): p. 1168-1175.
52. Engelking, L.J., et al., *Overexpression of Insig-1 in the livers of transgenic mice inhibits SREBP processing and reduces insulin-stimulated lipogenesis.* J Clin Invest, 2004. **113**(8): p. 1168-75.
53. Schoiswohl, G., et al., *Adipose triglyceride lipase plays a key role in the supply of the working muscle with fatty acids.* J Lipid Res, 2010. **51**(3): p. 490-9.
54. Haemmerle, G., et al., *Defective Lipolysis and Altered Energy Metabolism in Mice Lacking Adipose Triglyceride Lipase.* Science, 2006. **321**(5774): p. 734-737.
55. Wu, J.W., et al., *Deficiency of liver adipose triglyceride lipase in mice causes progressive hepatic steatosis.* Hepatology, 2011. **54**(1): p. 122-32.
56. Schweiger, M., et al., *Pharmacological inhibition of adipose triglyceride lipase corrects high-fat diet-induced insulin resistance and hepatosteatosis in mice.* Nat Commun, 2017. **8**: p. 14859.
57. Mayer, N., et al., *Development of small-molecule inhibitors targeting adipose triglyceride lipase.* Nat Chem Biol, 2013. **9**(12): p. 785-7.
58. Shao, W., C.E. Machamer, and P.J. Espenshade, *Fatostatin blocks ER exit of SCAP but inhibits cell growth in a SCAP-independent manner.* J Lipid Res, 2016. **57**(8): p. 1564-73.
59. Sheng, Z., et al., *Independent regulation of sterol regulatory element-binding proteins 1 and 2 in hamster liver.* Proc Natl Acad Sci U S A, 1995. **92**(4): p. 935-938.
60. Xianxin Hua, J.S., Michael S. Brown, and Joseph L. Goldstein, *Regulated Cleavage of Sterol Regulatory Element Binding Proteins Requires Sequences on Both Sides of the Endoplasmic Reticulum Membrane.* JBC, 1996. **271**(17): p. 10379-10384.
61. Lee, C.Y., et al., *Regulation of the Sre1 hypoxic transcription factor by oxygen-dependent control of DNA binding.* Mol Cell, 2011. **44**(2): p. 225-34.
62. Lee, J.N., et al., *Proteasomal degradation of ubiquitinated Insig proteins is determined by serine residues flanking ubiquitinated lysines.* Proc Natl Acad Sci U S A, 2006. **103**(13): p. 4958-63.
63. Shao, W. and P.J. Espenshade, *Expanding roles for SREBP in metabolism.* Cell Metab, 2012. **16**(4): p. 414-9.
64. Brown, M.S. and J.L. Goldstein, *Cholesterol feedback: from Schoenheimer's bottle to Scap's MELADL.* J Lipid Res, 2009. **50 Suppl**: p. S15-27.

65. Nagarajan, S.R., et al., *Lipid and glucose metabolism in hepatocyte cell lines and primary mouse hepatocytes: a comprehensive resource for in vitro studies of hepatic metabolism.* Am J Physiol Endocrinol Metab, 2019. **316**(4): p. E578-E589.