Regulation of *Elov*l and fatty acid metabolism

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Dedicated to the future reader
ABSTRACT

Fatty acids are important regulators in the control of mammalian energy homeostasis. They are ingested in the diet but a significant amount are also endogenously produced by de novo lipogenesis. Fatty acid elongation beyond 16 carbons (palmitic acid) can occur to generate very long chain fatty acids (VLCFA), a process that is initiated by the rate-limiting condensation reaction. To date, six mammalian enzymes responsible for this reaction, ELOVL1-6 (Elongation of very long chain fatty acid), have been characterized. All of them exert substrate specificity and tissue-specific gene expression. In this thesis, factors that regulate fatty acid metabolism and, in particular, fatty acid synthesis and elongation will be presented.

The enclosed papers discuss issues as to how *Elov*3 is regulated in liver and in different adipose depots and what effects ablation of this enzyme causes to lipid homeostasis. In contrast to the expression of several other lipogenic genes, *Elov*3 gene expression was not affected by fasting or refeeding. Instead, the gene expression was influenced by steroid hormones such as glucocorticoids and sex hormones. Also, hepatic *Elov*3 gene expression followed a circadian rhythm, present exclusively in sexually mature male mice. Interestingly, despite reduced levels of leptin, *Elov*3-ablated mice were shown to be resistant to diet induced weight gain, which seemed to be due to a decreased ratio between energy intake and energy expenditure. This phenotype was more pronounced in female mice.
This thesis is based on the following papers, which are referred to in the text by their respective Roman numerals I-IV.

Steroid hormones control circadian *Elovl3* expression in mouse liver.
*Endocrinology* 149(6):3158-3166.

Ablation of the very long chain fatty acid elongase ELOVL3 in mice leads to constrained lipid storage and resistance to diet-induced obesity.
*Manuscript*

III. Zadravec, D., **Brolinson, A.** and Jacobsson, A. (2009)
Fat depot specific analysis of *Elovl3*-ablated mice.
*Manuscript*

IV. Dallner, O.S., Chernogubova, E., **Brolinson, K.A.** and Bengtsson, T. (2006)
β3-adrenergic receptors stimulate glucose uptake in brown adipocytes by two mechanisms independently of GLUT4 translocation.
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ABBREVIATIONS

11β-HSD  11β-hydroxysteroid dehydrogenase
ACC     Acetyl-CoA carboxylase
AMPK    5’-AMP protein kinase
BAT     Brown adipose tissue
cBAT    Cold induced brown adipose tissue
CNS     Central nervous system
CPT     Carnitine palmitoyltransferase
ELOVL   Elongation of very long chain fatty acids
ER      Endoplasmic reticulum
FAS     Fatty acid synthase
GLUT    Glucose transporter
LXR     Liver X receptor
PPAR    Peroxisome proliferator-activated receptor
SCD     Stearoyl-CoA desaturase
SCN     Suprachiasmatic nucleus
SREBP   Sterol regulatory element-binding protein
VLCFA   Very long chain fatty acids
WAT     White adipose tissue
ZT      Zeitgeber time
Lipids, which are fat-soluble molecules, include a broad variety of compounds which are indisputable for life. These compounds include different fats such as oils, waxes, sterols, fat-soluble vitamins, glycerophospholipids, sphingolipids, mono-, di- and triglycerides. Fatty acids (FA) do not only serve as a major source of energy, but are also crucial structural components of membranes. Additionally, fatty acids may function as signaling molecules, thus exerting key biological functions, such as regulating fatty acid metabolism (Duplus and Forest, 2002). For example, polyunsaturated fatty acids (PUFAs) can bind to transcription factors as ligands and regulate *de novo* fatty acid synthesis by either activate or suppress transcription of proteins and enzymes involved in lipid metabolism (reviewed in (Jump and Clarke, 1999)). Fatty acids also regulate the properties of numerous membrane proteins by binding to them.

In times of caloric excess, fatty acids are synthesized and triglycerides (TG) are formed, resulting in the most important form of stored chemical energy. Dietary fatty acids are modulated in the digestive tract before reaching target tissues. In the intestine, digested triglycerides are broken down by pancreatic lipase into monoglycerides and free fatty acids. Together with bile salts they form a complex that can diffuse into the intestinal epithelial cells. Once there, new triglycerides are synthesized from the monoglycerol and free fatty acids. Lipoprotein particles called chylomicrons are now formed from triglycerides together with cholesterol, phospholipids and protein molecules. Via the lymphatic system, the chylomicrons reach the circulation, hence delivering fatty acids to various tissues such as liver and adipose tissue, in what is known as the exogenous pathway.

The major metabolic organ responsible for uptake, synthesis and release of fatty acids into the circulation is the liver. It is capable of converting carbohydrates into fatty acids and triglycerides, a process termed *de novo* lipogenesis (reviewed in (Dentin et al., 2005; Leonhardt and Langhans, 2004)). Only a small part of excess dietary carbohydrates are stored in the liver as glycogen while most of it is converted into fatty acids. Both *de novo* synthesized fatty acids and dietary fatty acids can be further modified in various ways inside the cell, including being oxidized, elongated and desaturated.

The so called endogenous pathway describes the journey of triglycerides from the liver to target tissues. Triglycerides resulting from hepatic lipogenesis can either be stored in the liver through incorporation into lipid droplets or packed into very low-density lipoprotein (VLDL) particles for further distribution to various tissues. Lipoproteins are particles that
constitute of a neutral lipid core, mainly comprised of triglycerides and cholesterol esters, surrounded by a monolayer consisting of unesterified cholesterol, phospholipids and specific proteins.

Storage of energy predominantly occurs in the form of fatty acids and triglycerides sequestered in lipid droplets. Lipid droplets are found in a broad variety of cells. Nevertheless, adipocytes are the far most important cells for the storage of energy in the form of lipid droplets. Adipocytes are gathered in adipose tissue, a metabolically active endocrine organ (reviewed in (Kershaw and Flier, 2004)). When energy expenditure exceeds energy intake, the breakdown of fatty acids provides the organism with energy, a process called lipolysis.

The breakdown of fatty acids up to 18 carbons in length takes place in the mitochondria while longer fatty acids need to be shortened in peroxisomes before further oxidation in mitochondria occurs. As increased energy demands lead to mobilization of stored energy from white adipose tissue (WAT), the adipocytes in turn signal to the brain to refill the fat depots, a process where we feel hunger.

Below this thesis will focus on factors such as transcriptional control, circadian rhythms, food intake and sexual dimorphism, factors that have been identified as players involved in the physiology of fatty acid synthesis and especially the mammalian fatty acid elongase family ELOVL (Elongation of very long chain fatty acids).

2. FATTY ACID SYNTHESIS

Lipids, carbohydrates and amino acids can all be metabolized into acetyl-CoA, thus serving as substrates for lipogenesis (Figure 1). The structure of the fatty acid molecule is essential for its function. The basic structure is comprised of a carboxylic acid head and a long saturated or unsaturated aliphatic tail, which can be of varying length. The synthesis of fatty acids involves acetyl-CoA which is carboxylated by the enzyme acetyl-CoA carboxylase (ACC) into malonyl-CoA. Malonyl-CoA is the two carbon donor in de novo lipogenesis resulting in a two carbon elongation of the acetyl-CoA molecule. This reaction is performed in the cytosol by fatty acid synthase (FAS) and can be repeated seven times in a cyclic manner forming palmitic acid (C16:0). In addition of being carbon donor in fatty acid synthesis, malonyl-CoA inhibits carnitine palmitoyltransferase 1 (CPT1) which is mediating the transport of acyl-CoA into the mitochondria where it can be β-oxidized (McGarry et al., 1977). Finally, fatty acids produced by FAS or taken up from the diet can be further modulated i.e. elongated or desaturated by the elongase and desaturase enzymes in the endoplasmatic reticulum (ER).
Figure 1. Fatty acid metabolism. Fatty acids can be absorbed from the diet or synthesised de novo from glucose or other metabolites by the production of acetyl-CoA, which is transformed into malonyl-CoA by the enzyme acetyl coenzyme A carboxylase (ACC). Malonyl-CoA is used as substrate by the fatty acid synthase (FAS) to produce palmitic acid. Palmitic acid can then be further desaturated or elongated by separate enzymes localized in the endoplasmatic reticulum (ER).

2.1. Acetyl-CoA carboxylase

The carboxylation of acetyl-CoA to malonyl-CoA is considered to be the rate-limiting step of the fatty acid synthesis pathway (Hillgartner et al., 1995; Kim, 1997). Two different isoforms of ACC have been identified, ACC1 and ACC2, encoded by two distinct genes. The former predominates in lipogenic tissues such as liver and adipose tissue while the latter is highly expressed in oxidative tissues such as heart and skeletal muscle, but also to some extent in liver and adipose tissue (Abu-Elheiga et al., 2003; Abu-Elheiga et al., 1997; Abu-Elheiga et al., 2001; Fukuda et al., 1992; J Ha et al., 1996). Ablation of ACC1 in mice is embryonically lethal, whereas ACC2-ablated mice are viable, although with a higher fatty acid oxidation rate.
in heart and muscle (Abu-Elheiga et al., 2001; Abu-Elheiga et al., 2005). Malonyl-CoA generated from ACC1 is used by FAS for fatty acid synthesis while malonyl-CoA produced by the ACC2 isoform acts as an inhibitor of CPT1 activity, hence controlling the transport of fatty acids into the mitochondria for β-oxidation (Abu-Elheiga et al., 2003; McGarry et al., 1977; McGarry and Foster, 1980). The activity of ACC1 is inhibited by phosphorylation, which is suggested to be mediated by 5’-AMP protein kinase (AMPK) when energy expenditure exceeds energy intake.

2.2. Fatty acid synthase

As described above, fatty acids up to 16 carbon atoms in length are synthesized in the cytosol by the multifunctional protein FAS which uses acetyl-CoA as a primer substrate and malonyl-CoA as a two carbon source to elongate acetyl-CoA (Wakil et al., 1983; Wakil, 1989). FAS function as a 540 kDa homodimer which harbors all catalytic sites needed for fatty acid elongation and were the substrates are handed from one catalytic site to the next (reviewed in Smith et al., 2003). The main reactions are condensation, reduction, dehydration and one further reduction (Figure 2).

Figure 2. Fatty acids up to C16 are synthesized in the cytosol by the multifunctional protein fatty acid synthase (FAS). Further elongation into long-, and very long chain fatty acids occurs in the endoplasmatic reticulum by four distinct enzymes, elongation of very long chain fatty acids (ELOVL), 3-ketoacyl-CoA reductase (KAR), PTPLA homolog involved in sphingolipid biosynthesis 1 (Phs1p) and trans-2,3-,enoyl-CoA reductase (TER). The main reactions for the two elongation complexes are however the same starting with a condensation reaction followed by a reduction, dehydration and then a second reduction.
2.3. Elongation of very long chain fatty acids

Fatty acids taken up from the diet as well as a significant amount of the fatty acids produced by FAS, undergo further elongation into long chain fatty acids (LCFA > C18) and very long chain fatty acids (VLCFA > C20). As early as in the nineteen sixties, Nugteren made significant advances in the rather unknown field of fatty acid chain elongation. It was at this point shown that the enzymatic chain elongation of fatty acids takes place in the microsomal fraction of rat liver. Nugteren also showed that malony-CoA is the two-carbon source in this reaction and that NADPH is preferentially used during the elongation process (Nugteren, 1965). Today it is known that elongation beyond 16 carbon atoms occurs in the endoplasmatic reticulum of most cells, where the elongation process is performed in four steps by four distinct membrane-bound enzymes (Cinti et al., 1992) (Figure 2). Three of the enzyme reactions are localized to the cytoplasmic side of the ER membranes, while the enzyme activity performing the dehydration reaction is suggested to be embedded in the membrane (Osei et al., 1989). In the first reaction, acyl-CoA and malonyl-CoA are condensed by a 3-keto acyl-CoA synthase, resulting in β-ketoacyl-CoA. The second step, which requires NADPH, is a reduction reaction. Here β-ketoacyl-CoA is converted to β-hydroxyacyl-CoA by a 3-ketoacyl-CoA reductase, β-hydroxyacyl-CoA is subsequently dehydrated in the third step, which is catalyzed by 3-hydroxy acyl-CoA dehydratase, resulting in enoyl-CoA. This molecule is then reduced by enoyl-CoA reductase, in the presence of NADPH, thereby completing the fourth and last step. The acyl-chain has now been extended by two carbons (Cinti et al., 1992) (Figure 3).

Figure 3. Long and very long chain fatty acid elongation cycle. Enzymatic steps of microsomal fatty acyl chain elongation by 2-carbon units. This reaction cycle can be repeated. Elongation of very long chain fatty acids (ELOVL), 3-ketoacyl-CoA reductase (KAR), PTPLA homolog involved in sphingolipid biosynthesis 1 (Phs1p) and trans-2,3,-enoyl-CoA reductase (TER).
About five years ago Moon and co-workers identified and characterised two mammalian enzymes, 3-ketoacyl-CoA reductase (KAR) and trans-2,3-enoyl-CoA reductase (TER) that were found to catalyse the reduction of 3-ketoacyl-CoA and trans-2,3-enoyl-CoA respectively (Moon and Horton, 2003). At that time, Ybr159p and Tsc13p had been identified as analogue reduction enzymes in yeast (Beaudoin et al., 2002; Kohlwein et al., 2001). Recently, Denic and Weissman have identified Phs1p as the VLCFA dehydratase, thus revealing the final missing component of the elongation cycle (Denic and Weissman, 2007).

More than a decade ago, deletion studies in yeast revealed information about the ELO (elongation) enzymes involved in the condensation reaction described above, which appear to be indispensable for VLCFA elongation (Oh et al., 1997; Toke and Martin, 1996). The elongases comprise several members from all species. They lack sequence homology to other known condensing enzyme domains of the FAS complex. More recent studies have revealed that the condensing domain in FAS contains a strictly conserved cysteine-containing catalytic triad which is missing in the elongases. All the members of the elongase family contain absolutely conserved signature sequence motifs located within either the juxta-cytosolic transmembrane helix regions or within a cytosolic loop. Denic and Weissman have analyzed these conserved motifs with respect to their possible involvement in catalysing the condensation reaction in yeast. They suggest that these motifs might be arranged into a catalytic ring, thereby forming the entrance of an intramembrane substrate-binding pocket, and that the conserved motif might interact with the phosphate moieties of CoA. Hence while the active site faces the cytosol, the length of the VLCFA is determined by a lysine more or less close to the luminal surface. The lysine residue is situated on the sixth (out of seven) transmembrane helix. The distance between the active site and the lysine residue determines the length of the fatty acid produced (Denic and Weissman, 2007).

Today, there are six mammalian homologues characterized of the elongase enzyme family (Figure 4) which comprises both enzymes that are ubiquitously expressed and also more tissue specific enzymes. These are termed ELOVL1 through ELOVL6 and just like the yeast ELO proteins they exhibit substrate specificity and control the first, rate-limiting condensation step of VLCFA elongation (Toke and Martin, 1996; Leonard et al., 2004; Jakobsson et al., 2006). Although the genes have been identified, the high hydrophobicity of the enzyme complex makes it difficult to purify and therefore to characterize in detail. Thus, most of the data regarding the mammalian ELOVL enzymes is derived from gene expression analysis and of studies of mouse mutants such as knockout mice which I will discuss further below.
2.3.1. ELOVL1

The *Elov1* gene was identified due to its sequence similarity to *Elov3* which was the first *Elov* gene to be identified. ELOVL1 has been postulated to serve as a “house-keeping elongase” due to its ubiquitous gene expression pattern in all murine tissues tested (Tvrdik et al., 2000). Yeast complementation studies have implied a role for ELOVL1 in the formation of saturated fatty acids up to 26 carbons in length and to play an important role in the formation of membrane lipids such as sphingolipids (Oh et al., 1997; Tvrdik et al., 2000). The myelin deficient mouse models Jimpy and Quaking have reduced C20 and C22 elongation activity which is paralleled by reduced *Elov1* gene expression in the brains of both mutants, supporting the important role of the saturated fatty acid product of ELOVL1 for sphingomyelin formation (Suneja et al., 1991; Tvrdik et al., 2000).
2.3.2. ELOVL2

The Elovl2 gene was also identified based on sequence similarity to Elovl3. In contrast to Elovl1, Elovl2 displays a more restricted gene expression pattern with the highest mRNA abundance in testis and liver and, although to a lesser extent, also in brain, kidney and white adipose tissue (Tvrdik et al., 2000). ELOVL2 is suggested to elongate polyunsaturated fatty acids such as 20:4,n-6 and 20:5,n-3 (Leonard et al., 2002; Wang et al., 2008b). Overexpression of Elovl2 in 3T3 preadipocytes augments both fatty acid uptake and lipid droplet formation pointing towards a lipogenic role for ELOVL2 (Kobayashi et al., 2007).

2.3.3. ELOVL3

Elovl3 was identified due to its profound increase in gene expression in mouse brown adipose tissue (BAT) upon cold stimulation of the animals (Tvrdik et al., 1997). At that time it was named Cig30 (cold-induced glycoprotein of 30 kDa), but based on its function the protein has now been renamed to Elovl3. In addition to cold exposure, the gene expression was also induced by injection of norepinephrine and by feeding mice with a calorie-rich diet confirming that the gene is under sympathetic control in brown adipose tissue. Enhanced Elovl3 gene expression has also been demonstrated during perinatal development, just before birth when brown adipose tissue is recruited. Together, these observations suggest a role for ELOVL3 in the recruitment of brown adipose tissue.

As for ELOVL1, complementation studies in yeast revealed that ELOVL3 has a putative role in elongation of saturated and monounsaturated fatty acids (MUFA) of up to 24 carbons in length (Tvrdik et al., 2000). In addition to being expressed in brown adipose tissue, Elovl3 is also expressed in the hair follicles of the skin and in liver where it in the latter case is under strong circadian control exclusively in male mice (Tvrdik et al., 1997) and (Paper I). Elovl3-ablated mice have a distinct skin phenotype with tousled fur and display general hyperplasia of the sebaceous glands as well as impaired water repulsion of the fur. The hair lipid content is disturbed, with an accumulation of eicosenoic acid (20:1) and reduced amount of 22-26 fatty acid species (Westerberg et al., 2004).

It has recently been demonstrated that Elovl3-ablated mice are unable to hyperrecruit their brown adipose tissue (Westerberg et al., 2006). This circumstance, in addition to impaired skin barrier function, and hence increased heat loss, causes increased muscle shivering in order to defend body temperature during cold exposure. Histology studies in brown adipose
tissue shows impaired fat accumulation in warm-acclimated "Elovl3"-ablated mice supporting the theory that ELOVL3 is important for maintaining lipid homeostasis for triglyceride and lipid droplet formation (Westerberg et al., 2006). Recently, we have shown that "Elovl3"-ablated mice are resistant to diet induced obesity which was more pronounced in females (Paper II). This will be discussed further in the fatty acid homeostasis section of the thesis below.

2.3.4. ELOVL4

Until recently, "Elovl4" has mainly been studied in humans because of its association with Stargardt-like macular dystrophy. These patients suffer from an eye disorder that leads to loss of vision, which is due to three independent mutations in the last exon (VI) of the "Elovl4" gene (Edwards et al., 2001; Grayson and Molday, 2005; Karan et al., 2005; Zhang et al., 2001). ELOVL4 has been considered to be involved in elongation of PUFAs in retina, brain, skin and testis (Mandal et al., 2004). A mouse model with a deletion in the "Elovl4" gene was recently generated, which leads to scaly, wrinkled skin, and the mice dies within a few hours after birth due to highly impaired skin barrier function (Vasireddy et al., 2007). The "Elovl4"-ablated mouse display a marked depletion of saturated and monounsaturated C28 and longer VLCFA in skin. Despite early death and skin abnormalities, no obvious abnormalities were found in internal organs (Cameron et al., 2007; Li et al., 2007; McMahon et al., 2007; Vasireddy et al., 2007). By overexpressing ELOVL4 in cultured cells Agbaga and co-workers have recently published results implying that ELOVL4 is required for the synthesis of C28 and C30 saturated VLCFA in skin and of C28 to C38 polyunsaturated VLCFA in retina (Agbaga et al., 2008).

2.3.5. ELOVL5

Although "Elovl5" is highly expressed in testis, the adrenal glands and liver, it has been found to be expressed, to some extent, in all tissues tested (Leonard et al., 2000). Based on gene expression studies in rat, the enzyme has been suggested to play an important role in liver development during the postnatal stage (Wang et al., 2005). ELOVL5 is suggested to be involved in elongation of polyunsaturated fatty acyl-CoA substrates of 18 and 20 carbons in length (Inagaki et al., 2002; Moon et al., 2001; Parker-Barnes et al., 2000; Wang et al., 2005). Adenoviral overexpression of "Elovl5" in primary rat hepatocytes resulted in increased...
elongation of arachidonic acid (20:4, n-6) and eicosapentaenoic acid (20:5, n-3) into adrenic acid (22:4, n-6) and docosapentaenoic acid (22:5, n-3) respectively (Wang et al., 2008b). The overexpression gave rise to altered fatty acid content, which in turn affected lipid and carbohydrate composition. Recently, Moon and co-workers presented an Elovl5-ablated mouse which develops hepatic steatosis with increased hepatic cholesterol and triglyceride levels due to increased activation of sterol regulatory element-binding protein-1c (SREBP-1c) and its target genes (Moon et al., 2008). The levels of arachidonic acid (20:4, n-6) and docosahexaenoic acid (22:6, n-3) were decreased in this mouse model. However, there was an increased elongation activity of ELOVL2 and ELOVL6, pointing towards compensation of these enzymes in the Elovl5-ablated mice. Interestingly, female Elovl5-ablated mice had fertility problems, which was not observed in male Elovl5-ablated mice.

2.3.6. ELOVL6

The majority of fatty acids in a cell have a length of C16 to C18 carbon atoms. However, the end product of FAS is palmitic acid (16:0). Palmitic and palmitoleic acid (16:1, n-7) acid can be further elongated by ELOVL6 to stearic acid (18:0) and oleic acid (18:1, n-9) respectively. Thus, ELOVL6 has a pivotal role in the elongation of saturated and monounsaturated long chain fatty acids (Matsuzaka et al., 2002; Moon et al., 2001). High mRNA levels were found in mouse liver and adipose tissue, as well as in a variety of other tissues. Recently it was shown that mice deficient for Elovl6 mated to leptin-deficient ob/ob mice had improved insulin sensitivity, although the mice remained obese and hepatosteatotic (Matsuzaka et al., 2007).

2.4. Fatty acid desaturation – SCD

Stearoyl-CoA desaturases (SCD) are the rate limiting microsomal enzymes involved in the biosynthesis of monounsaturated fatty acids from saturated fatty acids. SCD converts palmitic acid (16:0) and stearic acid (18:0) to palmitoleic acid (16:1, n-7) and oleic acid (18:1, n-9). The monounsaturated fatty acids produced by SCD are further used as substrates in the production of cholesterol esters, triglycerides, phospholipids and wax esters (reviewed in (Ntambi and Miyazaki, 2004)).

In mouse, there are at least three isoforms identified whereas in rat only two isoforms have been found. SCD1 is mainly found in white and brown adipose tissue and in liver, but
also in lipid producing glands, for example the preputial- and meibomian glands (Miyazaki et al., 2001b). In terms of gene expression in tissues, SCD2 differs from SCD1. SCD2 has for example not been detected in liver but is expressed in the brain (Kaestner et al., 1989). SCD1-ablated mice are lean and hypermetabolic and the ablation points towards the importance of SCD1 in de novo synthesis of triglycerides, cholesterol esters and wax esters which is required for accurate skin and eyelid function (Miyazaki et al., 2001a). Furthermore, ob/ob mice with targeted disruption of SCD1 are protected against insulin resistance and obesity due to increased fatty acid oxidation and activation of AMPK (Cohen et al., 2002).

3. FATTY ACID HOMEOSTASIS

Fatty acid metabolism is strictly controlled by a variety of factors that act in concert to maintain energy homeostasis within the whole organism, which if disturbed may cause devastating consequences such as lipodystrophy or obesity and other metabolic disturbances coupled to the metabolic syndrome. When energy consumption exceeds energy expenditure weight is gained. The equilibrium between energy consumption and expenditure is centrally controlled by the hypothalamus in the brain. Adipocytes secrete proteins that signal to the central nervous system (CNS) whether they are in a fed or fasted state. As a consequence we feel satiety or hunger. Leptin and adiponectin have emerged as important “adipokine” messengers between peripheral tissues and the CNS.

Leptin is a 16 kDa protein hormone secreted primarily from adipose tissue. It functions as a key regulator of energy expenditure and, via the hypothalamus, acts as a satiety factor, thus causing reduced food intake. It can also be linked to insulin sensitivity and is elevated in obese subjects (Maffei et al., 1995). Elovl3-ablated mice have significantly reduced serum leptin levels compared to wildtype mice, which was expected since they have reduced adiposity (Paper II) (Figure 5). Furthermore, mRNA levels of leptin were also shown to be reduced in brown adipose tissue and in inguinal as well as gonadal WAT of Elovl3-ablated mice (Paper III).
To date, adiponectin is the only “true” adipokine, since it is the only adipokine that is exclusively secreted from adipocytes (Scherer et al., 1995). In obese individuals, both serum and mRNA levels of adiponectin are decreased and administration of adiponectin to obese animal models reverses insulin resistance (Arita et al., 1999; Yamauchi et al., 2001). Modest overexpression of adiponectin in adipose tissue of ob/ob mice rescue insulin resistance by increased expansion of the adipose tissue (Kim et al., 2007). The authors suggest that adiponectin can act as a “starvation” signal promoting storage of triglycerides in adipose tissue. Surprisingly, Elov3-ablated mice have lower serum levels of adiponectin as compared to wild-type mice even though the Elov3-ablated mice have markedly reduced adiposity suggesting that lack of ELOVL3 fatty acids restrains the starvation-signaling pathway in these mice (Paper II).

Transcriptional control of genes encoding proteins involved in fatty acid metabolism is an indispensable tool in keeping lipid homeostasis. This, in turn, is regulated on a higher level by e.g. hormones, circadian rhythms and food intake. For example, normal meal distribution over the day gives rise to a pulsatile release of insulin which in turn causes cellular glucose uptake via specific glucose transporters (GLUT). Insulin acts on the transcription factor SREBP-1, hence inducing the transcription of lipogenic genes like for example GLUT4, FAS, SCD1 and Elovl6 (Im et al., 2006; Latasa et al., 2003). In addition, hormones such as glucocorticoids, triiodothyronine (T3) and growth hormone contribute strongly to the regulation of fatty acid metabolism. However, nothing has been shown on the regulation of Elovl, T3 and growth hormone Recently even “lipokines”, such as palmitoleic acid (16:1), have emerged as potent regulators (Cao et al., 2008). The synthetic glucocorticoid
Dexamethasone is known to enhance GLUT4 and lipoprotein lipase (LPL) gene expression in primary brown adipocytes, pointing towards an increased cellular surge for glucose and fatty acids (Tvrdik et al., 1997) under circumstances when heat is produced and fatty acid mobilization is increased in these cells. Glucocorticoids are also important mediators of the circadian rhythm in peripheral tissues which strongly effects feeding behavior hence fatty acid homeostasis. Also, the neurotransmitter norepinephrine (NE) takes part in the regulation of fatty acid metabolism and together with dexamethasone they highly stimulate Elovl3 gene expression in primary brown adipocytes (Tvrdik et al., 1997). Interestingly, we have shown that in addition to stimulate fatty acid uptake, norepinephrine also stimulate glucose uptake via increased gene expression of GLUT1 and not via the insulin sensitive GLUT4 (Paper IV) in brown adipose tissue.

Another aspect in the control of fatty acid metabolism is the gender perspective. Sex steroids like testosterone and estrogen play a big part in the discrepancies between genders, which in turn affects fatty acid metabolism differently. However, there is a vast requirement for more knowledge in this rather uninvestigated field. I will here describe and discuss the issues which up to date are known to be involved in the regulation of ELOVL and fatty acid homeostasis.

### 3.1. TRANSCRIPTIONAL CONTROL OF FATTY ACID METABOLISM

Similar to FAS, the elongation activity of the ELOVL enzymes seems to be mainly regulated on the transcriptional level and not by protein modifications.

It is evident that the presence of transcription factors, ligands and cofactors in a certain tissue at a certain time contribute to both spatial and temporal expression of a certain gene. However, the control of this interplay is quite complex and regarding the control of Elovl gene expression, the information on this is quite sparse. In the following section I will give a brief overview on the transcription factors sterol regulatory element-binding protein (SREBP), liver X receptor (LXR), peroxisome proliferator-activated receptor α and γ (PPARα and PPARγ) which have been shown to be involved in the regulation of fatty acid metabolism and Elovl gene expression (Table 1).
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<th>Gene</th>
<th>Expression pattern</th>
<th>Involvement of transcription factors</th>
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<tbody>
<tr>
<td>ACC1</td>
<td>Liver, adipose tissue</td>
<td>LXR (↑) (Liang et al., 2002)</td>
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<td>SREBP-1c (↑) (Shimano et al., 1997)</td>
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<td>PPARα (↑) (Patel et al., 2001)</td>
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<tr>
<td>ACC2</td>
<td>Heart, skeletal muscle, liver, adipose tissue</td>
<td>LXR (↑) (Liang et al., 2002)</td>
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<td>SREBP-1 (↑) (Liang et al., 2002)</td>
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<td>PPARα (↑) (Patel et al., 2001)</td>
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<td>Elov1</td>
<td>Ubiquitous</td>
<td>LXR (↑) (Jakobsson et al., 2005), (~) (Wang et al., 2006)</td>
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<td>SREBP (~) (Jakobsson et al., 2005)</td>
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<td>PPARα (~) (Jakobsson et al., 2005)</td>
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<td>Elov2</td>
<td>Testis, liver, brain, kidney, white adipose tissue</td>
<td>LXR (~) (Wang et al., 2006)</td>
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<td>SREBP-1a (↑) (Horton et al., 2003)</td>
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<td>Elov3</td>
<td>cBAT, liver, skin WAT</td>
<td>LXR (↓) (Jakobsson et al., 2005)</td>
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<td>SREBP-1a and 1c (↑) (Anzulovich et al., 2006)</td>
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<td>PPARα (↑) (Jakobsson et al., 2005)</td>
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<td>PPARγ (↑) (Jorgensen, J.A. 2007)</td>
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<td>Elov4</td>
<td>Retina, brain, Skin, testis</td>
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<td>LXR (~) (Wang et al., 2006), (↑) (Qin et al., 2009)</td>
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<td></td>
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<td>SREBP-1a (↑) (Horton et al., 2003)</td>
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<td>SREBP-1c (↑) (Qin et al., 2009)</td>
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<td>Elov6</td>
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<td>SREBP-1 (↑) (Moon et al., 2000)</td>
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Table 1. Gene expression and involvement of transcription factors in fatty acid elongation.
3.1.1. Sterol regulatory element-binding proteins

SREBPs regulate the expression of numerous genes involved in both synthesis and uptake of fatty acids and triglycerides as well as genes involved in cholesterol and phospholipid metabolism (Horton et al., 2002). The regulation of the amount of SREBP occurs both on the transcriptional and posttranscriptional level. After translation, the protein exists as precursor and as such it is localized to the membrane of the ER, where it forms a complex with the escort protein SREBP cleavage-activating protein (SCAP). Intracellular fatty acid and sterol levels regulate a proteolytic cleavage resulting in a nuclear translocation of SREBP. (Brown and Goldstein, 1997; Brown and Goldstein, 1999; Goldstein et al., 2002).

There are three different isoforms identified: SREBP-1a, -1c and -2. SREBP-1a and -1c are isoforms derived from the same gene with alternative transcription start sites, while SREBP2 is derived from a different gene (Hua et al., 1993; Shimomura et al., 1997; Yokoyama et al., 1993). SREBP-1c and SREBP-2 regulate genes involved in fatty acid metabolism and cholesterol synthesis respectively. The role of SREBP-1a is not as restricted in its function as the other two, however it mainly regulates genes that encode fatty acid synthesis (Horton et al., 2003a; Kim and Spiegelman, 1996). SREBP-1c has emerged as the main player in insulin mediated homeostasis in the liver (Zhang et al., 2003) where it acts as a transcriptional regulator of lipogenic genes such as the insulin sensitive glucose transporter GLUT4 (Im et al., 2006), ACC and FAS. Both ACC and FAS have sterol responsive element (SRE) in their promoter regions for binding of SREBP-1c (reviewed in (Dentin et al., 2005)). It has also been shown that Elovl6 is a direct target of SREBP-1c (Moon et al., 2001). The gene expression of Elovl2 and Elovl5, which are known PUFA elongases, is slightly induced in TgSREBP-1a liver (Horton et al., 2003b). Deletion of Elovl5 causes hepatic steatosis, which is suggested to be due to a high level of SREBP-1c as a post-transcriptional mechanism and not via induced transcription (Moon et al., 2008). This is explained by that the Elovl5-ablated mice have reduced levels of arachidonic acid (20:4,n-6) and docosahexaenoic acid (22:6,n-3), which are PUFAs that have been reported to reduce the amount of active (nuclear) SREBP-1c (Xu et al., 1999b). Regarding Elovl3, promoter analyzes in mouse hepatocyte cell line have revealed that both SREBP-1a and -1c have a stimulatory effect on Elovl3 gene expression (Anzulovich et al., 2006).
3.1.2. Liver X Receptor

LXR is a nuclear receptor existing in two isoforms: LXRα and LXRβ, both being involved in the regulation of lipid and cholesterol synthesis. LXR is activated by oxysterols and heterodimerises with retinoic X receptor (RXR) (Joseph et al., 2002; Repa et al., 2000). Downstream target genes of LXR are genes involved in fatty acid and triglyceride synthesis. For example, LXR/RXR can bind directly to and activate the FAS promoter and is also known to induce SREBP-1 (Joseph et al., 2002; Repa et al., 2000). Feeding mice a LXR agonist has been shown to induce fatty acid triglyceride synthesis by inducing genes via SREBP-1c in mouse liver (Liang et al., 2002). LXRα-ablated mice accumulate cholesterol in the liver when fed high fat diet and they are resistant to diet induced obesity (Kalaany et al., 2005). The PUFA inhibition of SREBP-1 described previously is independent of LXRα (Pawar et al., 2003). Since the LXR agonist (T0901317) down-regulates 11β-hydroxysteroid dehydrogenase 1 (11β-HSD-1) gene expression in mouse liver and in primary cultures of brown adipocytes (Jakobsson et al., 2005; Stulnig et al., 2002), it is involved in the control of peripheral glucocorticoid levels and its actions on lipid metabolism. Studies have shown that LXR-agonist which normally induces ACC and FAS, suppresses Elovl3 gene expression in a SREBP-1 independent way, but increase Elovl1 gene expression in primary cultures of brown adipocytes (Jakobsson et al., 2005). Whether or not LXR has an effect on Elovl3 gene expression in liver is not known. Stimulating rat primary hepatocytes with LXR agonist has no effect on Elovl1, Elovl2, Elovl5 or Elovl6 gene expression (Wang et al., 2006).

3.1.3. Peroxisome proliferator-activated receptors

PPARs act as transcription factors as being nuclear hormone receptors. They are ligand activated by peroxisome proliferators e.g. fibrates (Gebel et al., 1992) and certain saturated and polyunsaturated fatty acids (reviewed in (Desvergne and Wahli, 1999)). To date there are three PPAR isoforms described (α, δ/β and γ), which are encoded by separate genes (reviewed in (Schoonjans et al., 1996)) with specific expression patterns (reviewed in (Lee et al., 2003)). PPARs heterodimerize with RXR and alter the transcription of the target genes by binding to peroxisome proliferator response element (PPRE).

PPARα was first identified and described in 1990 by Issemann and Green as a steroid hormone receptor and it is involved in activating genes coding for enzymes active in catabolic
processes like β-oxidation, but is today also recognized as a potent regulator of de novo lipogenesis, gluconeogenesis and fatty acid desaturation (Isseman et al., 1990; Lee et al., 1995; Sampath et al., 2005; Wang et al., 2006). It is mainly expressed in liver but also in brown adipose tissue, kidney and heart. To date there is more knowledge about the role of PPARα than about what regulates the expression of the gene. However, it is implicated that fatty acids produced by FAS and dietary fat, rather than fat that is mobilized from white adipose tissue, activates PPARα to maintain glucose, lipid and cholesterol homeostasis (Chakravarthy et al., 2005).

Twenty carbon PUFAs like eicosapentaenoic acid (20:5,n-3) are strong activators of PPARα while PUFAs of 22 carbons, for example docosapentaenoic acid (22:5,n-6) and docosahexaenoic acid (22:6,n-3) are weak inducers of PPARα (Pawar and Jump, 2003). In contrast, the nuclear abundance of SREBP-1 is reduced by docosahexaenoic acid, giving that the mentioned PUFAs inhibit fatty acid synthesis and promote fatty acid oxidation.

Both ACC and FAS gene expression has been shown to be attenuated in PPARα-ablated mice (Patel et al., 2001). The cold-induced Elovl3 gene expression in brown adipose tissue has been shown to be under the control of PPARα, which implies a role for ELOVL3 to synthesize saturated VLCFAs when fatty acid oxidation is increased (Jakobsson et al., 2005). In contrast, the Elovl1 gene expression, which also is involved in the synthesis of saturated VLCFAs, was not affected by the presence of PPARα ligand. In contrast to brown adipose tissue, Elovl3 gene expression was shown to not be under the control of PPARα in liver (Paper I). Rat primary hepatocytes overexpressing Elovl2 and Elovl5, but not Elovl6, has been shown to attenuate PPARα regulated genes that are involved in cholesterol metabolism (Wang et al., 2008b). On the other hand, feeding rats with PPARα agonist gives increased hepatic gene expression of Elovl1, Elovl5 and Elovl6 (Wang et al., 2005). Later on, it was shown that PPARα is required for hepatic induction of Elovl5, Elovl6 and SCD1 in mice (Wang et al., 2006).

PPARγ, on the other hand, plays a pivotal role in adipogenesis and insulin sensitivity of white adipose tissue (Rosen et al., 1999). It exists in two isoforms, PPARγ1 and PPARγ2. PPARγ1 is expressed in a variety of tissues like white and brown adipose tissue, liver, macrophages, skeletal muscle and placenta while PPARγ2 gene expression is, under normal physiological conditions, mainly restricted to white and brown adipose tissue (Escher et al., 2001; Werman et al., 1997). However, PPARγ2 gene expression has been shown to be
inducible in liver and skeletal muscle as a consequence of disturbed lipid homeostasis (Medina-Gomez et al., 2005; Vidal-Puig et al., 1996).

Natural ligands for PPARγ are PUFAs, prostaglandins and leukotriens, which consequently activate PPARγ and stimulate the expression of genes involved in fatty acid storage and lipid droplet function (Matsusue et al., 2008) and (reviewed in (Lemberger et al., 1996)). Recently, there has been a growing body of evidence for the importance PPARγ in adipose tissue expandability and insulin sensitivity suggesting that PPARγ increases the lipid-buffering capacity of peripheral organs hence preventing lipotoxicity (Medina-Gomez et al., 2007). This was implicated by the use of the PPARγ2/ob (POKO) mouse, which has an ablation of PPARγ2 on ob/ob background. In accordance with impaired PPARγ activity, these mice also have markedly reduced adiponectin levels compared to wild-type and ob mice. Overexpression of the adipokine adiponectin in adipose tissue of obese ob/ob mice improve insulin sensitivity in these mice probably due to increased gene expression of PPARγ target genes initiating adipose tissue expansion (Kim et al., 2007).

Differentiation of adipocytes is initiated by exposing the cells to relative high doses of cAMP, glucocorticoids and insulin, which, together with PPARγ ligands, are agents also shown to be required for optimal Elovl3 gene expression in brown preadipocytes (Jorgensen et al., 2007). Interestingly, the peak of PPARγ ligand activity in 3T3-L1 cells coincides with the peak of Elovl3 gene expression (Wang et al., 2008a), supporting the notion that ELOVL3 fatty acids, together with PPARγ, are required for proper lipid expansion during adipogenesis.

In paper II we show that, in addition to altered lipogenic gene expression, the Elovl3-ablated mice have reduced PPARγ gene expression in liver and up regulated PPARγ protein in white adipose tissue implying a crosstalk between Elovl3 gene expression, PPARγ and lipogenesis (Paper II). It is to date not known if the other elongases are regulated by PPARγ.

3.2. CIRCADIAN CONTROL OF FATTY ACID METABOLISM

It has been postulated that a great part of the mammalian genome may be regulated by “the biological clock”. Circadian clock genes control transcription factors that contribute to keep a rhythmic expression going of downstream target genes (Gachon et al., 2004; Goto and Johnson, 1995; Sweeney and Hastings, 1960). The circadian timing system is regulated by a central (master) pacemaker and peripheral (slave) pacemakers. In mammals, the postulated central pacemaker is situated in the suprachiasmatic nucleus (SCN), which is located in the...
ventral part of the hypothalamus in the brain (Rusak and Zucker, 1979). The retinohypothalamic tract (RHT) transfers information about the light from the retina to the SCN and ensures that the daily oscillations are in synchrony with the environment. As a result, many of the physiological and behavioural processes occurring in mammals are oscillating on a daily basis and are referred to as circadian rhythms, driven by an endogenous circadian timing system. Zeitgeber time (ZT), meaning "time-giver", with ZT0 defined as lights on and ZT12 as lights off, is often used to describe time-points. The circadian clock controls energy homeostasis by regulating hormones and enzymes involved in glucose and lipid metabolism mainly via clock genes like Clock, Bmal, Per and Cry (reviewed in (Froy, 2007)). Clock-controlled genes have also been found in most peripheral tissues. Circadian rhythms are endogenously generated, but can be modulated by external cues, primarily by daylight as mentioned above, but also by hormonal fluctuations and by nutrients, and allow organisms to anticipate and prepare for precise and regular environmental changes.

For example, serum leptin levels, as well as leptin gene expression in adipocytes and leptin receptor gene expression in rats, is highest during the night (Xu et al., 1999a). It has been suggested that leptin can regulate the hypothalamic-pituitary-adrenal axis (HPA) and studies in humans and mice have shown an inverse relationship between the circadian rhythms of leptin and glucocorticoids (Ahima et al., 1996; Licinio et al., 1997; Sinha et al., 1996).

Interestingly, Elovl3 came up, among others, as a highly cycling gene in liver by microarray analysis on hepatic gene expression (Panda et al., 2002). The circadian gene expression was independent of light since it persisted during constant darkness and it was abolished in CLOCK-ablated mice pointing towards Elovl3 as a “true” circadian gene.

3.2.1. Glucocorticoids

We have shown that circadian hepatic Elovl3 gene expression is regulated by steroid hormones such as glucocorticoids and gender specific hormones (Paper I). Cortisol (hydrocortisone) is the main glucocorticoid hormone in humans while corticosterone is the major glucocorticoid in nocturnal rodents (Lemberger et al., 1996; Seckl and Walker, 2001). Glucocorticoids are synthesized from either dietary or endogenously synthesized cholesterol. The effect the hormone executes on peripheral tissues depends on an interplay between three main factors; namely the blood concentration and the binding of the hormone to plasma proteins, the amount of glucocorticoid receptors (GR) available in target cells, and the activity
of 11β-HSD, which catalyses the interconversion of active corticosterone to inactive 11-dehydrocorticosterone in target tissues. Glucocorticoid hormones form an active hormone-receptor complex with the glucocorticoid receptors in the cytosol and subsequently translocate into the nucleus where they function as a transcription factors (Berdanier, 1989; Sassi et al., 1998).

Glucocorticoids play a pivotal role in glucose and fatty acid metabolism. Overproduction of glucocorticoids induces insulin resistance and leads to redistribution of fat from the extremities (e.g. legs and arms) to visceral depots. This is also associated with an increased gluconeogenesis in the liver as well as decreased glucose uptake in adipose tissue, causing hyperglucosemia (Morton et al., 2001; Shrago et al., 1963).

Glucocorticoids are secreted in daily cycles (Damiola et al., 2000) and in nocturnal rodents the hormone level peak during late day and early evening (Figure 6). In humans, however, the diurnal variation is inverted and hence peaks early in the morning (Krieger et al., 1971). Serum corticosterone levels peak when insulin levels are low, i.e. in the fasted state (Le Minh et al., 2001). Glucocorticoids are predominantly secreted from the adrenal cortex as a response to stress or starvation. Stress conditions are characterized by energy mobilization and in liver mobilized fatty acids are β-oxidized and ketogenesis (formation of ketone bodies from fatty acids) is induced (Dolinsky et al., 2004; Hillgartner et al., 1995; McCann et al., 2000; Morton et al., 2001; Tilbrook et al., 2000).

![Figure 6. Schematic description of diurnal variations of FAS, Elovl3 and PPARα expression in liver tissue and plasma corticosterone in mice. FAS mRNA levels increase when the mice start their major food-intake and decrease during the inactive light phase (graph modified from (Patel et al. 2001). The Elovl3 expression peaks in the beginning of the light phase and reaches its lowest expression during the first half of the active dark phase (Paper I). PPARα expression peaks when the mice have their inactive period and is low during the active phase (graph modified from (Patel et al. 2001). Corticosterone levels in plasma peaks during the end of the light phase, when insulin levels are low (graph modified from (Le Minh et al. 2001). The white bar represents the light phase and the black bar represents the dark phase.](image)
3.2.2. Circadian control of ACC and FAS

There are reports with fairly ambiguous results concerning the potential diurnal rhythm of ACC. In several studies, hepatic ACC gene expression and activity has been shown not to follow an obvious diurnal rhythm (Fukuda et al., 1985; Gibbons et al., 2002; Knight et al., 2005; Patel et al., 2001). On the other hand, a marked diurnal rhythm in ACC activity has been reported with highest activity, i.e. decreased phosphorylation, during the dark period (Davies et al., 1992). The effects of glucocorticoids on ACC in liver tissue are scarcely elucidated. Administration of the synthetic glucocorticoid dexamethasone induced hepatic ACC activity, although in an insulin dependent manner (Diamant and Shafirr, 1975). This indicates that glucocorticoid-induced de novo lipogenesis mainly occurs in the fed state. On the contrary, Volpe and Marasa did not detect any change in liver ACC activity when glucocorticoids were administered to rats (Volpe and Marasa, 1975).

In livers of mice, FAS gene expression follows a diurnal rhythm with a nocturnal peak at the midpoint of the dark phase, accompanied with increased nocturnal fatty acid synthesis (Figure 6) (Fukuda and Iritani, 1991; Gibbons et al., 2002; Patel et al., 2001). This diurnal variation is more pronounced compared to the diurnal variation of ACC. Like ACC, FAS has also been shown to be induced by glucocorticoids in liver as well as in white and brown adipose tissues, lung, heart, and spleen (Soncini et al., 1995).

3.2.3. Circadian control of ELOVL and SCD

The circadian rhythm seen for Elovl3 is very different from the gene expression pattern seen for most other lipogenic genes, such as ACC, FAS, SCD1 and Elovl6, which are responding to fasting and refeeding (Bianchi et al., 1990; Ntambi, 1992; Wang et al., 2005). Male hepatic Elovl3 mRNA increases early in the morning at ZT20, peaks at about ZT2 and does not decrease until the end of the light period at ZT10, hence giving an acrophase (the time at which the peak of a rhythm occurs) at the beginning of the light period (Figure 6) (Paper I). In addition, since the expression is not affected by fasting and refeeding it implies non-insulin regulated circadian mechanism. As mentioned earlier, the circadian rhythm of Elovl3 gene expression in CLOCK-ablated mice was totally abolished (Anzulovich et al., 2006; Panda et al., 2002). However, overexpression of the circadian BMAL/CLOCK did not result in a transactivation of the Elovl3 promoter, although the promoter contains one perfect and three
noncanonical E-boxes, which is a well-known BMAL/CLOCK-responsive region (Anzulovich et al., 2006). *Elovl3* was also used as a gene model to elucidate homeostatic links between the circadian clock and nutritional status. Overexpression of *RevErba*, a transcription factor that is likely to be involved in providing clock-dependent information, resulted in a decreased *Elovl3* promoter activity, while overexpression of SREBP-1, which is a transcription factor involved in nutritional homeostasis, resulted in increased activity (Anzulovich et al., 2006). In contrast to *Elovl3*, *Elovl1* and *Elovl2* do not show any strong indications of following a circadian rhythm (Paper I). Microarray analysis on hepatic gene expression has shown that *Elovl6* has lower expression during the beginning of the light period as compared to the beginning of the dark period, independent of glucocorticoids (Oishi et al., 2005b). *Elovl5* was shown to have slightly higher gene expression during the beginning of the light phase. This difference was attenuated in adrenalectomized mice (Oishi et al., 2005b). Interestingly, the *Elovl3* gene expression in this study was vaguely lowered in adrenalectomized mice.

According to Diczfalusy and co-workers, hepatic SCD1 gene expression follows a diurnal rhythm and peaks, just like FAS, in the middle of the dark phase. Thus the desaturation of saturated fatty acids appears to be highest when the animals have high levels of energy (Diczfalusy et al., 1995). It has been shown that SCD1 mRNA levels are down-regulated by the synthetic glucocorticoid dexamethasone in rat sertoli cells (Saether et al., 2003). It has also been shown that dexamethasone enhances the SCD1 activity from saturated precursors into oleic acid in HTC rat hepatoma cells (Marra and de Alaniz, 1995). Whether there is a connection between SCD1 gene expression in liver and glucocorticoids remains to be further elucidated.

### 3.2.4. Circadian control of transcription factors

Regarding transcription factors, Gibbons and co-workers have analyzed hepatic mRNA levels of SREBP-1c for putative differences in the gene expression over the diurnal cycle. However, despite diurnal variation of target genes as FAS, they report no detectable difference between the SREBP-1c gene expression at the midpoint of the dark and the midpoint of light phase (Gibbons et al., 2002). On the contrary, Anzulovich and co-workers show that SREBP-1 gene expression follows a diurnal rhythm in liver with highest levels at the end of the light and beginning of the dark period (Anzulovich et al., 2006), which is in parallel with the active phase of the mouse. It is not known if LXR gene expression follows a diurnal rhythm.
PPARα gene expression in liver follows a diurnal rhythm that is different of that seen for ACC, FAS and SREBP-1. The highest levels are before the end of the light period (Figure 6) (Lemberger et al., 1996; Patel et al., 2001). The circadian rhythm of hepatic PPARα mRNA level is abolished in homozygous Clock mutants (Oishi et al., 2005a) but it is not affected by induced diabetes or adrenalectomy in mice, stressing the importance of the master pacemaker in SCN even under conditions when lipid homeostasis is impaired in peripheral tissues. Administration of the synthetic glucocorticoid dexamethasone to primary cultures of rat hepatocytes or injected intraperitoneal (IP) into rats in vivo has been shown to induce hepatic PPARα gene expression. Furthermore, stress, as a glucocorticoid releasing factor, has also been shown to induce PPARα gene expression in liver (Lemberger et al., 1994; Lemberger et al., 1996) suggesting that the physiological role of stress-induced PPARα gene expression is to release energy through β-oxidation of fatty acids. Somewhat surprising, experiments by Patel and co-workers show that the diurnal rhythm of ACC and FAS mRNA in liver is reduced in PPARα-null mice (Patel et al., 2001) while mice fed a PPARα ligand for 7 days had induced hepatic gene expression of both ACC and FAS (Knight et al., 2005). Interestingly, even if Elovl3 gene expression in brown adipose tissue is regulated by PPARα (Jakobsson et al., 2005), we have obtained results that indicate that the diurnal variation of hepatic Elovl3 gene expression is independent of PPARα since the variation is sustained in PPARα KO mice (Paper I).

PPARδ is linked to basal lipid metabolism preferentially in liver and has been found to have a rhythmic gene expression pattern with highest hepatic expression levels at ZT20, which is at the end of the dark period (Yang et al., 2006).

Hepatic PPARγ gene expression follows a diurnal rhythm with the highest level at the end of the light period, just before PPARα reaches its highest gene expression (Patel et al., 2001; Tontonoz et al., 1994; Yang et al., 2006). It is still unknown what factors control the diurnal gene expression of PPARδ and PPARγ.

3.3. INFLUENCE OF FOOD INTAKE ON FATTY ACID METABOLISM

There are nutritionally regulated factors reported to have the ability to phase-shift peripheral tissue-oscillators or timekeepers without affecting the SCN pacemaker (reviewed in (Davidson et al., 2004)). This was illustrated by an experiment with altered feeding time which was not able to affect the phase of the SCN, but acted specifically on oscillating genes
in peripheral tissues (Damiola et al., 2000). Restricted feeding, i.e. giving mice access to food only during the light phase, resulted in an altered diurnal variation of glucocorticoids that regulates nutritional homeostasis in peripheral tissues (Balsalobre et al., 2000). Interestingly, it has been reported that not only restricted feeding, but also serving high fat diet to the animals can alter the function of the circadian clock in mice (Kohsaka et al., 2007). High calorie diet gave not only rise to changes in the rhythm of locomotor activity, it also altered expression and rhythm of circadian clock genes, nuclear receptors that have clock-controlled genes as their target, and genes controlling energy homeostasis in the hypothalamus, liver and white adipose tissue. The importance of a well functioning clock machinery in this respect was emphasized in CLOCK-ablated mice, which become hyperphagic, obese and have elevated plasma leptin levels (Oishi et al., 2006; Turek et al., 2005). Furthermore, human plasma adiponectin concentrations as well as adiponectin gene expression in the perigonadal adipose tissue in mice display a diurnal variation, which is abolished in obese subjects (Ando et al., 2005; Calvani et al., 2004; Yildiz et al., 2004).

3.3.1. Diet control of ACC and FAS

There are numerous studies in the field of diet and its effects on ACC and FAS. Together with several other lipogenic genes, they are shown to be under hormonal and nutritional regulation (Yin et al., 2000). Many of the lipogenic enzymes in liver, including ACC and FAS, are known to be increased when feeding rats with high-carbohydrate fat-free diet (Bianchi et al., 1990). In contrast, the corresponding genes were not induced, or even suppressed, when feeding rats a PUFA rich diet (Fukuda et al., 1992; Toussant et al., 1981). Male rats that were food-deprived overnight and subsequently refed with either fat-free or 10% corn oil diet (PUFA-rich) had increased gene expression of ACC and FAS upon refeeding. However, the gene expression was not induced to the same extent by the PUFA-rich diet as compared to fat-free diet (Iritani et al., 2000). Studies on mice have shown parallel results, for example Liang et al. showed a more than 6-fold increase in ACC mRNA levels in mice liver when animals were refed a high carbohydrate/low fat diet (Liang et al., 2002). Studies performed in mouse liver showed no detectable FAS mRNA when animals were fasted for 48 hours. However, in refed mice with fat-free, high-carbohydrate diet for 16 hours, the mRNA level was increased 20-fold (Paulauskis and Sul, 1988). Transgenic mice with the FAS promoter fused to a chloramphenicol acetyltransferase (CAT) reporter gene showed an increased CAT activity in liver when animals were refed a high-carbohydrate, fat-free diet (Soncini et al., 1995). These
results imply that the synthesis of fatty acids for storage is more pronounced when animals are depleted of dietary lipids.

### 3.3.2. Diet control of ELOVL and SCD

In contrast to ACC and FAS, data on the effect of diet intake and fatty acid elongation beyond C16 is relatively limited. High-fat diet and n-3 PUFA-enriched diets have been implicated in altered hepatic *Elovl2* and *Elovl5* gene expression in mice (Wang et al., 2006). *Elovl2* gene expression is up-regulated, while *Elovl5* gene expression is suppressed when mice are fed high-fat diet, resulting in a decreased ratio of arachidonic acid (20:4,n-6) to linoleic acid (18:2,n-6). As mentioned above, feeding animals with diets supplemented with n-3 PUFAs inhibits *de novo* lipogenesis, including SCD1 and FAS. In accordance with being SREBP-1 target genes, PUFAs also significantly suppress *Elovl5* and *Elovl6* gene expression (Matsuzaka et al., 2002; Nakamura and Nara, 2004; Wang et al., 2005). It has been shown in several studies that *Elovl6* gene expression is suppressed by fasting and induced by refeeding (Matsuzaka et al., 2002; Wang et al., 2005) and that the regulation of *Elovl6* mimics FAS and SCD1 gene expression in a SREBP-1 controlled fashion. It has recently been shown that the effect of PUFAs on FAS, *Elovl5* and *Elovl6* gene expression goes via SREBP-1, via a complex feedback mechanism (Moon et al., 2008; Qin et al., 2009, in press). Interestingly, *Elovl5* deficiency leads to increased levels of SREBP-1 in liver resulting in hepatosteatosis (Moon et al., 2008).

Studies involving *Elovl3* have revealed that cafeteria diet induces the gene expression of *Elovl3* in brown adipose tissue but high fat diet does suppress *Elovl3* gene expression in liver (Tvrdik et al., 1997) and (Brolinson, unpublished data). The increased expression in brown adipose tissue is a consequence of increased sympathetic signaling. Fasting for 17 hours, followed by refeeding, did not alter the *Elovl3* or *Elovl1* gene expression patterns in liver indicating that *Elovl1* and *Elovl3* are not insulin sensitive (Paper I). In contrast, conditional feeding for nine days, meaning that mice were exclusively fed during the day, significantly shifted the acrophase of *Elovl3* by about twelve hours (Paper I). A similar effect was seen when Anzulovich and co-workers subjected mice to restricted feeding, i.e. the mice were fed only between ZT5 and ZT9, which is in the second half of the light period, when the mice normally have a low food intake. One week of this feeding regime altered the *Elovl3* gene expression so that the acrophase was shifted and occurred at the beginning of the dark period (Anzulovich et al., 2006).
SCD1 is an interesting target in the context of insulin resistance and diabetes as it has been found to be under strong nutritional and hormonal control (Dobrzyn et al., 2004; Ntambi et al., 2002). It is known to be induced by several kinds of hormones, such as insulin, estrogens, androgens and growth hormone, and suppressed by thyroid hormone and leptin (Cohen et al., 2002; Ntambi and Miyazaki, 2004). The first study on SCD1 gene expression and the effects of fasting and refeeding in mice was published by Ntambi and co-workers in 1988 (Ntambi et al., 1988). SCD1 gene expression in liver was highly induced when mice were starved and refed a fat-free, high-carbohydrate diet, whereas no SCD2 gene expression could be detected at all. In contrast, SCD1 gene expression did not increase when animals were refed chow diet (Ntambi, 1992). SCD1-ablated mice on ob/ob background are protected against insulin resistance and obesity due to increased fatty acid oxidation and activation of AMPK (Cohen et al., 2002).

3.3.3. Diet control of transcription factors

During times of carbohydrate excess, insulin stimulates hepatic lipogenesis via the induction of LXR and SREBP-1 (Chen et al., 2004). Anzulovich and co-workers have shown that the diurnal rhythm of SREBP-1 was shifted when mice were subjected to restricted feeding and exclusively fed between ZT5 and ZT9 for one week (Anzulovich et al., 2006). Elovl6 was shown to be induced in transgenic mice overexpressing SREBP-1, while fasting and refeeding, suppressed and induced respectively, the Elovl6 gene expression in a SREBP-1 dependent manner (Matsuzaka et al., 2002; Moon et al., 2001). High fat diet stimulate the gene expression of both SREBP-1a and -1c in liver and also refeeding high carbohydrate/low fat diet after starvation gives rise to increased gene expression of SREBP-1a and -1c and their target genes, such as ACC, FAS, SCD1 and Elovl6 (Liang et al., 2002; Lin et al., 2005; Matsuzaka et al., 2007). When SREBP-1-ablated mice are starved and refed, there is a marked reduction of ACC, FAS and SCD1 hepatic gene expression compared to wild-type animals, hence supporting the notion that SREBP-1 is involved in the nutritional regulation of fatty acid synthesis (Shimano et al., 1999).

PPARα gene expression can be induced or repressed in liver by fasting or refeeding, respectively. This food-induced regulation seems to be only partly dependent on glucocorticoids (Lemberger et al., 1996). One could speculate that the PPARα response to stress or starvation is dependent on glucocorticoids in order to produce glucose by stimulation of gluconeogenic enzymes and release energy through β-oxidation, while in the fed state,
when insulin is present, the decrease in PPARα gene expression is not dependent on glucocorticoids. Wang and co-workers have shown that rats fed with a high carbohydrate and olive oil diet supplemented with the PPARα agonist WY14,643 for seven days have induced hepatic Elovl1, Elovl5 and Elovl6 gene expression (Wang et al., 2005). However, this induction could not be reproduced in rat primary hepatocytes upon stimulation with the mentioned PPARα agonist. Adenovirus-mediated induction of hepatic Elovl5 expression in mice resulted in an attenuated fasting-refeeding responses of PPARα target genes (Wang et al., 2008b) as a result of reduced levels of eicosapentaenoic acid (20:5,n-3), which is a suggested PPARα agonist. PPARα gene expression was reduced in Elovl6-ablated mice supporting the shift towards induced lipogenesis in these mice (Matsuzaka et al., 2007).

As compared to PPARα, relatively few studies are available on how fasting and refeeding effects PPARγ and PPARδ. In contrast to PPARα, PPARγ gene expression and protein amount is decreased by fasting in white and brown adipose tissue (Escher et al., 2001; Vidal-Puig et al., 1996) by unknown mechanism. Moreover, PPARγ2 gene expression was shown to be increased in mice fed high fat diet (Vidal-Puig et al., 1996) and PPARγ2-ablated male, but not female mice, are insulin resistant both on chow and high fat diet (Medina-Gomez et al., 2005) pointing towards an important role for PPARγ2 in maintenance of carbohydrate metabolism. PPARδ gene expression is also decreased upon fasting and restored when refed in liver and kidney, also by unknown mechanism (Escher et al., 2001). We have seen that high fat diet results in a decrease of PPARγ gene expression in white adipose tissue of Elovl3-ablated mice (Paper II).

3.4. GENDER AND FATTY ACID METABOLISM

Together with already mentioned factors, sexual dimorphism also plays an important role in fatty acid metabolism. It is well known that in humans, fat is accumulated differently between males and female. Also, there are major differences in gene expression profiles between different adipose tissue depots in humans (Snijder et al., 2003; Zhang et al., 2007) as well as in mice (Flachs et al., 2005), which supports a functional difference between different adipose depots. In general, human visceral WAT is recognized as being a more metabolic active tissue depot and associated with increased metabolic risk than subcutaneous WAT. Regarding brown adipose tissue, no sexual dimorphism has been observed.

Concerning sexual dimorphism and Elovl3, there is a pronounced sexual dimorphism of the gene expression in mouse liver with high levels in males and almost undetectable gene
expression in female liver (Anzulovich et al., 2006) and (Paper I). In addition, there is no visible gene expression of Elovl3 in sexually immature male mice (Paper I). Furthermore, Elovl3 ablation led to resistance to diet-induced obesity which was more pronounced in female mice (Paper II). Interestingly, we have found sexual dimorphism in Elovl3 gene expression upon cold exposure in gonadal WAT, whereas no discrepancies between genders were found in inguinal WAT (Paper III). Moreover, Elovl3 ablation had no effect on triglyceride content in male inguinal WAT, while there was a reduced amount in females (Paper III).

Steroid hormones are classified into different groups depending on the receptors to which they bind. There are glucocorticoids (discussed in section 3.2.1), mineralocorticoids, androgens, estrogens, and progestagens. Steroid hormones are lipid molecules that generally originate from cholesterol and are synthesized in the adrenal glands and in the gonads.

Testosterone is an androgen produced mainly in the Leydig cells of the testes of males. However, it should be noted that the ovaries of females also produce and secrete testosterone, although at a much lower level. The adrenal cortex does also produce small amounts of testosterone. The hormone is mainly recognised as being important for male sexual development and behaviour, however, they also exert effects in a variety of organs throughout the body. For example, adipocytes express the androgen receptor and they are known to affect fat accumulation and distribution (Belanger et al., 2002; Mayes and Watson, 2004). Our laboratory has shown that immature and castrated male mice have undetectable levels of hepatic Elovl3 mRNA (Paper I).

Several reports underline the existence of a diurnal rhythm of androgens and estrogens in humans as well as rodents, although the physiological significance of this, if any, is not understood. The oscillation of testosterone secretion display a maximal secretion in late evening and minimal secretion in early morning in rodents and the opposite secretion pattern in humans. (Bjorkhem et al., 1975; Kalra and Kalra, 1977; Sjoberg et al., 1979).

Testosterone can be further converted into the estrogens, of which the most potent is estradiol-17β. Adult male rats display a diurnal variation of nuclear estrogen receptors in liver as well as a diurnal variation in serum levels of estradiol. Maximal levels are seen in the morning and the lowest levels in the evening. Estrogen is known to play an important role in glucose metabolism with ovariectomy leading to increased body weight and impaired glucose tolerance (Ahmed-Sorour and Bailey, 1980; Bailey and Ahmed-Sorour, 1980). Estrogen receptor-α knockout (ERKO) female mice have higher fasting blood glucose and insulin levels (Bryzgalova et al., 2006). Also, the mice have increased plasma leptin concentration
while the adiponectin concentration is decreased. Genes involved in hepatic lipogenesis like are upregulated in the ERKO mice, although lipid transport genes are downregulated. In the same study, they show that Elovl3 gene expression is highly induced in the ERKO female mice, which implies that estrogen suppresses Elovl3 gene expression under normal conditions. However, our analysis of ovariectomized mice suggests that reduced estrogen levels are not enough to stimulate Elovl3 gene expression in female mice (Paper I).

3.4.1. Sexual dimorphism, transcription factors and target genes

To elucidate the role of gender in SREBP-1c and target genes in rat liver, Améen and co-workers have compared mRNA levels of SREBP-1c, FAS, ACC and SCD1 between sexes. They found higher mRNA levels of SREBP-1c and FAS in females whereas no sex differences were detected for ACC1 and SCD1 mRNA (Ameen et al., 2004).

A decade ago, Costet and co-workers reported that PPARα deficiency gives rise to progressive dyslipidemia with obesity and steatosis as a result (Costet et al., 1998). PPARα-ablated females display an earlier onset of obesity and accumulate more white adipose tissue, as well as brown adipose tissue, than males. Female mice developed a weight discrepancy as early as at age five weeks, while male mice do not differ in weight until they reach an age of more than five months. So, the presence, or absence, of PPARα affects lipid metabolism in a sexually dimorphic fashion. In addition, PPARα is suggested to be regulated in a gender-specific fashion in liver, with slightly higher levels expressed in female livers (Tai et al., 2003). However, in another study published at about the same time, no difference in PPARα gene expression was observed between male and female liver tissue from mice (Yoon et al., 2002). Furthermore, gender specificity has been demonstrated on the level of transcriptional activity, with less gene expression in females compared to males (Ciana et al., 2007). Also, it has been shown that male rats have higher levels of PPARα gene expression and higher levels of PPARα protein in the liver compared to female rats (Jalouli et al., 2003). Hypophysectomized rats have a higher PPARα gene expression in liver, a circumstance which is shown to be independent of gender, leading to the conclusion that the ablation of pituitary-dependent hormones abolishes the sexually dimorphic phenotype (Jalouli et al., 2003).
4. SUMMARY AND CONCLUSIONS

In this thesis, I have summarized the field of fatty acid elongases and how they contribute to fatty acid homeostasis. It is clear that the daily rhythm plays a major role in mammalian life, since we regulate our feeding time and wake/sleep hours after the light, both if we are night active or day active.

Paper I describes the regulation of Elovl3 gene expression in mouse liver. The distinct diurnal variation was exclusively found in sexually mature male mice, a fact that led us to suspect steroids as a regulator of Elovl3. This was later confirmed by the observation of completely abolished Elovl3 gene expression in castrated and immature male mice. Another interesting aspect of Paper I is that, in sharp contrast to other lipogenic genes like ACC, FAS, SCD1 and Elovl6, the gene expression of Elovl3 was not affected by fasting or refeeding. Instead restricted feeding for a longer period of time was needed to affect the hepatic Elovl3 gene expression, which made us consider glucocorticoids as players in the regulation of Elovl3. Also, these results point towards Elovl3 gene expression not being regulated by sudden changes in glucose and insulin levels but rather by factors controlling lipid homeostasis in a more long term perspective. Glucocorticoids are known to regulate lipogenic genes via PPARα and it has also been shown that both glucocorticoids and PPARα induce Elovl3 gene expression in brown adipocytes (Jakobsson et al., 2005). However, despite the fact that hepatic Elovl3 gene expression was induced by glucocorticoids, the diurnal expression was PPARα independent. Although, we show that peroxisomal fatty acid oxidation is somewhat coupled to the regulation of Elovl3 gene expression as it was elevated in the peroxisomal ABCD2 knockout mice, suggesting that the level of VLCFA oxidation might directly or indirectly modulate regulation of Elovl3 expression in mouse liver. In addition, X-linked adrenoluekodystrophy, caused by a mutation in the gene encoding the peroxisomal fatty acid transporter ABCD1 (a close homologue to ABCD2), is characterized by impaired peroxisomal β-oxidation and accumulation of saturated and monounsaturated VLCFAs in plasma and tissues (Valianpour et al., 2003). Interestingly, our preliminary data suggests that increased Elovl3 expression in ABCD1-ablated mice is PPARα dependent since Elovl3 expression is normal in ABCD1/PPARα-ablated mice (Figure 7).
In paper II, we show that the ablation of \textit{Elovl3} in mice leads to impaired lipid storage and resistance to diet-induced obesity, particularly in female mice. We present a genotype that, despite low levels of leptin, does not eat more than the wildtype mice. This is accompanied by impaired hepatic lipogenic expression and triglyceride secretion, as well as increased lipogenic expression in the adipose tissue. Interestingly, despite this, adipose tissue expansion is constrained. Although \textit{Elovl3} mRNA levels are almost undetectable in female mouse liver, the histological analysis in \textit{Elovl3}-ablated mice revealed that liver was significantly affected. Therefore we conclude that VLCFAs produced by ELOVL3, even at very low levels, are of great importance for proper lipid homeostasis. Another interesting aspect of paper II is the discovered strain difference. It is widely accepted that the C57BL/6 mouse strain is metabolically different from the 129Sv mouse strain. In contrast to C57BL/6, 129Sv mice are not prone to develop diet induced obesity. Thus the two strains respond differently to a high fat diet challenge. When the \textit{Elovl3}-ablated mice were back-crossed to the 129Sv strain, we observed an even more distinct metabolic phenotype combined with severe lipodystrophy in both male and female mice. The female mice were extremely lean and showed a dramatic reduction in fat content, as well as total body weight, which occasionally resulted in infertility. These results underline the importance of genetic background in regard of fatty acid metabolism and obesity.

In paper III we extend the observation on disturbed triglyceride formation in \textit{Elovl3}-ablated mice to also include different depots of adipose tissue. Here too we found a pronounced difference between genders, a theme that has become a red thread throughout my studies on \textit{Elovl3}. In this context it is worth mentioning that prior to our studies, relatively few comparative studies on how the genes, which are discussed in this thesis, are expressed and regulated regarding gender specificity. Furthermore, paper III establishes a role of
ELOVL3 in the dynamics of the lipid droplet in white adipose tissue, especially during physiological challenges such as increased energy expenditure.

Paper IV deals with glucose uptake in brown adipocytes during sympathetic stimulation. Brown adipose tissue is activated by norepinephrine under stressful situations, such as cold exposure. Under these circumstances, glucose is taken up to refill cellular energy and there is a high turnover of fatty acids used for heat production. Here we show that norepinephrine stimulates gene expression of GLUT1, and not the classical lipogenic isoform GLUT4, in mature brown adipocytes. One reason for this could be a difference in function: While GLUT1 is responsible for supplying the cells with energy in stressful situations, GLUT4, which is insulin sensitive, might supply the cells with glucose aimed for lipogenesis under conditions when dietary glucose is exceeding energy expenditure.

We know that mammals can produce fatty acids of varying length and degree of saturation de novo. However, the importance of the de novo synthesized fatty acids compared to the fatty acids that we consume is not known. The role of the products of ELOVL3, which are believed to be saturated and monounsaturated (C20 to C24) VLCFAs, is far from elucidated. Although, the effect of the Elolvl3 ablation seen in mice points towards a role for VLCFAs produced by ELOVL3 to act as powerful signaling molecules. One area of interest would be to learn more about how different diets affect the de novo fatty acid synthesis and especially how “unhealthy” saturated and monounsaturated fatty acids affect Elolvl3 gene expression and lipid homeostasis.
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